ALCOHOL RESEARCH
Current Reviews

THE JOURNAL OF THE NATIONAL INSTITUTE ON ALCOHOL ABUSE AND ALCOHOLISM

Curated Collection
Genetics

Compiled June 2024

Curated Collection PDFs are updated by journal staff on a yearly basis. More recent articles for this Collection may be available on the *ARCR* website.

National Institute on Alcohol Abuse and Alcoholism

TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

TABLE OF CONTENTS (CONTINUED)

TABLE OF CONTENTS (CONTINUED)

Biology, Genetics, and Environment

Underlying Factors Influencing Alcohol Metabolism

Tamara L. Wall, Ph.D.; Susan E. Luczak, Ph.D.; and Susanne Hiller-Sturmhöfel, Ph.D.

Gene variants encoding several of the alcohol-metabolizing enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), are among the largest genetic associations with risk for alcohol dependence. Certain genetic variants (i.e., alleles)—particularly the ADH1B*2, ADH1B*3, ADH1C*1, *and* ALDH2*2 *alleles—have been associated with lower rates of alcohol dependence. These alleles may lead to an accumulation of acetaldehyde during alcohol metabolism, which can result in heightened subjective and objective effects. The prevalence of these alleles differs among ethnic groups;* ADH1B*2 *is found frequently in northeast Asians and occasionally Caucasians,* ADH1B*3 *is found predominantly in people of African ancestry,* ADH1C*1 *varies substantially across populations, and* ALDH2*2 *is found almost exclusively in northeast Asians. Differences in the prevalence of these alleles may account at least in part for ethnic differences in alcohol consumption and alcohol use disorder (AUD). However, these alleles do not act in isolation to influence the risk of AUD. For example, the gene effects of* ALDH2*2 *and* ADH1B*2 *seem to interact. Moreover, other factors have been found to influence the extent to which these alleles affect a person's alcohol involvement, including developmental stage, individual characteristics (e.g., ethnicity, antisocial behavior, and behavioral undercontrol), and environmental factors (e.g., culture, religion, family environment, and childhood adversity).*

Key words: Alcohol dependence; alcohol use disorder (AUD); alcohol metabolism; alcohol-metabolizing enzymes; genetic factors; environmental factors; biological factors; gene variants; alcohol dehydrogenase (ADH); aldehyde dehydrogenase (ALDH); alleles; acetaldehyde; Asians; Caucasians; Africans; Asian-American; African-American

Tamara L. Wall, Ph.D., is a professor in the Department of Psychiatry at the University of California, San Diego, and associate chief of the Psychology Service at the Veterans Affairs San Diego Healthcare System, San Diego, California.

Susan E. Luczak, Ph.D., is an associate research professor at the University of Southern California, Los Angeles, California.

Susanne Hiller-Sturmhöfel, Ph.D., is senior science editor at Alcohol Research: Current Reviews.

Epidemiological studies have demonstrated that drinking patterns and the prevalence of alcohol-related adverse consequences, including alcohol use disorder (AUD), differ substantially among racial/ethnic groups in the United States. For example, analyses comparing drinking patterns and their consequences among Whites, Blacks, Asians, and Hispanics found the following: Whites have the highest risk and Asians have the lowest risk of AUD among these ethnic groups; Hispanics have higher rates and Asians have lower rates of heavy drinking than do Whites; and Hispanics and Blacks are more likely to have health and social problems from drinking than are Whites and Asians (Chartier and Caetano 2010). Other studies have found subgroup differences within racial/ethnic groups for alcohol-related problems; for example, individuals of Korean ancestry have higher rates of AUD than those of Chinese ancestry (Helzer et al. 1990; Luczak et al. 2004).

These differences among racial/ethnic/ancestry groups result from a variety of biological, genetic, and environmental influences, some of which relate to the metabolism of alcohol and are explored in this article. Genes encoding several variants of alcohol-metabolizing enzymes are among the largest genetic associations with the risk for alcohol dependence (Li 2000). This article briefly reviews how alcohol is metabolized in the body and describes ethnic differences in some of the genes encoding the enzymes involved in alcohol metabolism, as well as the mechanism by which these genes are thought to give rise to differences in rates of alcohol dependence. The article also summarizes what is known about

potential individual and environmental influences that may moderate the effects of these gene variants.

Alcohol Metabolism

The key enzymes involved in alcohol metabolism in the liver are alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). ADH mediates (i.e., catalyzes) the oxidation of beverage alcohol (ethanol) into acetaldehyde. Acetaldehyde then is further metabolized by ALDH into acetate. These two reactions need to be properly coordinated in the body because accumulation of acetaldehyde can lead to heightened responses as well as unpleasant reactions, such as flushing, nausea, vomiting, hypotension, and/or rapid heartbeat (i.e., tachycardia). Variant forms of several ADH and ALDH enzymes exist and are encoded by an individual's genes. These variants (i.e., alleles) produce enzymes with different properties, resulting in potential differences in the rates with which alcohol or acetaldehyde are metabolized. As a result, these variants also may influence a person's response to alcohol, drinking behavior, and consequent risk of developing an AUD. People possessing certain ADH or ALDH alleles have significantly lower rates of alcohol dependence. The following sections review four of the best-studied ADH and ALDH variants—*ADH1B*2* (rs1229984), *ADH1B*3* (rs2066702), *ADH1C*1* (rs698), and *ALDH2*2* (rs671)—and their associations with a variety of alcohol-related factors or phenotypes. The table reports the allele frequencies of these genes in different populations.

ADH Variants

To date, seven different ADH genes—*ADH1A, ADH1B, ADH1C, ADH4, ADH5, ADH6,* and *ADH7*—have been identified clustered together on the long arm of chromosome 4 (Edenberg 2007). Of these, the *ADH1A*, *ADH1B*, and *ADH1C* genes encode the majority of the ADH enzymes that metabolize alcohol in the liver. Several genome-wide association studies of alcohol dependence have found significant results in the region of chromosome 4q that includes the ADH gene cluster in a variety of ethnically diverse samples (e.g., Gelernter et al. 2014). The ADH gene with the largest effect size with alcohol dependence is *ADH1B*. Significant associations have been found for the *ADH1B*2* allele and alcohol dependence in Asian populations (Li et al. 2012*a*; Luczak et al. 2006*a*), as well as in European and African-American populations (Bierut et al. 2012; Whitfield 1997, 2002). Whitfield (2002) found that Europeans with one *ADH1B*2* allele were about half as likely (odds ratio [OR] = 0.47) to be alcohol dependent as individuals without this genetic variant (*ADH1B*1/*1* genotype). In a large meta-analysis of Asian, European, African, Hispanic, and Native-American samples, individuals with an *ADH1B*2* allele overall were about half as likely to be alcohol dependent

Table Gene Frequencies of Specific Alleles of the Genes Encoding Alcohol Dehydrogenase (ADH) and Aldehyde Dehydrogenase (ALDH) in Different Ethnic Populations

Allele	rs Number	Frequency in Different Populations		
ADH1B*2	***rs1229984***		**A allele**	**G allele**
		European	0.000–0.008	0.992–1.000
		Asian	0.739–0.771	0.229–0.261
		Sub-Saharan African	0.000	1.000
		African American	0.000	1.000
ADH1B*3	***rs2066702***		**C allele**	**T allele**
		European	1.000	0.000
		Asian	1.000	0.000
		Sub-Saharan African	0.500–0.783	0.217–0.500
		African American	0.733	0.267
ADH1C*1	***rs698***		**C allele**	**T allele**
		European	0.523–0.527	0.473–0.477
		Asian	0.927–0.975	0.025–0.073
		Sub-Saharan African	0.938–0.958	0.042–0.062
		African American	0.800	0.200
ALDH2*2	***rs671***		**C allele**	**T allele**
		European	0.000	1.000
		Asian	0.110–0.282	0.718–0.890
		Sub-Saharan African	0.000	1.000
		African American	0.000	1.000

SOURCE: dbSNP Database (www.ncbi.nlm.nih.gov/snp).

as those without this genetic variant (OR = 0.49) (Li et al. 2012*a*). The protective association is also greater for individuals with two *ADH1B*2* alleles (Li et al. 2012*a*; Luczak et al. 2006*a*). When subgroup analyses were conducted, the associations were larger in Asian populations (Li et al. 2012*a*). This is likely a result of the combined effects of the *ADH1B*2* and *ALDH2*2* alleles, as expanded upon below (Luczak et al. 2006*a*).

A second *ADH1B* gene variant, the *ADH1B*3* allele, has been related to lower rates of alcohol dependence in many but not all association studies (Edenberg 2007; Edenberg et al. 2006, 2010; Ehlers et al. 2001, 2007; Gizer et al. 2011; Luo et al. 2006; Wall et al. 1997*a*). Significant associations for the *ADH1B*3* allele and alcohol dependence primarily have been found in individuals of African ancestry where this genetic variant is most prevalent (Edenberg et al. 2006; Luo et al. 2006).

A variant of the *ADH1C* gene, the *ADH1C*1* allele, also has been well studied with respect to alcohol dependence, but the results have been inconsistent because of limited sample sizes, ethnic variation, and the close proximity of the *ADH1B* and *ADH1C* genes. Some studies showed that *ADH1C*1* and *ADH1B*2* are in linkage disequilibrium, suggesting that associations of *ADH1C*1* with alcohol dependence may be attributed to correlation with *ADH1B*2* (Borras et al. 2000; Chen et al. 1999*a*; Osier et al. 1999). A large meta-analysis of Asian, European, African, and Native-American samples found that individuals with an *ADH1C*1* allele overall were about one-third as likely to be alcohol dependent as those without this genetic variant (OR = 0.66) and also demonstrated a larger effect (OR = 0.48) in Asian populations (Li et al. 2012*b*). Furthermore, linkage disequilibrium analyses located the *ADH1C* gene in a different haplotype block than the *ADH1B* gene, suggesting the associations may be independent of one another, even though the two genes are close together.

The proposed mechanism by which these ADH alleles lead to lower rates of alcohol dependence relate to differences in the characteristics of the enzymes that they ultimately encode. The *ADH1B*2* and *ADH1B*3* alleles are thought to encode enzymes that oxidize ethanol at an increased rate compared with enzymes encoded by the more common *ADH1B*1* allele, resulting in faster acetaldehyde production. Because this increased production may lead to the accumulation of acetaldehyde and potentially more intense and/or unpleasant alcohol reactions (e.g., a flushing response), people carrying these alleles may be less likely to drink alcohol, particularly at high levels, and accordingly they also may be less likely to develop an AUD (Wall 2005; Wall et al. 2013). Similarly, the *ADH1C*1* allele is thought to encode an enzyme that accelerates the conversion rate of alcohol into acetaldehyde relative to the *ADH1C*2* allele and thus may lead to acetaldehyde buildup after alcohol consumption, thereby promoting reduced alcohol consumption and ultimately protection against AUD (Li et al. 2012*b*).

The findings assessing this proposed mechanism of action—that *ADH1B* and *ADH1C* variations reduce alcohol dependence risk through elevated acetaldehyde levels, heightened responses to alcohol, and reduced drinking—have been inconsistent. *ADH1B*2*, *ADH1B*3*, and *ADH1C*1* have not been associated with elevations in acetaldehyde, although acetaldehyde is difficult to measure in the low concentrations expected from these alleles. Many but not all studies have found that *ADH1B*2* is associated with increased sensitivity to alcohol (i.e., increased flushing and associated symptoms; see Wall et al. 2013 for review). The *ADH1B*3* allele has been associated with a faster rate of alcohol elimination and a more intense response to alcohol in individuals of African ancestry (McCarthy et al. 2010; Thomasson et al. 1995).

ALDH Variants

The acetaldehyde generated by the ADH-mediated oxidation of ethanol is further oxidized by two main ALDH enzymes—ALDH1 and ALDH2—encoded by different genes. With regard to ALDH, the *ALDH2*2* allele has shown the largest association with alcohol dependence. A meta-analysis of studies of Asian samples (Luczak et al. 2006*a*) indicated that having one *ALDH2*2* allele was associated with a four- to fivefold reduction in alcohol dependence (OR = 0.22), and having two *ALDH2*2* alleles was associated with an eight- to ninefold reduction in alcohol dependence (OR = 0.12). This meta-analysis also examined the effect of *ALDH2*2* and *ADH1B*2* alleles in combination on the risk for alcohol dependence (Luczak et al. 2006*a*). In *ALDH2*1/*1* individuals (i.e., *ALDH2*1* homozygotes), one *ADH1B*2* allele was associated with about one-fourth (OR = 0.26) and two *ADH1B*2* alleles were associated with about one-fifth (OR = 0.20) the risk of alcohol dependence compared with individuals with no *ADH1B*2* alleles. In *ALDH2*1/*2* individuals (people who carry one *ALDH2*2* allele and one *ALDH2*1* allele; i.e., who are heterozygous), one *ADH1B*2* allele was associated with about one-sixth (OR = 0.17) and two *ADH1B*2* alleles were associated with about one-eleventh (OR = 0.09) the risk of alcohol dependence compared with individuals with no *ADH1B*2* alleles. These results suggest both *ALDH2* and *ADH1B* each contribute unique protective effects on alcohol dependence, and the level of protection may be even stronger in conjunction than alone (i.e., a gene × gene interaction exists).

A similar mechanism of action has been proposed for how *ALDH2*2* results in lower rates of alcohol dependence (Wall 2005; Wall et al. 2013). According to this model, *ALDH2*2*

encodes a deficient protein subunit that has low or no activity. As a result, acetaldehyde generated by the actions of ADH cannot be readily metabolized and accumulates in the body. Consistent with this assumption, in vitro and in vivo studies have demonstrated that compared with the enzyme activity generated in cells or organisms homozygous for *ALDH2*1* (i.e., *ALDH2*1/*1* genotype), those who are heterozygous show only 12 to 20 percent of the enzyme activity and elevated acetaldehyde levels, and those who are homozygous for *ALDH2*2* show no enzyme activity and even higher acetaldehyde levels (Bosron and Li 1986; Wall et al. 1997*b*). Consequently, people who are homozygous for *ALDH2*2* experience acetaldehyde buildup even after consuming only small amounts of alcohol. As a result, these individuals rarely consume large amounts of alcohol, and there are very few documented cases of people with this genotype having alcohol dependence (Chen et al. 1999*b*; Luczak et al. 2004).

Because of the accumulation of acetaldehyde, people carrying the *ALDH2*2* allele are thought to experience heightened responses to alcohol. This has been confirmed in self-report and alcohol-challenge studies. Thus, in self-report studies *ALDH2*2* has been related to indicators of alcohol sensitivity, such as alcohol-induced flushing and other symptoms (e.g., nausea, headaches, and palpitations). Similarly, numerous alcohol-challenge studies found that people who are heterozygous for *ALDH2*2* experience flushing as well as changes in pulse rate, hormone levels, psychomotor performance, and neurophysiological reactivity compared with people homozygous for *ALDH2*1* who had the same blood alcohol concentrations. People who are homozygous for *ALDH2*2* experience even more intense subjective and objective reactions to alcohol (see Wall et al. 2013).

As a result of this heightened sensitivity to alcohol, people with the *ALDH2*2* allele may have lower positive and higher negative expectancies about alcohol's effects. Alcohol expectancies are thought to be mediators between the biological factors that determine the physiological consequences of alcohol consumption and a person's actual alcohol use. Thus, people who are highly sensitive to alcohol's unpleasant effects because they carry the *ALDH2*2* allele may be less likely to drink because they do not expect alcohol to have pleasant, reinforcing effects and instead may expect it to have unpleasant, aversive ones. Several studies examining the association between *ALDH2*2* and alcohol expectancies support this hypothesis. Two studies (McCarthy et al. 2000, 2001) found that *ALDH2*2* was associated with reduced positive expectancies but was unrelated to negative expectancies. In another analysis (Hendershot et al. 2009*b*), people with *ALDH2*2* alleles reported greater negative expectancies and thought that alcohol had greater physiological effects than did people without the allele.

The greater sensitivity to alcohol and the resulting altered alcohol expectancies then are likely to lead to lower rates of drinking and of heavy drinking. Thus, several studies have found that people with one *ALDH2*2* allele showed lower quantity and frequency of alcohol use and engaged in less binge drinking than did people without this allele; the presence of two *ALDH2*2* alleles exacerbated these effects (see Wall et al. 2013). Reduced consumption, in turn, leads to fewer alcohol-related adverse consequences, as indicated by lower scores on questionnaires measuring hazardous alcohol use and alcohol-related problems (Hendershot et al. 2009*a*, 2011). Similarly, hangovers and blackouts as consequences of heavy drinking also are inversely associated with *ALDH2*2* (Luczak et al. 2006*b*; Wall et al. 2000). A longitudinal study found that *ALDH2*2* changes the association between alcohol consumption and problems over time, with *ALDH2*2* group differences in alcohol-related problems fully accounted for by differences in frequency of binge drinking (Luczak et al. 2014).

Similar to the results from meta-analyses showing that the *ALDH2* and *ADH1B* genes may have an interactive effect on alcohol dependence (Luczak et al. 2006*a*), some self-report and alcohol-challenge data in Asians suggest that the effects of *ADH1B*2* may be stronger in individuals with *ALDH2*1/*2* genotype (e.g., Chen et al. 1999*b*; Cook et al. 2005; Luczak et al. 2006*b*; Takeshita et al. 1996, 2001). For example, in one study of Asians who carried the *ADH1B*2* allele, a heightened sensitivity to alcohol was reported only if they also carried the *ALDH2*2* allele, whereas no increase in sensitivity was reported by people carrying *ADH1B*2* in combination with only *ALDH2*1* alleles (Luczak et al. 2011). Similarly, an alcohol-challenge study only found an increased response to alcohol in people with *ADH1B*2* who also were heterozygous for *ALDH2*2* (Cook et al. 2005). These results suggest that the effects of *ADH1B*2* may be felt more strongly in Asians who already have some heightened sensitivity to alcohol from possessing one *ALDH2*2* allele, but additional research is needed to confirm these findings.

Ethnic Differences in Prevalence of *ADH1B*, *ADH1C*, and *ALDH2* Alleles

Prevalence of ADH1B and ADH1C Alleles

The *ADH1B*2* allele is found in 80 percent or more of northeast Asians (i.e., Chinese, Japanese, and Koreans) and about 50 percent of Russians and Jews, but only in 10 percent or less of Caucasians of European ancestry (Goedde et al. 1992; Osier et al. 2002). However, within the large Asian ethnic group, variations in the prevalence of the *ADH1B*2* allele exist among subpopulations (Eng et al. 2007).

The *ADH1B*3* allele is found predominantly in people of African ancestry (about 30 percent) and in much lower prevalence in certain Native Americans (i.e., Mission Indians), likely because of admixture (Bosron et al. 1983; Edenberg et al. 2006; Wall et al. 1997*a*, 2003). This allele rarely has been found in Asians and Whites.

The *ADH1C*1* allele varies substantially across different populations. It is highly prevalent in Asian and African groups (80 percent or more) and lower in Caucasians of European ancestry (about 50 percent) (Eng et al. 2007; Li et al. 2012*b*).

Prevalence of ALDH2 Alleles

*ALDH2*2* is found almost exclusively in northeastern Asian populations, albeit with varying prevalences among different Asian ethnicities (see Eng et al. 2007). For example, among Han Chinese, overall approximately one-third of individuals possess at least one *ALDH2*2* allele, with different studies determining prevalence ranging from 20 to 47 percent of participants. In contrast, *ALDH2*2* was much less commonly found among Chinese and Taiwanese natives. Studies of Japanese identified prevalence rates of 41 to 52 percent for the *ALDH2*2* allele, whereas analyses of Koreans found *ALDH2*2* prevalence of 29 to 37 percent. In other Asian ethnicities (e.g., Thais), the *ALDH2*2* allele is much less common and is found only in 10 percent or less of individuals. In all cases, only a small proportion of the individuals were homozygous for this allele (about 5 percent); most were heterozygous (Eng et al. 2007).

Moderators of the Effects of *ADH1B*2* and *ALDH2*2*

Although the studies described above demonstrate that *ADH1B* and *ALDH2* variants influence the risk of AUD, it also is clear these genes and their alleles do not act in isolation. The effects of the *ADH1B*2* allele on a person's risk of AUD also depend on the person's *ALDH2* genotype. Thus, Asians who carry the *ALDH2*2* allele show a greater protective effect (i.e., a lower risk of alcohol dependence) from the *ADH1B*2* allele than do people who only carry the functional *ALDH2*1* allele (Luczak et al. 2006*a*). However, numerous additional factors may influence the extent to which *ALDH2*2* and *ADH1B*2* affect a person's risk of alcohol involvement and AUD. Even the design of the studies assessing the associations between genotypes and AUD risk may influence the results. Thus, results from a meta-analysis study found that both the diagnostic system used in a study and the recruitment strategy used to identify study participants moderated the effects of *ALDH2*2* on risk of alcohol dependence (Luczak et al. 2006*a*). For example, studies that used the more stringent criteria of the *International Code of Diseases, 10th Edition* (ICD–10) to establish an AUD diagnosis rather than the less stringent criteria of the *Diagnostic and Statistical Manual of Mental Disorders, 3rd Edition, Revised* (DSM–III–R) revealed a greater protective effect of *ALDH2*2*. Similarly, studies in which participants were recruited from treatment settings showed greater protective effects of *ALDH2*2* than did studies involving recruitment of community samples, Thus, these findings demonstrate the importance of methodological issues that must be considered when examining the influence of moderators of gene effects. Only by accounting for these potential moderators will researchers be able to further understand the influences of these alleles and their interactions with other variables on alcohol-related behaviors and the risk of AUD. Other possible moderators of these gene effects include the following:

- Developmental stage;
- Individual characteristics, such as ethnicity, antisocial behavior, and behavioral undercontrol; and
- Environmental factors, such as culture, religion, family environment, and childhood adversity.

These factors are discussed in the following sections. Because *ALDH2*2* has the largest effect on alcohol dependence and because it is found almost exclusively in Asian populations, most of this discussion will focus on this gene and these ethnic groups.

Developmental Stage

The magnitude of *ALDH2*2* effects on alcohol use phenotypes has been shown to change over the course of development. In particular, associations of *ALDH2*2* with alcohol-related measures become stronger over the course of adolescence and young adulthood as alcohol use increases (Doran et al. 2007; Irons et al. 2007, 2012; Luczak et al. 2014). These findings are consistent with twin studies and studies of other candidate genes where genetic influences on alcohol phenotypes increase with age (Dick et al. 2006; Rose and Dick 2005).

Furthermore, although *ALDH2*2* protects against the development of alcohol dependence, the protection is not complete. In the presence of alcohol dependence or at lower levels of alcohol use, individuals with *ALDH2*2* alleles are more vulnerable to alcohol-related pathologies—particularly head and neck cancers, but also liver disease, pancreatitis, and Alzheimer's disease—consistent with a role of acetaldehyde in the pathogenesis of organ damage (Brennan et al. 2004; Brooks et al. 2009; Hao et al. 2011; Lewis and Smith 2005; Yang et al. 2010; Zhang et al. 2010; Zintzaras et al. 2006). Thus, the influence of *ALDH2*2* seems to change over the course of drinking; that is, *ALDH2*2* is protective at one stage of alcohol use (i.e., progression to heavy drinking) but becomes a risk factor at another stage (i.e., progression to alcohol-related medical problems). Prospective studies are needed to determine how

gene effects may change over the lifespan.

Individual Characteristics

Ethnicity

A study comparing Korean Americans and Chinese Americans examined whether differences in the prevalence of the *ALDH2*2* allele mediated ethnic differences in AUD and whether the effect of *ALDH2*2* was moderated by ethnicity (Luczak et al. 2004). These analyses found that *ALDH2*2* was a significant mediator of protection against alcohol dependence across different ethnic groups. However, no significant interaction existed between *ALDH2*2* and ethnicity. Another study, in contrast, found an interaction between *ALDH2*2*, ethnicity (i.e. Korean vs. Chinese), and alcohol dependence (Luczak et al. 2001). Chinese with an *ALDH2*2* allele were about one-quarter as likely to be alcohol dependent as those without the allele, whereas among the Koreans those with *ALDH2*2* were half as likely to be alcohol dependent. This finding suggests that *ALDH2*2* may have a stronger protective effect in Chinese than in Koreans. However, additional studies are needed to further explore this issue to conclusively determine the interplay between *ALDH2*2* and ethnicity, as well as other factors that might underlie ethnic differences.

Antisocial Behavior

Antisocial behavior and conduct disorder (CD) consistently have been identified as risk factors for alcohol use and AUD (see Krueger et al. 2002; Waldman and Slutske 2000). In both genders, symptoms of antisocial behavior and CD precede alcohol-related problems (Disney et al. 1999; Slutske et al. 1998). The prevalence of antisocial behavior as indicated by a diagnosis of antisocial personality disorder (ASPD) and CD differs among men and women and also shows racial/ethnic differences. In all populations studied, the prevalence for these conditions is significantly higher among men than among women (e.g., Lee et al. 1990; Luczak et al. 2004). Ethnic differences have been demonstrated particularly among Asian populations. For example, the rates of ASPD were substantially higher among South Koreans (1.6 percent) (Lee et al. 1990) than among Taiwanese (0.1 to 0.2 percent) (Hwu et al. 1989). Similarly, the prevalence of CD was higher among Korean-American college students (29 percent of men and 2 percent of women) than among Chinese-American college students (9 percent of men and 2 percent of women) (Luczak et al. 2004).

Several studies have analyzed whether differences in prevalence of protective alleles of alcohol-metabolizing enzymes and ASPD/CD could account for differences in the prevalence of AUD in different populations. A study assessing the relationship between *ALDH2*2*, CD, and alcohol dependence in Korean Americans and Chinese Americans found that although CD was a significant mediator of alcohol dependence, no significant interaction existed between CD and *ALDH2*2*. In other words, both *ALDH2*2* and CD influenced the risk of alcohol dependence, but these effects were independent of each other (Luczak et al. 2004). Other studies, however, have suggested that ASPD might interact with *ALDH2*2* to influence alcohol dependence. A study comparing *ALDH2* and *ADH1B* allele status in Taiwanese with and without ASPD and/or alcohol dependence found that *ALDH2*2* showed reduced association with alcohol dependence in people with ASPD compared with people without ASPD. *ADH1B*2* also no longer showed any association with alcohol dependence in antisocial alcoholics (Lu et al. 2005). Another study found that the prevalence of ASPD was higher in alcoholics with the *ALDH2*2* allele than in alcoholics without this allele (Iwahashi 1995). These findings suggest that the protective effects of *ALDH2*2* may be less strong in people with more antisocial behavior.

Behavioral Undercontrol

One of the personality traits known to predict alcohol and other drug use and abuse is behavioral undercontrol, a personality trait characterized by impulsivity, sensation seeking, and disinhibition (Sher et al. 2000). It also can explain, at least in part, the association between CD and AUD discussed above—that is, people with behavioral undercontrol also are more likely to be diagnosed with CD (Slutske et al. 2002). Researchers have investigated whether the increase in AUD risk conferred by behavioral undercontrol interacts with the reduction in risk conferred by *ALDH2*2*. One study (Doran et al. 2007) examined whether *ALDH2* status and the levels of behavioral undercontrol influenced the risk of binge drinking over a 2-week period in 18- to 29-year-old college students. The study found that, as expected, *ALDH2*2* reduced the risk of binge drinking, whereas behavioral undercontrol increased binge-drinking frequency. However, behavioral undercontrol did not seem to moderate the effects of *ALDH2*2*; instead, the effects of both factors were additive. This finding may be explained by the fact that behavioral undercontrol seems to act primarily at the level of alcohol use initiation (i.e., people with high levels of impulsivity and sensation seeking may be particularly likely to try alcohol and other drugs). In contrast, *ALDH2*2* influences not alcohol use initiation but continued use (i.e., people with *ALDH2*2* are less likely to continue using alcohol because they experience more intense effects).

Environmental Factors

Culture

Cultural influences, such as societal beliefs regarding alcohol use, which

are shaped by traditions, religious beliefs, and other philosophies widely acknowledged within a society, also shape drinking behaviors. For example, both Chinese and Korean cultures are influenced by Confucian philosophy, which emphasizes drinking in moderation (Bond and Hwang 1986; Cheng 1980). In addition, however, in Korean culture it also is important, especially for men, to socialize and drink heavily, which may result in greater acceptance of heavy drinking and alcohol problems (Cho and Faulkner 1993; Higuchi et al. 1996; Park et al. 1998*a,b*). Such cultural differences may contribute to the observed higher prevalence of AUD in people of South Korean heritage compared with those of Chinese or Taiwanese heritage (Helzer et al. 1990; Luczak et al. 2004). However, as mentioned previously, differences in the prevalence of *ALDH2*2* and *ADH1B*2* between different Asian ethnic groups also may account for at least part of the difference in AUD prevalence.

Further support for the relationship between culture and drinking behavior comes from observations that changes in cultural influences over time also may be followed by changes in drinking behaviors. Such developments, which have been observed in several Asian countries, also may moderate the influence of biological protective factors such as *ALDH2*2*. For example, a Japanese study found that between 1979 and 1992, when alcohol consumption became more culturally accepted and social pressure to drink increased, the proportion of Japanese patients who received treatment for alcohol dependence and carried the *ALDH2*2* allele increased from 2.5 percent to 13 percent, indicating that the protective effects of *ALDH2*2* had declined (Higuchi et al. 1994). Along the same lines, increasing acculturation of Asian Americans to American culture led to more heavy drinking and binge drinking (Hendershot et al. 2005). However, the extent of this effect was influenced by ethnicity. Thus, greater levels of acculturation in the United States may increase binge-drinking risk among people of Chinese origin but not among those of Korean origin.

Religion

Higher levels of religious behavior (e.g., commitment, affiliation, and service attendance, primarily with Christian religions) have been associated with lower alcohol use and related problems in the United States (e.g., Cochran et al. 1988; Midanik and Clark 1994; Wechsler et al. 1998). Similar analyses have been conducted with Asian and Asian-American populations, with different results depending on the population studied. Thus, whereas religious affiliation and involvement, particularly with Protestant denominations, was related to lower rates of alcohol involvement among Korean Americans (Lubben et al. 1989), the findings were inconsistent for Chinese Americans (Chi et al. 1988, 1989). In another study, religious affiliation as measured by service attendance was related to lower rates of binge drinking in Koreans regardless of their religion; among Chinese, however, such a relationship was found only among those affiliated with Western religions (Luczak et al. 2003).

Because twin studies have identified gene–environment interactions of religiosity with alcohol use behavior (Heath et al. 1999; Koopmans et al. 1999), researchers also have investigated potential interactions with *ALDH2*2* status. These analyses found that religiosity moderated the association of *ALDH2*2* with binge drinking (Luczak et al. 2003). Specifically, religious service attendance was related to binge drinking only in people homozygous for *ALDH2*1*, but not in those with at least one *ALDH2*2* allele, suggesting that the protective effect of *ALDH2*2* may be less strong in people with higher levels of religiosity.

Family Environment

Adoption studies can be especially informative for disentangling genetic influences from those of social environment. In particular, studies of adoptees can help determine if effects may be due to genetic factors or modeling behavior in the adoptive family environment. A study of adopted adolescents and young adults of Asian descent found that the effect of *ALDH2*2* was moderated by environmental influences of parental alcohol use and misuse as well as sibling alcohol use. Specifically, high parental alcohol use and misuse reduced the protective effect of *ALDH2*2* on alcohol phenotypes, whereas low parental alcohol use and misuse enhanced the effect of the allele (Irons et al. 2012). In a similar fashion, sibling alcohol use also appeared to moderate the effect of *ALDH2*2* on an adoptee's drinking behavior (Irons et al. 2007).

Childhood Adversity

Many but not all studies have shown that exposure to adverse events in childhood, such as sexual, emotional, and physical abuse, is a risk factor for developing an AUD in adulthood (Keyes et al. 2011). In a sample of Israeli adults with a relatively high prevalence of the *ADH1B*2* allele (47 percent either heterozygous or homozygous), a history of childhood adversity moderated the influence of *ADH1B*2* on alcohol-related phenotypes (Meyers et al. 2015). There was a stronger effect of *ADH1B*2* on AUD severity and the maximum number of drinks consumed in a day in individuals who had a history of childhood adversity compared with those who did not. Thus, *ADH1B*2* seems to exert a stronger effect in individuals whose risk for drinking is increased by their childhood adversity, although longitudinal studies are needed to confirm this finding.

Conclusions

Variations in the alcohol-metabolizing enzymes ADH and ALDH and the genes encoding them are associated with alcohol-related behaviors and the risk of AUD. In particular, the *ADH1B*2*, *ADH1B*3*, *ADH1C*1,* and *ALDH2*2* alleles have shown protective associations with alcohol dependence. The *ADH1B*2*, *ADH1C*1*, and *ALDH2*2* alleles have high prevalence in Asian populations and the *ADH1B*3* and *ADH1C*1* alleles in African populations, which may contribute to the differences in AUD prevalence observed among larger racial groups (i.e., Whites, Blacks, and Asians). Moreover, the prevalence of these alleles varies among different Asian subpopulations and may account at least in part for the different rates of AUD among those populations.

However, it also is clear that these alleles alone cannot explain all the differences in AUD prevalence between racial and ethnic groups; individual and environmental factors also play a role. In studies of Asian populations, some of these factors demonstrate additive effects to those imparted by *ADH1B*2* and *ALDH2*2*. In other cases, however, these additional factors interact with and moderate the effects of these alleles. In addition, a gene–gene moderating effect appears to exist between *ADH1B*2* and *ALDH2*2*, such that among people of Asian descent the effects of *ADH1B*2* may be larger in those who also carry *ALDH2*2*. Further exploration of the interactions between various genetic, individual, and environmental factors influencing drinking behavior and thus risk of AUD is necessary to fully understand how drinking behavior is shaped across developmental stages, which individual characteristics place people at risk for alcohol-related problems or AUD, when and where individuals are at most or least risk, and how preventive measures and interventions can reduce risk.

Financial Disclosure

The authors declare that they have no competing financial interests.

References

Bierut, L.J.; Goate, A.M.; Breslau, N.; et al. ADH1B is associated with alcohol dependence and alcohol consumption in populations of European and African ancestry. *Molecular Psychiatry* 17(4):445–450, 2012. PMID: 21968928

Bond, M.H., and Hwang, K.-K. The social psychology of Chinese people. In: Bond, M.H., Ed. *The Psychology of Chinese People.* Hong Kong: Oxford University Press, pp. 213–266, 1986.

Borras, E.; Coutelle, C.; Rosell, A.; et al. Genetic polymorphism of alcohol dehydrogenase in Europeans: The ADH2*2 allele decreases the risk for alcoholism and is associated with ADH3*1. *Hepatology* 31(4):984–989, 2000. PMID: 10733556

Bosron, W.F., and Li, T.-K. Genetic polymorphism of human alcohol and aldehyde dehydrogenases, and their relationship to alcohol metabolism and alcoholism. *Hepatology* 6(3):502–510, 1986. PMID: 3519419

Bosron, W.F.; Magnes, L.J.; and Li, T.-K. Human liver alcohol dehydrogenase: $ADH_{Indianapolis}$ results from genetic polymorphism at the ADH2 gene locus. *Biochemical Genetics* 21(7–8):735–744, 1983. PMID: 6354175

Brennan, P.; Lewis, S.; Hashibe, M.; et al. Pooled analysis of alcohol dehydgrogenase genotypes and head and neck cancer: A HuGE review. *American Journal of Epidemiology* 159(1):1–16, 2004. PMID: 14693654

Brooks, P.J.; Enoch, M.-A.; Goldman, D.; et al. The alcohol flushing response: An unrecognized risk factor for esophageal cancer from alcohol consumption. *PLoS Medicine* 6(3):e50, 2009. PMID: 19320537

Chartier, K., and Caetano, R. Ethnicity and health disparities in alcohol research. *Alcohol Research & Health* 33(1–2):152–160, 2010. PMID: 21209793

Chen, C.-C.; Lu, R.-B.; Chen, Y.-C.; et al. Interaction between the functional polymorphisms of the alcohol-metabolism genes in protection against alcoholism. *American Journal of Human Genetics* 65(3):795–807, 1999*a.* PMID: 10441588

Chen, Y.-C.; Lu, R.-B.; Peng, G.S.; et al. Alcohol metabolism and cardiovascular response in an alcohol patient homozygous for the ALDH2*2 variant gene allele. *Alcoholism: Clinical and Experimental Research* 23(12):1853–1860, 1999*b.* PMID: 10630602

Cheng, T.K. *The World of the Chinese: The Struggle for Human Unity.* Hong Kong: Chinese University Press, 1980.

Chi, I.; Kitano, H.H.; and Lubben, J.E. Male Chinese drinking behavior in Los Angeles. *Journal of Studies on Alcohol* 49(1):21–25, 1988. PMID: 3347072

Chi, I.; Lubben, J.E.; and Kitano, H.H. Differences in drinking behavior among three Asian-American groups. *Journal of Studies on Alcohol* 50(1):15–23, 1989. PMID: 2927118

Cho, Y.I., and Faulkner, W.R. Conceptions of alcoholism among Koreans and Americans. *International Journal of the Addictions* 28(8):681–694, 1993. PMID: 8349386

Cochran, J.K.; Beeghley, L.; and Bock, E.W. Religiosity and alcohol behavior: An exploration of reference group theory. *Sociological Forum* 3:257–277, 1988.

Cook, T.A.; Luczak, S.E.; Shea, S.H.; et al. Associations of ALDH2 and ADH1B genotypes with response to alcohol in Asian Americans. *Journal of Studies on Alcohol* 66(2):196–204, 2005. PMID: 15957670

Dick, D.M.; Bierut, L.; Hinrichs, A; et al. The role of GABRA2 in risk for conduct disorder and alcohol and drug dependence across developmental stages. *Behavior Genetics* 36(4):577–590, 2006. PMID: 16557364

Disney, E.R.; Elkins, I.J.; McGue, M.; and Iacono, W.G. Effects of ADHD, conduct disorder and gender on substance use and abuse in adolescence. *American Journal of Psychiatry* 156(10):1515–1521, 1999. PMID: 10518160

Doran, N.; Myers, M.G.; Luczak, S.E.; et al. Stability of heavy episodic drinking in Chinese- and Korean-American college students: Effects of ALDH2 gene status and behavioral undercontrol. *Journal of Studies on Alcohol and Drugs* 68(6):789–797, 2007. PMID: 17960296

Edenberg, H.J. The genetics of alcohol metabolism: Role of alcohol dehydrogenase and aldehyde dehydrogenase variants. *Alcohol Research & Health* 31:5–13, 2007. PMID: 17718394

Edenberg, H.J.; Koller, D.L.; Xuei, X.; et al. Genome-wide association study of alcohol dependence implicates a region on chromosome 11. *Alcoholism: Clinical and Experimental Research* 34(5):840–852, 2010. PMID: 20201924

Edenberg, H.J.; Xuei, X.; Chen, H.J.; et al. Association of alcohol dehydrogenase genes with alcohol dependence: A comprehensive analysis. *Human Molecular Genetics* 15(9):1539–1549, 2006. PMID: 16571603

Ehlers, C.L.; Gilder, D.A.; Harris, L.; and Carr, L. Association of the ADH2*3 allele with a negative family history of alcoholism in African American young adults. *Alcoholism: Clinical and Experimental Research* 25(12):1773–1777, 2001. PMID: 11781511

Ehlers, C.L.; Montane-Jaime, K.; Moore, S.; et al. Association of the ADHIB*3 allele with alcohol-related phenotypes in Trinidad. *Alcoholism: Clinical and Experimental Research* 31(2):216–220, 2007. PMID: 17250612

Eng, M.Y.; Luczak, S.E.; and Wall, T.L. ALDH2, ADH1B, and ADH1C genotypes in Asians: A literature review. *Alcohol Research & Health* 30(1):22–27, 2007. PMID: 17718397

Gelernter, J.; Kranzler, H.R.; Sherva, R.; et al. Genome-wide association study of alcohol dependence and alcohol dependence: Significant findings in African- and European-Americans including novel risk loci. *Molecular Psychiatry* 19(1):41–49, 2014. PMID: 24166409

Gizer, I.; Edenberg, H.J.; Gilder, D.A.; et al. Association of alcohol dehydrogenase genes with alcohol-related

phenotypes in a Native American community sample. *Alcoholism: Clinical and Experimental Research* 35(11):2008–2018, 2011. PMID: 21635275

Goedde, H.W.; Agarwal, D.P.; Fritze, G.; et al. Distribution of ADH2 and ALDH2 genotypes in different populations. *Human Genetics* 88(3):344–346, 1992. PMID: 1733836

Hao, P.-P.; Chen, Y.-G.; Wang, J.-L.; et al. Meta-analysis of aldehyde dehydrogenase 2 gene polymorphism and Alzheimer's disease in East Asians. *Canadian Journal of Neurological Sciences* 38(3):500–506, 2011. PMID: 21515512

Heath, A.C.; Madden, P.A.; Bucholz, K.K.; et al. Genetic differences in alcohol sensitivity and the inheritance of alcoholism risk. *Psychological Medicine* 29(5):1069–1081, 1999. PMID: 10576299

Helzer, J.E.; Canino, G.J.; Yeh, E.-K.; et al. Alcoholism—North America and Asia. A comparison of population surveys with the Diagnostic Interview Schedule. *Archives of General Psychiatry* 47(4):313–319, 1990. PMID: 2322082

Hendershot, C.S.; Collins, S.E.; George, W.H.; et al. Associations of the ALDH2 and ADH1B genotypes with alcohol-related phenotypes in Asian young adults. *Alcoholism: Clinical and Experimental Research* 33(5):839–847, 2009*a*. PMID: 19298323

Hendershot, C.S.; MacPherson, L.; Myers, M.G.; et al. Psychosocial, cultural and genetic influences on alcohol use in Asian American youth. *Journal of Studies on Alcohol* 66(2):185–195, 2005. PMID: 15957669

Hendershot, C.S.; Neighbors, C.; George, W.H.; et al. ALDH2, ADH1B and alcohol expectancies: Integrating genetic and learning perspectives. *Psychology of Addictive Behaviors* 23(3):452–463, 2009*b*. PMID: 19769429

Hendershot, C.S.; Witkiewitz, K.; George, W.H.; et al. Evaluating a cognitive model of ALDH2 and drinking behavior. *Alcoholism: Clinical and Experimental Research* 35(1):91–98, 2011. PMID: 21039630

Higuchi, S.; Matsushita, S.; Imazeki, H.; et al. Aldehyde dehydrogenase genotypes in Japanese alcoholics. *Lancet* 343(8899):741–742, 1994. PMID: 7907720

Higuchi, S.; Matsushita, S.; Muramatsu, T.; et al. Alcohol and aldehyde dehydrogenase genotypes and drinking behavior in Japanese. *Alcoholism: Clinical and Experimental Research* 20(3):493–497, 1996. PMID: 8727242

Hwu, H.-G.; Yeh, E.-K.; and Chang, L.-Y. Prevalence of psychiatric disorders in Taiwan defined by the Chinese Diagnostic Interview Schedule. *Acta Psychiatrica Scandinavica* 79(2):136–147, 1989. PMID: 2923007

Irons, D.E.; McGue, M.; Iacono, W.G.; and Oetting, W.S. Mendelian randomization: A novel test of the gateway hypothesis and models of gene-environment interplay. *Development and Psychopathology* 19(4):1181–1195, 2007. PMID: 17931442

Irons, D.E.; Iacono, W.G.; Oetting, W.S.; and McGue, M. Developmental trajectory and environmental moderation of the effect of ALDH2 polymorphism on alcohol use. *Alcoholism: Clinical and Experimental Research* 36(11):1882–91, 2012. PMID: 22563891

Iwahashi, K.; Matsuo, Y.; Suwaki, H.; et al. CYP2E1 and ALDH2 genotypes and alcohol dependence in Japanese. *Alcoholism: Clinical and Experimental Research* 19(3):564–566, 1995. PMID: 7573775

Keyes, K.M.; Hatzenbuehler, M.L.; and Hasin, D.S. Stressful life experiences, alcohol consumption, and alcohol use disorder: The epidemiologic evidence for four main types of stressors. *Psychopharmacology* 218(1):1–17, 2011. PMID: 21373787

Koopmans, J.R.; Slutske, W.S.; van Baal, G.C.; and Boomsma, D.I. The influence of religion on alcohol use initiation: Evidence for genotype X environment interaction. *Behavior Genetics* 29(6):445–453, 1999. PMID: 10857249

Krueger, R.F.; Hicks, B.M.; Patrick, C.J.; et al. Etiological connections among substance dependence, antisocial behavior, and personality: Modeling the externalizing spectrum. *Journal of Abnormal Psychology* 111(3):411–424, 2002. PMID: 12150417

Lee, C.K.; Kwak, Y.S.; Yamamoto, J.; et al. Psychiatric epidemiology in Korea, Part I: Gender and age differences in Seoul. *Journal of Nervous and Mental Disease* 178(4):242–246, 1990. PMID: 2319232

Lewis, S.J., and Smith, G.D. Alcohol, ALDH2, and esophageal cancer: A meta-analysis which illustrates the potentials and limitations of a Mendelian randomization approach. *Cancer Epidemiology, Biomarkers & Prevention* 14(8):1967–1971, 2005. PMID: 16103445

Li, D; Zhao, H; and Gelernter, J. Strong protective effect of the aldehyde dehydrogenase gene (ALDH2) 504lys (*2) allele against alcoholism and alcohol-induced medical diseases in Asians. *Human Genetics* 131(5): 725–737, 2012*a*. PMID: 22102315

Li, D.; Zhao, H.; and Gerlenter, J. Further clarification of the contribution of the ADH1C gene to vulnerability to alcoholism and selected liver diseases. *Human Genetics* 131(8):1361–1374, 2012*b*. PMID: 22476623

Li, T.-K. Pharmacogenetics of responses to alcohol and genes that influence alcohol drinking. *Journal of Studies on Alcohol* 61(1):5–12, 2000. PMID: 10627090

Lu, R.B.; Ko, H.C.; Lee, J.F.; et al. No alcoholism-protection effects of ADH1B*2 allele in antisocial alcoholics among Han Chinese in Taiwan. *Alcoholism: Clinical and Experimental Research* 29(12):2101–2107, 2005. PMID: 16385179

Lubben, J.E.; Chi, I.; and Kitano, H.H. The relative influence of selected social factors on Korean drinking behavior in Los Angeles. *Advances in Alcohol & Substance Abuse* 8:1–17, 1989. PMID: 2711913

Luczak, S.E.; Glatt, S.J.; and Wall, T.L. Meta-analyses of ALDH2 and ADH1B with alcohol dependence in Asians. *Psychological Bulletin* 132(4):607–621, 2006*a*. PMID: 16822169

Luczak, S.E.; Corbett, K.; Oh, C.; et al. Religious influences on heavy episodic drinking in Chinese-American and Korean-American college students. *Journal of Studies on Alcohol* 64(4):467–471, 2003. PMID: 12921188

Luczak, S.E.; Elvine-Kreis, B.; Shea, S.H.; et al. Genetic risk for alcoholism relates to level of response to alcohol in Asian-American men and women. *Journal of Studies on Alcohol* 63(1):74–82, 2002. PMID: 11925062

Luczak, S.E.; Pandika, D.; Shea, S.H.; et al. ALDH2 and ADH1B interactions in retrospective reports of low-dose reactions and initial sensitivity to alcohol in Asian American college students. *Alcoholism: Clinical and Experimental Research* 35(7):1238–1245, 2011. PMID: 21355870

Luczak, S.E.; Shea, S.H.; Hsueh, A.C.; et al. ALDH2*2 is associated with a decreased likelihood of alcohol-induced blackouts in Asian-American college students. *Journal of Studies on Alcohol* 67(3):349–353, 2006*b*. PMID: 16608143

Luczak, S.E.; Wall, T.L.; Cook, T.A.; et al. ALDH2 status and conduct disorder mediate the relationship between ethnicity and alcohol dependence in Chinese, Korean, and White American college students. *Journal of Abnormal Psychology* 113(2):271–278, 2004. PMID: 15122947

Luczak, S.E.; Wall, T.L.; Shea, S.H.; et al. Binge drinking in Chinese, Korean, and White college students: Genetic and ethnic group differences. *Psychology of Addictive Behaviors* 15(4):306–309, 2001. PMID: 11767261

Luczak, S.E.; Yanrell, L.M.; Prescott, C.A.; et al. Effects of ALDH2*2 on alcohol problem trajectories of Asian American college students. *Journal of Abnormal Psychology* 123(1):130–140, 2014. PMID: 24661165

Luo, X.; Kranzler, H.R.; Zuo, L.; et al. Diplotype trend regression analysis of the ADH gene cluster and the ALDH2 gene: Multiple significant associations with alcohol dependence. *American Journal of Human Genetics* 78(6):973–987, 2006. PMID: 16685648

McCarthy, D.M.; Brown, S.A.; Carr, L.G.; and Wall, T.L. ALDH2 status, alcohol expectancies, and alcohol response: Preliminary evidence for a mediation model. *Alcoholism: Clinical and Experimental Research* 25(11): 1558–1563, 2001. PMID: 11707629

McCarthy, D.M.; Wall, T.L.; Brown, S.A.; and Carr, L.G. Integrating biological and behavioral factors in alcohol use risk: The role of ALDH2 status and alcohol expectancies in a sample of Asian Americans. *Experimental and Clinical Psychopharmacology* 8(2):168–175, 2000. PMID: 10843299

McCarthy, D.M.; Pederson, S.L.; Lobos, E.A.; et al. ADH1B*3 and response to alcohol in African Americans. *Alcoholism: Clinical and Experimental Research* 34(7):1274–1281, 2010. PMID: 20477764

Meyers, J.L.; Shmulewitz, D.; Wall, M.M.; et al. Childhood adversity moderates the effect of ADH1B on risk for alcohol-related phenotypes in Jewish Israeli drinkers. *Addiction Biology,* 20(1):205-214, 2015. PMID: 24164917

Midanik, L.T., and Clark, W.B. The demographic distribution of US drinking patterns in 1990: Description and trends from 1984. *American Journal of Public Health* 84(8):1218–1222, 1994. PMID: 8059875

Osier, M.; Pakstis, A. J.; Kidd, J. R.; et al. Linkage disequilibrium at the ADH2 and ADH3 loci and risk of alcoholism. *American Journal of Human Genetics* 64(4):1147–1157, 1999. PMID: 10090900

Osier, M.V.; Pakstis, A.J.; Soodyall, H.; et al. A global perspective on genetic variation at the ADH genes reveals unusual patterns of linkage disequilibrium and diversity. *American Journal of Human Genetics* 71(1): 84–99, 2002. PMID: 12050823

Park, J.Y.; Danko, G.P.; Wong, S.Y.; et al. Religious affiliation, religious involvement, and alcohol use in Korea. *Cultural Diversity and Mental Health* 4(4):291–296, 1998*a*. PMID: 9818517

Park, S.C.; Oh, S.I.; and Lee, M.S. Korean status of alcoholics and alcohol-related health problems. *Alcoholism: Clinical and Experimental Research* 22(3 Suppl):170S–172S, 1998*b*. PMID: 9622398

Rose, R.J., and Dick, D.M. Gene–environment interplay in adolescent drinking behavior. *Alcohol Research and Health* 28:222–229, 2005.

Sher, K.J.; Bartholow, B.D.; and Wood, M.D. Personality and substance use disorders: A prospective study. *Journal of Consulting and Clinical Psychology* 68(5): 818–829, 2000. PMID: 11068968

Slutske, W.S.; Heath, A.C.; Dinwiddie, S.H.; et al. Common genetic risk factors for conduct disorder and alcohol dependence. *Journal of Abnormal Psychology* 107(3):363–374, 1998. PMID: 9715572

Slutske, W.S.; Heath, A.C.; Madden, P.A.; et al. Personality and genetic risk for alcohol dependence. *Journal of Abnormal Psychology* 111(1):124–133, 2002. PMID: 11871377

Takeshita, T.; Mao, X.-Q.; and Morimoto, K. The contribution of polymorphism in the alcohol dehydrogenase beta subunit to alcohol sensitivity in a Japanese population. *Human Genetics* 97(4):409–413, 1996. PMID: 8834233

Takeshita, T.; Yang, X.; and Morimoto, K. Association of the ADH2 genotypes with skin responses after ethanol exposure in Japanese male university students. *Alcoholism: Clinical and Experimental Research* 25(9):1264–1269, 2001. PMID: 11584144

Thomasson, H.R.; Beard, J.D.; and Li, T.K. ADH2 gene polymorphisms are determinants of alcohol pharmacokinetics. *Alcoholism: Clinical and Experimental Research* 19(6):1495–1499, 1995. PMID: 8749816

Waldman, I.D., and Slutske, W.S. Antisocial behavior and alcoholism: A behavioral genetic perspective on comorbidity. *Clinical Psychology Review* 20(2):255–287, 2000. PMID: 10721500

Wall, T.L. Genetic associations of alcohol and aldehyde dehydrogenase with alcohol dependence and their mechanisms of action. *Therapeutic Drug Monitoring* 27(6):700–703, 2005. PMID: 16404797

Wall, T.L.; Carr, L.G.; and Ehlers, C.L. Protective association of genetic variation in alcohol dehydrogenase with alcohol dependence in Native American Mission Indians. *American Journal of Psychiatry* 160(1):41–46, 2003. PMID: 12505800

Wall, T.L.; Luczak, S.; Orlowska, D.; and Pandika, D. Differential metabolism as an intermediate phenotype of risk for alcohol use disorder: Alcohol and aldehyde dehydrogenase variants. In: MacKillop, J., and Munafo, M.R., Eds. *Genetic Influences on Addiction: An Intermediate Phenotype Approach.* Cambridge, MA: MIT Press, pp. 41–63, 2013.

Wall, T.L.; Garcia-Andrade, C.; Thomasson, H.R.; et al. Alcohol dehydrogenase polymorphisms in Native Americans: Identification of the ADH2*3 allele. *Alcohol and Alcoholism* 32(2):129–132, 1997*a*. PMID: 9105506

Wall, T.L.; Horn, S.M.; Johnson, M.L.; et al. Hangover symptoms in Asian Americans with variations in the aldehyde dehydrogenase (ALDH2) gene. *Journal of Studies on Alcohol* 61(1):13–17, 2000. PMID: 10627091

Wall, T.L.; Peterson, C.M.; Peterson, K.P.; et al. Alcohol metabolism in Asian-American men with genetic polymorphisms of aldehyde dehydrogenase. *Annals of Internal Medicine* 127(5):376–379, 1997*b*. PMID: 9273829

Wechsler, H.; Dowdall, G.W.; Maenner, G.; et al. Changes in binge drinking and related problems among American college students between 1993 and 1997: Results of the Harvard School of Public Health College Alcohol Study. *Journal of American College Health* 47(2):57–68, 1998. PMID: 9782661

Whitfield, J.B. Meta-analysis of the effects of alcohol dehydrogenase genotype on alcohol dependence and alcoholic liver disease. *Alcohol and Alcoholism* 32(5): 613–619, 1997. PMID: 9373704

Whitfield, J.B. Alcohol dehydrogenase and alcohol dependence: Variation in genotype-associated risk between populations. *American Journal of Human Genetics* 71(5):1247–1251, 2002. PMID: 12452180

Yang, S.-J.; Yokoyama, A.; Yokoyama, T.; et al. Relationship between genetic polymorphisms of ALDH2 and ADH1B and esophageal cancer risk: A meta-analysis. *World Journal of Gastroenterology* 16(33):4210–4220, 2010. PMID: 20806441

Zhang, G.-H.; Mai, R.-Q.; and Huang, B. Meta-analysis of ADH1B and ALDH2 polymorphisms and esophageal cancer risk in China. *World Journal of Gastroenterology* 16(47):6020–6025, 2010. PMID: 21157980

Zintzaras, E.; Stefanidis, I.; Santos, M.; and Vidal, F. Do alcohol-metabolizing enzyme gene polymorphisms increase the risk of alcoholism and alcoholic liver disease? *Hepatology* 43(2):352–361, 2006. PMID: 16440362

Advances in Human Neuroconnectivity Research

Applications for Understanding Familial History Risk for Alcoholism

Anita Cservenka, Ph.D.; Gabriela Alarcón; Scott A. Jones; and Bonnie J. Nagel, Ph.D.

Recent advances in brain imaging have allowed researchers to further study the networks connecting brain regions. Specifically, research examining the functioning of these networks in groups with a genetic predisposition for alcoholism has found atypical circuitry in the brains of such individuals. Further research with larger sample sizes and multimodal method integration are necessary to confirm these intriguing findings.

Key words: Alcoholism; genetic vulnerability; genetic risk factors; brain; brain function; brain imaging; neuroimaging; neuron; neural network; neuroconnectivity; neurobiology; functional magnetic resonance imaging; psychophysiological interactions; neuroscience

Advances in human neuroimaging have expanded our ability to understand the functioning of the brain, with particular recent advances fostering our analytic capacity to examine networks between the brain's nerve cells (i.e., neurons) and neuroconnectivity (i.e., neural networks). Relevant to the field of alcoholism, several researchers recently have applied these strategies to groups at genetic risk for alcoholism, in hopes of identifying neurobiological, and specifically neuroconnectivity, phenotypes underlying this risk. This article provides an overview of the methods used to study connectivity and highlights research detailing the application of these methods to studying populations at risk for alcoholism.

Neuroconnectivity Methods

Task-Based Connectivity

With the aim of understanding network functioning in the brain, one analytic strategy has been to examine the correlations between activation in regionally disparate brain regions during functional magnetic resonance imaging (fMRI).[1] To do this, investigators typically have correlated average signal change in two or more regions of interest (ROIs) during a task, with the assumption that higher correlations reflect greater connectivity between regions (i.e., they are simultaneously showing significant changes in neural activation). Although this approach is confounded by variations in the underlying baseline intrinsic connectivity of the brain, it has nonetheless been used to demonstrate evidence of altered neuroconnectivity patterns in specific populations, as detailed below.

Psychophysiological interactions (PPI) analysis is another functional connectivity method used to analyze the coupling of neuronal activity between distinct brain regions while an individual is engaged in a task. This is different than other functional connectivity methods (e.g., resting-state functional connectivity) in that it allows one to assess the impact of task condition (or context) on the functional connectivity of two distinct brain regions. Friston and colleagues (1997) first described PPI as the statistical processing (i.e., regression) of neuronal activity in one brain region (the target region) onto the neuronal activity in a second (seed) region, with the slope of this regression being indicative of the relationship between the activity in these two regions. Comparing the slope of this regression during two distinct task conditions is the crux of PPI analysis. With PPI, seed regions can either be defined by functional subsets of data (i.e., masks) created in group-level analysis by selecting the volume elements (i.e., voxels)—three-dimensional elements that make up an image—that are most active during the task condition, or by a priori selection of a particular anatomical brain ROI (O'Reilly et al. 2012). The neural activity over time from this seed region is then multiplied by the regressor representing task-related activity and entered into the individual subject model to identify brain regions or voxels whose activity is synchronous with activity in the seed region. Recently, a more generalized form of PPI analysis allowed for the use of more than two task conditions in the same PPI model (McLaren et al. 2012). This is especially pertinent when analyzing tasks that have two or more distinct experimental conditions, as well as a control (baseline) condition in which no stimulus is occurring, typical of many fMRI tasks currently employed. This generalized form of PPI allows for better within-subject model fit and prevents

[1] fMRI measures brain activity by detecting changes in blood oxygenation and flow in different areas of the brain.

Anita Cservenka, Ph.D., is a postdoctoral fellow; Gabriela Alarcón is a graduate student; Scott A. Jones is a graduate student; and Bonnie J. Nagel, Ph.D., is associate professor of psychiatry and behavioral neuroscience, all at Oregon Health & Science University, Portland, Oregon.

having to collapse data across multiple conditions. Furthermore, PPI also can be used to compare functional connectivity between groups using group status, instead of context, as the regressor (O'Reilly et al. 2012) and could be useful for comparing groups with and without family history of alcoholism.

Connectivity Without Task Engagement

The functional connectivity of the brain also has been measured using resting-state functional connectivity (RSFC) during fMRI. With this technique, functional connectivity is measured by correlating blood-oxygen-level–dependent (BOLD) signal, an indirect measure of neuronal activity, across the brain in an individual who is resting. Regions of the brain are thought to be functionally connected if they share a temporally correlated neurophysiologic response. Spontaneous brain fluctuations persist across a variety of states, such as sleep or anesthesia, as well as in animal species (Kannurpatti et al. 2008; Kojima et al. 2009), suggesting that spontaneous BOLD correlations are an intrinsic property of brain activity (Fox and Raichle 2007). RSFC has led to the identification of numerous sets of functionally connected brain regions, termed networks, including the default mode, fronto-parietal, dorsal attention, and ventral attention networks (Fox and Raichle 2007). These networks have been identified with a myriad of techniques, the most common of which, seed-based correlation analysis (SCA) and independent components analysis (ICA), are described below. Furthermore, analysis of complex networks using mathematical approaches (e.g., graph theory) provides insight into local and global properties of specific and whole-brain network organization (Rubinov and Sporns 2010). Such analysis can be useful for identifying differences between populations, such as those at risk for alcoholism and healthy control subjects.

SCA and ICA

SCA requires a priori selection of a voxel, cluster, or anatomical region, usually based on previous fMRI literature delineating relevant regions of activation or through anatomical delineation. From this a priori selection, time series data are extracted and used as regressors in a linear correlation (or general linear model) analysis from which whole-brain, voxel-wise functional connectivity maps are derived that co-vary with the seed region (for more details see Cole et al. 2010). This approach shows networks of regions that are most strongly functionally connected with the seed voxel or ROI. Conversely, ICA is a data-driven method of analysis that uses whole-brain data to obtain spatially independent and additive components, while assuming statistical independence of non-Gaussian source signals. Networks identified with ICA are compatible with networks found using seed-based methods and typically include less artifactual effects from noise. Additionally, this method can be effective because it eliminates some inherent bias in selecting seed regions (Cole et al. 2010).

Complex network analysis elucidates properties of neural networks beyond simple local correlations established through SCA and ICA. Complex network analysis originated from graph theory but is distinct because it deals with biological networks that are large and complex, like the brain. Nodes and links make up a complex network, which is neither random nor ordered. Nodes typically represent brain regions, whereas links can be represented by anatomical or functional connections. Nodes typically span the entirety of the cortex and do not overlap, whereas links can be unidirectional or bidirectional, or binary, or weighted and represent size, density, or coherence and magnitudes of correlations, or causal interactions in anatomical and functional networks, respectively. The relationships between nodes and edges, in turn, define the network's topology, which is amenable to descriptive analyses that explore local and global aspects of a network's organization (Sporns 2014). Node degree, clustering, and modularity are commonly applied measures.

Structural Connectivity

Neuroanatomic connectivity often is characterized with diffusion tensor imaging (DTI), which provides an indirect measure of white matter[2] integrity, including myelination and axonal coherence[3] (Hagmann et al. 2006). DTI assesses diffusion of water molecules in brain tissue. In white matter, water diffusion is restricted and preferentially diffuses along axonal bundles that make up white matter tracts. This restricted diffusion is called anisotropic. Conversely, diffusion of water molecules is isotropic, or less directionally restricted, in other tissues, such as gray matter, indicating more random diffusion. By measuring fractional anisotropy (FA) in the brain, which reflects the degree to which water diffusion is constrained, researchers can draw inferences regarding the underlying white matter microstructure. Because of limitations of DTI in accurately characterizing diffusion in regions with crossing fibers (e.g., regions of prefrontal white matter), researchers must cautiously interpret findings.

Applications of Neuroconnectivity Analyses to Studies of Risk for Alcoholism

Family History of Alcoholism and Connectivity

One major risk factor for developing an alcohol use disorder (AUD) is having a family history of alcoholism (Lieb et al. 2002; Schuckit 1985). Neuroimaging research has identified various structural and functional brain differences

[2] White matter consists of axons surrounded by a protective fatty substance (i.e., myelin) that carry information between brain cells, or neurons, which make up the brain's gray matter.

[3] Axons are the nerve fibers that carry information between neurons.

between youth with familial alcoholism and their peers using volumetric analyses, DTI, and task-based fMRI, which may suggest that there are neural markers of risk, even in the absence of heavy alcohol use (Cservenka and Nagel 2012; Cservenka et al. 2012; Herting et al. 2010; Mackiewicz Seghete et al. 2013; Schweinsburg et al. 2004; Silveri et al. 2011; Spadoni et al. 2013) and in samples with minimal abuse and dependence diagnoses (Hill et al. 2001, 2007, 2011). The available neuroconnectivity tools have been critical for identifying atypical functional connections in at-risk youth, as described below. This avenue of MRI research holds promise for characterizing brain network coherence in studies of familial and genetic risk for alcoholism and is valuable for the examination of connectivity characteristics that could predict future alcohol abuse. Assessing neuroconnectivity in those at risk for alcoholism who have not yet consumed alcohol heavily allows for the distinction between phenotypes related to risk for developing alcoholism and those that could be present as a result of alcohol-induced alterations in brain networks. This advantage may allow future prevention strategies to target their efforts toward risk phenotypes that increase vulnerability for alcoholism, prior to initiation of heavy use.

Task-Based Connectivity

Task-based functional connectivity has been used in two studies of youth with family history of alcoholism to examine connectivity during working-memory tasks. In a substance-naïve sample of 12- to 14-year-olds, Wetherill and colleagues (2012) examined functional connectivity in working-memory–relevant brain regions, including the bilateral dorsolateral prefrontal cortex (DLPFC) and the posterior parietal cortex (PPC). The BOLD time series were correlated among these seed regions during participant performance of a 6-dot version of the visual working-memory (VWM) task, in which youth had to identify whether dots were the same or different colors after a delay. All fronto-parietal connections examined exhibited weaker synchrony in youth with a family history of alcoholism (FHP) compared with their peers, despite comparable task performance between the groups. Additionally, within the FHP group, there was a significant correlation between number of missed responses and functional connectivity between the PPC and DLPFC. These findings suggest that even in the absence of alcohol or substance use, youth with familial alcoholism already exhibit similar deficits in functional connections in important executive functioning pathways as those seen in alcoholics (Schulte et al. 2012).

Some hypotheses regarding familial risk for alcoholism propose that youth with a family history of AUD may be at greater risk for alcohol abuse as a result of a developmental delay (Corral et al. 2003; Hill et al. 2001). This hypothesis was tested in a functional connectivity study that examined spatial working-memory task connectivity between predefined ROIs in FHP subjects and family history–negative (FHN) youth and compared these functional connectivity patterns to those of an older group of adolescents (Spadoni et al. 2013). Using structural equation modeling, the results showed that the FHP groups differed in connectivity in the right superior parietal lobule to left middle frontal gyrus pathway and that removal of this pathway from the model resulted in a much poorer fit for the FHP group than the FHN youth. These findings suggested that FHP youth differed from their peers in working-memory–related connectivity and that the overall fit of the model for the functional connections among working-memory–related brain regions more closely resembled older adolescents in the FHN sample. These two studies indicate that neural markers for alcoholism may be present during early adolescence when alcohol or substance use has not been initiated and that these patterns may represent a developmental delay in brain network maturity. It will be interesting for future studies to conduct graph theory analyses to examine specific metrics that may differentiate FHP and FHN adolescents in fronto-parietal executive functioning systems.

Alcoholics exhibit abnormalities in reward-related structures and atypical reward processing (Makris et al. 2008; Wrase et al. 2007), suggesting that incentive motivational systems may, in part, relate to the risk for alcohol abuse. A study of young adults used the monetary incentive delay (MID) task[2] to examine functional connectivity of the ventral striatum during incentive versus neutral trials in the MID task (Weiland et al. 2013). FHP young adults showed opposite patterns of connectivity from their peers, such that they exhibited positive functional connectivity between the ventral striatum and sensorimotor cortex, as well as default mode network regions, whereas FHN youth displayed negative functional connectivity between these regions. Additionally, positive functional connectivity between the ventral striatum and supplementary sensorimotor area (SSMA) in FHP youth was positively related to self-reported sensation seeking. A mediation analyses showed that the connectivity between the nucleus accumbens (NAcc) and SSMA mediated the significant association between sensation seeking and alcohol use in the FHP group. It is possible that increased connectivity between reward-related regions and regions involved in motor control could be maladaptive in at-risk youth. The authors proposed that this increased connectivity may represent enhanced and atypical connections between regions involved in reward salience and those important for motor preparation and action. This, in turn, could potentiate actions that involve reward-related behaviors, such as alcohol use.

Fronto-cerebellar abnormalities, including atypical connectivity between the frontal lobes and cerebellum, consistently have been reported in alcoholics (Chanraud et al. 2010; Desmond et al. 2003; Rogers et al. 2012; Sullivan et al. 2003). Because these studies often do not account for preexisting risk factors in adults with AUD, such as family history risk, Herting and colleagues (2011) examined

[4] In the monetary incentive delay task, participants respond within a time window and are potentially rewarded for the response depending on their reaction time.

fronto-cerebellar integrity in FHP youth who had no experience with alcohol to examine whether preexisting atypical connectivity of these regions may be a premorbid neural risk feature. Seed-based connectivity of these regions was examined during a variety of fMRI tasks performed by participants in the scanner, that were later averaged. The results from this study suggested weaker fronto-cerebellar connectivity in FHP youth compared with their peers, indicating that previous findings reported in alcoholics may in part be attributed to preexisting risk for alcohol abuse. Additional work is necessary to examine how the integrity of these systems relates to behavioral correlates. This will further increase understanding of the specific deficits that may be associated with weaker integrity of these functional connections, which are likely associated with executive functioning, as such functions have been reported to be mediated by fronto-cerebellar systems (Diamond 2000). Importantly, some of the networks that show FHP-associated alterations in functional connectivity include brain regions where volumetric differences have been identified in FHP individuals, such as the cerebellum (Hill et al. 2007, 2011). This suggests that the underlying basis for altered BOLD synchrony between these regions may be related to premorbid anatomical differences in these structures. Additional work using multimodal integration of structure and functional connectivity methods is needed to better understand these relationships.

Resting-State Functional Connectivity

Although more research on familial risk for alcoholism and brain connectivity has focused on functional connections present across task-related BOLD response, recent investigations have examined the intrinsic functional connectivity of brain regions in FHP youth (see figure), specifically using seed-based resting-state connectivity methods. Brain regions and networks that play important roles in reward and emotional processing (e.g., the NAcc and amygdala) often have been the focus of alcoholism research, as task-based neuroimaging studies suggest aberrant brain activity in these areas (Marinkovic et al. 2009; Wrase et al. 2007). Using anatomically defined ROIs of the NAcc on a subject-specific basis, Cservenka and colleagues (2014*a*) found significant differences in the synchrony of both left and right NAcc with other regions of the brain in FHP youth compared with their peers. Specifically, differences were most pronounced in connectivity of the ventral striatum with regions of the frontal lobe. FHP youth had less negative connectivity (or less segregation) between the NAcc and cognitive control regions of the frontal cortex, including bilateral inferior frontal gyri, than their peers. The authors suggested that because reward and executive functioning networks are not as distinctly segregated in FHP youth, this may lead to miscommunication between these regions. Furthermore, this study found that FHP youth had disrupted integration between the NAcc and orbitofrontal cortex (OFC), whereas these regions showed positive connectivity in FHN youth. The authors suggested that reward-related brain areas may be more weakly integrated in FHP youth, which may result in a dissociation between reward response in the brain (mediated by NAcc) and determining the value of rewards (mediated by OFC). Again, it is important to note that alterations in resting state synchrony between the NAcc and OFC may be related to underlying volumetric differences in these regions in FHP individuals. Disruptions in OFC laterality have been previously reported in at-risk youth/young adults (Hill et al. 2009). Associations between functional connectivity and relationships with brain structure require further study.

Recently, another study used RSFC to examine intrinsic connectivity of the amygdala in FHP adolescents and found relationships between behavior in an emotion-cognition task and functional connectivity between the left amygdala and left superior frontal gyrus (SFG) (Cservenka et al. 2014*b*). Weaker connectivity between amygdala and left SFG was associated with poorer impulse control in the context of emotional stimuli in the FHP group. The authors believe that segregation of cognitive and emotional circuitry in at-risk youth may be a marker of weaker cognitive control in FHP adolescents when they are in emotionally laden situations. Because FHN youth displayed mostly positive synchrony between these regions, which was unrelated to task performance, these findings could indicate that once connectivity is established in these regions, it may no longer aberrantly affect behavior. Given discrepancies between children and adults reported in the typical patterns of positive and negative functional connectivity between the amygdala and the frontal lobe (Qin et al. 2012; Roy et al. 2009), more work is needed to determine how the integrity of fronto-limbic circuitry is related to risk for alcoholism.

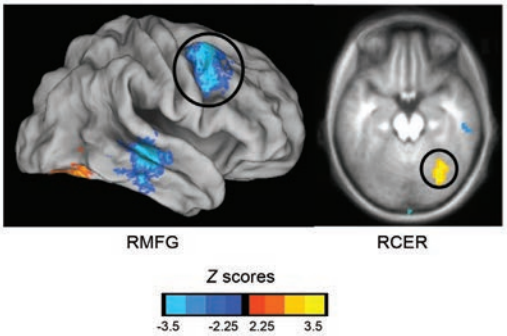


Figure FHP youth have significant differences in right amygdalar resting state functional connectivity patterns compared with FHN youth in frontal and cerebellar regions. This indicates atypical connectivity with executive functioning brain regions in at-risk adolescents compared with controls. RMFG = right middle frontal gyrus; RCER = right cerebellum.

SOURCE: Cservenka et al. 2014*b*.

Another finding from this study was an opposite pattern of functional connectivity between the amygdala and cerebellum in FHP youth compared with the pattern observed in their FHN peers (both greater and reduced connectivity, depending on the side of the brain). These results are interesting given previously reported weaker fronto-cerebellar connectivity in FHP youth (Herting et al. 2011), when BOLD signal was averaged across a variety of fMRI tasks. Not only may fronto-cerebellar connectivity be altered in FHP youth prior to heavy alcohol use, but connectivity of these regions with affect-related areas at rest also may be atypical. Interestingly, both reduced contralateral fronto-cerebellar and amygdalar-cerebellar connectivity was found across both studies (Cservenka et al. 2014*b*; Herting et al. 2011), which supports weaker interhemispheric connectivity between frontal and limbic brain regions with the cerebellum in FHP youth compared with their peers. These findings suggest that both top-down and bottom-up connections with the cerebellum show reduced synchrony across hemispheres in at-risk individuals, a phenotype that merits further exploration, especially given other reports of smaller amygdalar volumes in FHP youth (Hill et al. 2001, 2013*b*).

DTI

A number of DTI studies have identified white-matter pathways that are altered in high-risk youth and adults (Acheson et al. 2014; Herting et al. 2010; Hill et al. 2013*a*), suggesting that differences in functional connectivity may be related to atypical structural integrity of white matter in FHP individuals. The first study to do so examined white-matter integrity in alcohol-naïve FHP adolescents compared with age- and gender-matched FHN youth and found reduced FA in the superior and inferior longitudinal fasciculi, as well as the anterior superior corona radiata in FHP youth (Herting et al. 2010). Further, reduced FA mediated the relationship between familial alcoholism and reaction times on a delay-discounting task. Because many of these pathways are implicated in connections between brain regions involved in higher-order executive functioning (Seghete et al. 2013; Treit et al. 2013), the findings may reflect either a developmental delay in maturation of white matter, or more lasting deficits in white matter integrity in FHP individuals. Because executive functioning deficits have been observed in both alcoholics (Noel et al. 2007; Verdejo-Garcia et al. 2006) and offspring of alcoholics (Gierski et al. 2013; Harden and Pihl 1995; Nigg et al. 2004), it is plausible that premorbid weaknesses in top-down cognitive functioning and associated neurocircuitry could increase risk for maladaptive decisions regarding alcohol use.

Another DTI study found risk by alcohol exposure effects related to reduced FA in some of the same white-matter pathways previously reported to be altered in FHP youth, including superior and inferior longitudinal fasciculi (Hill et al. 2013*a*). Because this study was conducted in adults, it is possible that developmental timing is a key factor in determining whether risk effects alone are observed and how alcohol exposure may further compromise these vulnerable pathways.

Additional support for lower FA in a variety of white-matter tracts in frontal and parietal regions was recently reported in a large sample of 80 FHP youth, who also had lower FA, compared with their peers in anterior, superior, and posterior corona radiata (Acheson et al. 2014), with the first two pathways exhibiting similar reductions in FA to previously reported findings (Herting et al. 2010). In some cases, studies that have found reduced fronto-parietal functional connectivity in FHP youth have not found reductions in white matter integrity in fronto-parietal pathways (Wetherill et al. 2012). The dissociation between functional and structural connectivity was interpreted as delays in synaptic transmission, rather than compromised myelination of white-matter pathways (Wetherill et al. 2012). However, as a result of the small sample sizes, results need to be replicated.

Conclusions and Future Directions

As shown, several studies have used neuroconnectivity methods to identify atypical circuitry in the brains of those at familial risk for alcoholism, albeit generally with small sample sizes, which is a limitation of the available research. Overall, these studies have demonstrated abnormalities in connectivity between frontal regions with parietal, ventral striatal, cerebellar, and limbic regions of the brain in these populations, suggesting that these methods may be particularly useful in uncovering neurobiological risk phenotypes. Larger sample sizes and multi-modal method integration are critical to confirm these intriguing findings. Although studies of family history risk for alcoholism have reported atypical functional connectivity using seed-based resting-state and task-based connectivity approaches, none have used graph theory to examine network characteristics of alcoholism-related risk, an analytic strategy which may prove particularly useful for increasing our understanding of the interactions between these networks. Given recent work documenting the amenability of brain functioning to change in response to treatment (Feldstein Ewing et al. 2011), identification of neuroconnectivity treatment targets may substantially increase our capacity to intervene with at-risk populations in a neurobiologically targeted manner.

Financial Disclosure

The authors declare that they have no competing financial interests.

References

Acheson, A.,; Wijtenburg, S.A.; Rowland, L.M.; et al. Assessment of whole brain white matter integrity in youths and young adults with a family history of substance-use disorders. *Human Brain Mapping* 35(11):5401–5413, 2014. PMID: 24867528

Chanraud, S.; Pitel, A.L.; Rohlfing, T.; et al. Dual tasking and working memory in alcoholism: Relation to frontocerebellar circuitry. *Neuropsychopharmacology* 35(9):1868–1878, 2010. PMID: 20410871

Cole, D. M.; Smith, S.M.; and Beckmann, C.F. Advances and pitfalls in the analysis and interpretation of resting-state fMRI data. *Frontiers in Systems Neuroscience* 4:8, 2010. PMID: 20407579

Corral, M.; Holguin, S.R.; and Cadaveira, F. Neuropsychological characteristics of young children from high-density alcoholism families: A three-year follow-up. *Journal of Studies on Alcohol and Drugs* 64(2):195–199, 2003. PMID: 12713192

Cservenka, A., and Nagel, B.J. Risky decision-making: An fMRI study of youth at high risk for alcoholism. *Alcoholism: Clinical and Experimental Research* 36(4):604–615, 2012. PMID: 22250647

Cservenka, A.; Casimo, K.; Fair, D.A.; and Nagel, B.J. Resting state functional connectivity of the nucleus accumbens in youth with a family history of alcoholism. *Psychiatry Research* 221(3):210–219, 2014*a*. PMID: 24440571

Cservenka, A.; Fair, D.A.; and Nagel, B.J. Emotional processing and brain activity in youth at high risk for alcoholism. *Alcoholism: Clinical and Experimental Research* 38(7):1912–1923, 2014*b*. PMID: 24890898

Cservenka, A.; Herting, M.M.; and Nagel, B.J. Atypical frontal lobe activity during verbal working memory in youth with a family history of alcoholism. *Drug and Alcohol Dependence* 123(1–3):98–104, 2012. PMID: 22088655

Desmond, J.E.; Chen, S.H.; DeRosa, E.; et al. Increased frontocerebellar activation in alcoholics during verbal working memory: An fMRI study. *NeuroImage* 19(4):1510–1520, 2003. PMID: 12948707

Diamond, A. Close interrelation of motor development and cognitive development and of the cerebellum and prefrontal cortex. *Child Development* 71(1):44–56, 2000. PMID: 10836557

Feldstein Ewing, S.W.; Filbey, F.M.; Sabbineni, A.; et al. How psychosocial alcohol interventions work: A preliminary look at what fMRI can tell us. *Alcoholism: Clinical and Experimental Research* 35(4):643–651, 2011. PMID: 21223301

Fox, M.D., and Raichle, M.E. Spontaneous fluctuations in brain activity observed with functional magnetic resonance imaging. *Nature Reviews. Neuroscience* 8(9):700–711, 2007. PMID: 17704812

Friston, K.J.; Buechel, C.; Fink, G.R.; et al. Psychophysiological and modulatory interactions in neuroimaging. *NeuroImage* 6(3):218–229, 1997. PMID: 9344826

Gierski, F.; Hubsch, B.; Stephaniak, N.; et al. Executive functions in adult offspring of alcohol-dependent probands: Toward a cognitive endophenotype? *Alcoholism: Clinical and Experimental Research* 37(Suppl. 1):E356–E363, 2013. PMID: 23240659

Hagmann, P.; Jonasson, L.; Maeder, P.; et al. Understanding diffusion MR imaging techniques: From scalar diffusion-weighted imaging to diffusion tensor imaging and beyond. *Radiographics* 26(Suppl. 1):S205–S223, 2006. PMID: 17050517

Harden, P.W., and Pihl, R.O. Cognitive function, cardiovascular reactivity, and behavior in boys at high risk for alcoholism. *Journal of Abnormal Psychology* 104(1):94–103, 1995. PMID: 7897058

Herting, M.M.; Fair, D.; and Nagel, B.J. Altered fronto-cerebellar connectivity in alcohol-naive youth with a family history of alcoholism. *NeuroImage* 54(4):2582–2589, 2011. PMID: 20970506

Herting, M.M.; Schwartz, D.; Mitchell, S.H.; and Nagel, B.J. Delay discounting behavior and white matter microstructure abnormalities in youth with a family history of alcoholism. *Alcoholism: Clinical and Experimental Research* 34(9):1590–1602, 2010. PMID: 20586754

Hill, S.Y.; De Bellis, M.D.; Keshavan, M.S.; et al. Right amygdala volume in adolescent and young adult offspring from families at high risk for developing alcoholism. *Biological Psychiatry* 49(11):894–905, 2001. PMID: 11377407

Hill, S.Y.; Muddasani, S.; Prasad, K.; et al. Cerebellar volume in offspring from multiplex alcohol dependence families. *Biological Psychiatry* 61(1):41–47, 2007. PMID: 16533498

Hill, S.Y.; Terwilliger, R.; and McDermott, M. White matter microstructure, alcohol exposure, and familial risk for alcohol dependence. *Psychiatry Research* 212(1):43–53, 2013*a*. PMID: 23473988

Hill, S.Y.; Wang, S.; Carter, H.; et al. Cerebellum volume in high-risk offspring from multiplex alcohol dependence families: Association with allelic variation in GABRA2 and BDNF. *Psychiatry Research* 194(3):304–313, 2011. PMID: 22047728

Hill, S.Y.; Wang, S.; Carter, H.; et al. Amygdala volume in offspring from multiplex for alcohol dependence families: The moderating influence of childhood environment and 5-HTTLPR variation. *Journal of Alcoholism and Drug Dependence* S1(001):1–9, 2013*b*.

Hill, S.Y.; Wang, S.; Kostelnik, B.; et al. Disruption of orbitofrontal cortex laterality in offspring from multiplex alcohol dependence families. *Biological Psychiatry* 65(2):129–136, 2009. PMID: 18986649

Kannurpatti, S.S.; Biswal, B.B.; Kim, Y.R.; and Rosen, B.R. Spatio-temporal characteristics of low-frequency BOLD signal fluctuations in isoflurane-anesthetized rat brain. *NeuroImage* 40(4):1738–1747, 2008. PMID: 18339559

Kojima, T.; Onoe, H.; Hikosaka, K.; et al. Default mode of brain activity demonstrated by positron emission tomography imaging in awake monkeys: Higher rest-related than working memory-related activity in medial cortical areas. *Journal of Neuroscience* 29(46):14463–14471, 2009. PMID: 19923280

Lieb, R.; Merikangas, K.R.; Höfler, M.; et al. Parental alcohol use disorders and alcohol use and disorders in offspring: A community study. *Psychological Medicine* 32(1):63–78, 2002. PMID: 11883731

Mackiewicz Seghete, K.L.; Cservenka, A.; Herting, M.M.; and Nagel, B.J. Atypical spatial working memory and task-general brain activity in adolescents with a family history of alcoholism. *Alcoholism: Clinical and Experimental Research* 37(3):390–398, 2013. PMID: 23078615

Makris, N.; Oscar-Berman, M.; Jaffin, S.K.; et al. Decreased volume of the brain reward system in alcoholism. *Biological Psychiatry* 64(3):192–202, 2008. PMID: 18374900

Marinkovic, K.; Oscar-Berman, M.; Urban, T.; et al. Alcoholism and dampened temporal limbic activation to emotional faces. *Alcoholism: Clinical and Experimental Research* 33(11):1880–1892, 2009. PMID: 19673745

McLaren, D.G.; Ries, M.L.; Xu, G.; and Johnson, S.C. A generalized form of context-dependent psychophysiological interactions (gPPI): A comparison to standard approaches. *NeuroImage* 61(4):1277–1286, 2012. PMID: 22484411

Nigg, J.T.; Glass, J.M.; Wong, M.M.; et al. Neuropsychological executive functioning in children at elevated risk for alcoholism: Findings in early adolescence. *Journal of Abnormal Psychology* 113(2):302–314, 2004. PMID: 15122950

Noel, X.; Bechara, A.; Dan, B.; et al. Response inhibition deficit is involved in poor decision making under risk in nonamnesic individuals with alcoholism. *Neuropsychology* 21(6):778–786, 2007. PMID: 17983291

O'Reilly, J.X.; Woolrich, M.W.; Behrens, T.E.; et al. Tools of the trade: Psychophysiological interactions and functional connectivity. *Social Cognitive and Affective Neuroscience* 7(5):604–609, 2012. PMID: 22569188

Qin, S.; Young, C.B.; Supekar, K.; et al. Immature integration and segregation of emotion-related brain circuitry in young children. *Proceedings of the National Academy of Sciences of the United States of America* 109(20):7941–7946, 2012. PMID: 22547826

Rogers, B.P.; Parks, M.H.; Nickel, M.K.; et al. Reduced fronto-cerebellar functional connectivity in chronic alcoholic patients. *Alcoholism: Clinical and Experimental Research* 36(2):294–301, 2012. PMID: 22085135

Roy, A.K.; Shehzad, Z.; Margulies, D.S.; et al. Functional connectivity of the human amygdala using resting state fMRI. *NeuroImage* 45(2):614–626, 2009. PMID: 19110061

Rubinov, M., and Sporns, O. Complex network measures of brain connectivity: Uses and interpretations. *NeuroImage* 52(3):1059–1069, 2010. PMID: 19819337

Schuckit, M.A. Genetics and the risk for alcoholism. *JAMA* 254(18):2614–2617, 1985. PMID: 4057470

Schulte, T.; Muller-Oehring, E.M.; Sullivan, E.V.; and Pfefferbaum, A. Synchrony of corticostriatal-midbrain activation enables normal inhibitory control and conflict processing in recovering alcoholic men. *Biological Psychiatry* 71(3):269–278, 2012. PMID: 22137506

Schweinsburg, A.D.; Paulus, M.P.; Barlett, V.C.; et al. An fMRI study of response inhibition in youths with a family history of alcoholism. *Annals of the New York Academy of Sciences* 1021:391–394, 2004. PMID: 15251915

Seghete, K.L.; Herting, M.M.; and Nagel, B.J. White matter microstructure correlates of inhibition and task-switching in adolescents. *Brain Research* 1527:15–28, 2013. PMID: 23811486

Silveri, M.M.; Rogowska, J.; McCaffrey, A.; and Yurgelun-Todd, D.A. Adolescents at risk for alcohol abuse demonstrate altered frontal lobe activation during Stroop performance. *Alcoholism: Clinical and Experimental Research* 35(2):218–228, 2011. PMID: 21073483

Spadoni, A.D.; Simmons, A.N.; Yang, T.T.; and Tapert, S.F. Family history of alcohol use disorders and neuromaturation: A functional connectivity study with adolescents. *American Journal of Drug and Alcohol Abuse* 39(6):356–364, 2013. PMID: 24200205

Sporns, O. Contributions and challenges for network models in cognitive neuroscience. *Nature Neuroscience* 17(5):652–660, 2014. PMID: 24686784

Sullivan, E.V.; Harding, A.J.; Pentney, R.; et al. Disruption of frontocerebellar circuitry and function in alcoholism. *Alcoholism: Clinical and Experimental Research* 27(2):301–309, 2003. PMID: 12605080

Treit, S.; Chen, Z.; Rasmussen, C.; and Beaulieu, C. White matter correlates of cognitive inhibition during development: A diffusion tensor imaging study. *Neuroscience* 276:87–97, 2014. PMID: 24355493

Verdejo-Garcia, A.; Bechara, A.; Recknor, E.C.; and Pérez-García, M. Executive dysfunction in substance dependent individuals during drug use and abstinence: An examination of the behavioral, cognitive and emotional correlates of addiction. *Journal of the International Neuropsychological Society* 12(3):405–415, 2006. PMID: 16903133

Weiland, B.J.; Welsh, R.C.; Yau, W.Y.; et al. Accumbens functional connectivity during reward mediates sensation-seeking and alcohol use in high-risk youth. *Drug and Alcohol Dependence* 128(1–2):130–139, 2013. PMID: 22958950

Wetherill, R.R.; Bava, S.; Thompson, W.K.; et al. Frontoparietal connectivity in substance-naive youth with and without a family history of alcoholism. *Brain Research* 1432:66–73, 2012. PMID: 22138427

Wrase, J.; Schlagenhauf, F.; Kienast, T.; et al. Dysfunction of reward processing correlates with alcohol craving in detoxified alcoholics. *NeuroImage* 35(2):787–794, 2007. PMID: 17291784

Epigenetics Glossary

Adipogenesis: Process of cell differentiation by which fat cells (i.e., adipocytes) are generated from precursor cells.

AMP/ATP ratio: Ratio of adenosine monophosphate (AMP) to adenosine triphosphate (ATP) in the cell; ATP supplies the energy needed for many biochemical reactions; therefore, the AMP/ATP ratio reflects the cell's energy level.

Apoptosis: Series of biochemical reactions occurring in a cell whereby cells that are damaged or no longer needed undergo a process of self-destruction; also known as programmed cell death or cell suicide.

Astrocytes: Characteristic star-shaped *glial cells* in the brain and spinal cord that support the endothelial cells which form the blood–brain barrier, provide nutrients to the nervous tissue, and play a principal role in the repair and scarring process of the brain and spinal cord following traumatic injuries.

Caloric restriction: A dietary regimen characterized by a low calorie intake while maintaining adequate nutrition (i.e., sufficient levels of proteins, vitamins, and minerals).

Cerebellum: Region of the brain that controls motor function and plays a role in sensory perception.

Centromere: The central part of a chromosome where the two "arms" of the chromosome are attached to each other.

Competitive inhibitor: Any molecule that can bind to a receptor or an enzyme and prevent binding of the molecule that normally interacts with the receptor or enzyme, thereby inhibiting normal receptor or enzyme function.

Electron transport chain: An electron transport system located in the *mitochondria*, in which electrons released by *NADH* are passed on to a series of other molecules that first accept the electrons and then pass them on to the next molecule in the chain. The electrons ultimately are transferred to oxygen to generate water. These successive reactions provide enough energy to drive the synthesis of ATP molecules.

Endoparasitic sequences: DNA sequences that are repeated multiple times in the genome; a type of *transposable element*.

Endoplasmic reticulum: A system of folded membranes in the cell that loop back and forth, spreading throughout the cytoplasm and providing a large surface area for cell reactions.

Epigenome: Entirety of all epigenetic changes in a cell, tissue, or organism.

Folate: Vitamin B9; is needed by the body to synthesize, repair, and methylate DNA; alcohol consumption can lead to folate deficiency.

Folate cycle: A series of biochemical reactions in which one-carbon units (e.g., methyl groups) are transferred from folate or, more specifically, its derivative tetrahydrofolate, to other molecules.

Genotype: The complete genetic makeup of an individual organism that is determined by the specific variants (i.e., alleles) of each gene carried by the individual. Differences in alleles among individuals account for the differences in *phenotype* observed among those individuals.

Glial cells: Cells that provide support and protection for neurons.

Glutathione (GSH): An antioxidant molecule found naturally in the body, composed of three amino acids (i.e., glutamate, cysteine, and glycine).

Heterozygous: Carrying two different variants (i.e., alleles) of a given gene.

Hippocampus: Brain region shaped like a curved ridge found within the cerebral hemisphere that functions in consolidation of new memories; also thought to play a role in alcohol withdrawal seizures.

Homeostasis: The maintenance of a stable internal state or condition (e.g., body temperature or blood pressure) in a living organism.

Homozygous: Carrying two copies of the same variant (i.e., allele) of a given gene.

Hypoglycemia: Lower-than-normal blood sugar levels.

Epigenetics Glossary *continued*

Hypomethylation: Lower-than-normal levels of epigenetic methylation of the DNA.

Imprinting: A genetic phenomenon by which certain genes are marked by biochemical modifications after conception so that only the gene copy inherited from one of the parents is expressed whereas the imprinted gene copy is silenced; for example, for certain genes only the copy inherited from the father will be expressed.

Microglia: Type of *glial cell* that acts as the first and main form of active immune defense in the central nervous system.

Microsatellite: Highly variable DNA regions found every few thousand nucleotides in the DNA that can be used to determine from which parent or ancestor a specific DNA sequence has been inherited. Microsatellites typically consist of short sequences of 1 to 6 nucleotides that can be repeated 10 to 100 times. Each person or animal has a specific pattern of microsatellites that can be used to determine inheritance patterns.

Mitochondria: Structures within cells that generate most of the cells' energy through the production of adenosine triphosphate (ATP), a molecule that provides the energy needed for many key metabolic reactions.

Messenger RNA (mRNA): Key intermediary molecule generated when a gene is expressed (i.e., when the information encoded in the gene is converted into a protein product by the cell); mRNA levels for a gene are used as an indicator of how "active" the gene is (i.e., how much of the protein is produced).

Myelin: A white fatty material composed chiefly of alternating layers of lipids and lipoproteins that encloses the long extensions (i.e., axons) of myelinated nerve fibers.

Nicotinamide adenine dinucleotide (NAD): NAD is a molecule that binds with hydrogen atoms and becomes reduced NAD (NADH), during alcohol metabolism and other chemical reactions in the cell. NAD and NADH move hydrogen atoms back and forth with other molecules in the cell, thus helping to maintain balance between oxidation and reduction in the cell.

NADH/NAD$^+$ ratio: The ratio of reduced *NAD* to oxidized *NAD*; changes in this ratio can promote or interfere with certain biochemical reactions that require either NAD$^+$ or NADH as cofactors.

Neoplastic transformation: Process by which normal cells are transformed into malignant tumor cells.

Non-coding RNA (ncRNA): RNA molecules that are not used as a template to produce proteins.

Oncogene: A gene that has the potential to cause cancer, especially if it becomes mutated or expressed at high levels.

One-carbon metabolism: Network of biochemical reactions in which a chemical unit containing one carbon atom (e.g., a methyl group) is transferred through several steps from a donor to another compound, such as DNA.

Orthologue: Genes in different species that evolved from a common ancestral gene by speciation. Normally, orthologue genes retain the same function throughout the course of evolution.

Oxidative stress: An imbalance between oxidants (e.g., *reactive oxygen species [ROS]*) and antioxidants (e.g., *glutathione [GSH]*) that can lead to excessive oxidation and cell damage.

Phenotype: The observable structural or functional characteristics of an individual organism that result from the interaction of its genetic makeup (i.e., *genotype*) with environmental factors.

Polymorphism: The presence of two or more variants (i.e., alleles) of a gene or other DNA sequence in a population.

Epigenetics Glossary *continued*

Promoter: A DNA segment located at the start of a gene's coding sequence that provides a binding site for the enzymes that initiate the first step in the process of gene expression (i.e., *transcription*).

Quantitative trait locus (QTL): A DNA region that is associated with a quantitative trait—a *phenotype* that varies in the degree to which it is present (e.g., sensitivity to alcohol or height) and which typically is determined by more than one gene—and which may contain one of the genes contributing to that trait.

Reactive oxygen species (ROS): Highly reactive oxygen–containing free radicals that are generated during oxidative metabolism. ROS can react with and damage lipids, proteins, and DNA in cells, causing *oxidative stress.* Common ROS include hydrogen peroxide, superoxide radicals, and hydroxyl radicals.

Recombinant inbred strains: Sets of animal strains that all are derived from the same two parental inbred strains and which each carry a specific combination of the parental genes; within each RI strain all animals are genetically identical.

Redox/Redox state: Shorthand for reduction/oxidation reactions. The term redox state is often used to describe the balance of *NAD* and NADH in a biological system such as a cell or organ. An abnormal redox state can develop in a variety of deleterious situations.

Retrotransposon: DNA segment that can duplicate itself and thus multiply in the genome; during this process, the original DNA sequence first copies itself into RNA and then back into DNA, which is then incorporated back into the genome; retrotransposons make up a substantial portion of the genome.

RNA splicing: The removal of noncoding sequences (i.e., introns) from the sequence of an *mRNA* following *transcription* to form an uninterrupted coding sequence.

S-adenosylmethionine (SAM): Compound that serves as the principal donor of methyl groups for methylation reactions.

Somatic mutation: Alterations of the DNA that occur after conception in any of the cells of the body except the germ cells and which therefore cannot be passed on to offspring.

Substrate: A molecule that is acted upon by an enzyme.

Teratogenesis: The development of malformations or defects in a developing embryo or fetus.

Transcription: Biochemical process in which an intermediary molecule called *messenger RNA* is generated based on the genetic information of the DNA.

Transcription factor: Protein regulating the *transcription* of a gene; consists of at least two functional domains: a DNA-binding domain and an activating domain.

Transposable elements: A DNA segment that can change its position within the genome; *retrotransposons* are a type of transposable element.

Tricarboxylic acid (TCA) cycle: A series of biochemical reactions that serve to generate energy from the metabolism of acetyl-CoA, which in turn is derived from the metabolism of sugars, fats, and proteins; also called citric acid cycle.

Epigenetic Targets for Reversing Immune Defects Caused by Alcohol Exposure

Brenda J. Curtis, Ph.D.; Anita Zahs, Ph.D.; and Elizabeth J. Kovacs, Ph.D.

Brenda J. Curtis, Ph.D., *is a postdoctoral fellow,* **Anita Zahs, Ph.D.,** *is a postdoctoral fellow, and* **Elizabeth J. Kovacs, Ph.D.,** *is a professor and vice chair of research in the Department of Surgery, director of research in the Burn & Shock Trauma Institute, and director of the Alcohol Research Program at Loyola University, Chicago, Illinois.*

Alcohol consumption alters factors that modify gene expression without changing the DNA code (i.e., epigenetic modulators) in many organ systems, including the immune system. Alcohol enhances the risk for developing several serious medical conditions related to immune system dysfunction, including acute respiratory distress syndrome (ARDS), liver cancer, and alcoholic liver disease (ALD). Binge and chronic drinking also render patients more susceptible to many infectious pathogens and advance the progression of HIV infection by weakening both innate and adaptive immunity. Epigenetic mechanisms play a pivotal role in these processes. For example, alcohol-induced epigenetic variations alter the developmental pathways of several types of immune cells (e.g., granulocytes, macrophages, and T-lymphocytes) and through these and other mechanisms promote exaggerated inflammatory responses. In addition, epigenetic mechanisms may underlie alcohol's ability to interfere with the barrier functions of the gut and respiratory systems, which also contribute to the heightened risk of infections. Better understanding of alcohol's effects on these epigenetic processes may help researchers identify new targets for the development of novel medications to prevent or ameliorate alcohol's detrimental effects on the immune system. **KEY WORDS: Alcohol consumption; alcohol exposure; alcoholism; chronic drinking; binge drinking; epigenetics; epigenetic mechanisms; epigenetic targets; DNA code; immune system; immune cells; innate immunity; adaptive immunity; infections; inflammation; gut; respiratory system; acute respiratory syndrome (ARDS); liver cancer; alcoholic liver disease (ALD)**

Extensive clinical and experimental data suggest that alcohol consumption has dose-dependent modulatory effects on the immune system that influence the two arms of the immune response (i.e., innate and adaptive immune responses). In many other organ systems, such as the brain and liver, alcohol consumption has been shown to alter factors that can modify gene expression without changing the DNA code (i.e., epigenetic modulators) and which play critical roles in mediating alcohol's effects. However, very few studies have focused on the effects of alcohol-mediated epigenetic alterations on immunity. Because chronic alcohol consumption is correlated with an exacerbated state of chronic inflammation (which is part of the innate immune response), researchers can apply knowledge of how epigenetic factors are dysregulated in inflammatory and autoimmune disorders to identify potential epigenetic targets that can be used to develop therapies for treating alcohol-abusing patients. This review summarizes how inflammatory mediators and both innate and adaptive immune responses are modulated by moderate, binge, and chronic alcohol consumption. The discussion further identifies and highlights exciting potential avenues to explore epigenetic regulation of these immune responses.

Epigenetics: An Overview

All cells within an organism carry identical genetic information in the form of DNA, yet a multitude of individual cell types arises during the course of development. These individualized cellular morphologies, characteristics, and functions result from the unique gene expression profiles of the different cell types. Regulation of gene expression profiles is critical not only during development, but also for cellular

proliferation, differentiation, environmental adaptation, stress, and immune responses throughout the individual's lifetime and is largely dependent on epigenetic mechanisms. An epigenetic trait is a stably heritable observable characteristic (i.e., phenotype) that results from changes in a chromosome without alterations in the DNA sequence (Berger et al. 2009). Epigenetic regulation can involve a variety of chemical modifications of the DNA (e.g., methylation) and the histone proteins around which the DNA is wrapped (e.g., methylation, acetylation, phosphorylation, ubiquitinylation, ADP-ribosylation, and sumoylation), as well as the actions of small molecules called noncoding microRNAs (miRNAs). Of these mechanisms, higher-than-normal DNA methylation (i.e., hypermethylation) and miRNAs generally are correlated with decreased protein production through gene-silencing mechanisms and posttranscriptional regulation (Carthew and Sontheimer 2009). Age, environment, and exposure to drugs and other toxins (e.g., alcohol) can directly influence the epigenetic profile of the organism (Feil and Fraga 2012).

It is well documented that alcohol exposure prior to an injury or infection dampens the immune system, resulting in a range of adverse outcomes, such as delaying infection clearance, extending hospital stays, and increasing morbidity and mortality compared with nonintoxicated patients (for a review, see Messingham et al. 2002). This has led to the development of the "two-hit hypothesis," where the first hit (i.e., alcohol exposure) exaggerates the organism's physiological responses to the second hit (i.e., injury or infection). Epigenetic memory may be a contributing factor in this process.

So how does the epigenetic memory work? Throughout evolution, eukaryotic cells have adapted so that a vast amount of genetic material has become organized and compacted into the nucleus by forming a higher-order structure known as chromatin. The basic building block of chromatin is the nucleosome, which comprises 147 base pairs of DNA wrapped around a core of eight small histone proteins. Nucleosomes undergo dynamic relaxation and condensation in the nucleus, a process requiring the activities of two sets of molecules:

- ATP-dependent chromatin remodeling complexes that physically tighten or loosen histone–DNA contacts; and
- Epigenetic modifying enzymes that add or remove posttranslational covalent modifications from the tails of the histone proteins, thus either allowing or preventing access of nuclear factors to the DNA that are needed for gene transcription.

Also known as the histone code, the intricate combination of covalent modifications on the histones directly influences DNA–histone binding by altering electrical charge and providing a specific docking signal for recruitment of chromatin-modifying complexes and transcriptional machinery to either block or promote active gene transcription (Jenuwein and Allis 2001; Strahl and Allis 2000). Some covalent modifications are typically associated with the same effect on transcription; for example, histone acetylation generally is associated with active gene transcription (Turner 2000). The effects of histone methylation are much more complex. Thus, the degree of methylation (i.e., mono-, di-, or trimethylation); the particular histone protein, and, more specifically, lysine residue(s) being modified (e.g., H3K4,[1] H3K9, H3K27, H3K36, H3K79, or H4K20); and the degree of chromatin condensation (i.e., condensed heterochromatin versus relaxed euchromatin) all play a role. Likewise, the part of the gene where the DNA or histone modification occurs (i.e., the genomic location)—that is, whether it occurs in a promoter, enhancer, or the gene body—influences whether a gene will be actively transcribed after lysine methylation (Bannister and Kouzarides 2005; Heintzman et al. 2007; Martin and Zhang 2005).

Specific enzymes are responsible for adding or removing acetyl or methyl moieties from histone tails. Histone acetyl transferases (HATs) and histone deacetylases (HDACs) add and remove acetyl groups, respectively. Similarly, methylation is tightly regulated by enzymes that add methyl groups to (i.e., methyltransferases) or remove methyl groups from (i.e., demethylases) specific lysine residues (Shilatifard 2006). So far, 18 HDACs have been identified and subdivided into four classes. Classes I, II, and IV require Zn^{2+} for enzymatic activity, whereas class III HDACs, also known as sirtuins, utilize a mechanism that requires the cofactor nicotinamide adenine dinucleotide (NAD^+) (Shakespear et al. 2011).

Several approaches may potentially be used to prevent or correct the epigenetic effects of alcohol consumption, such as alcohol-mediated immune defects. For example, inhibition of HDACs by molecular HDAC inhibitors (HDACis), alteration of DNA methylation on cytosine residues, or miRNA modulation all represent branches of possible therapeutic targets for restoring immune defects caused by alcohol exposure. These approaches will be discussed later in this review.

Epigenetics and Alcohol

Beverage alcohol (i.e., ethanol) is predominantly metabolized by the enzymes alcohol dehydrogenase (ADH), cytochrome p450 (CYP 450), and aldehyde dehydrogenase (ALDH) in the liver (Dey and Cederbaum 2006). This process produces oxidative metabolites such as acetaldehyde, acetate, acetyl-CoA, and reactive oxygen species (ROS), as well as nonoxidative products, such as phosphatidylethanol (PEth) and fatty acid ethyl ester (FAEE) (Best and Laposata 2003; Shukla and Aroor 2006; Shukla et al. 2001). Many of these products or metabolites can

[1] The standard nomenclature for histone modifications is to indicate both the histone protein (e.g., histone 3) and the specific amino acid affected. For example, in H3K4, the fourth lysine (abbreviated as K) of histone 3 is affected by epigenetic modification.

induce tissue-specific epigenetic changes (Choudhury and Shukla 2008; Shukla and Aroor 2006). Ethanol exposure leads to epigenetic alterations through several mechanisms, including enhancing the enzymatic activity of HATs; altering substrate availability for histone acetylation, DNA, and histone methylation; or by influencing miRNA production. For example, studies found the following:

- Ethanol exposure enhances the activity of a HAT called p300 in the liver of rats fed a chronic ethanol diet, which leads to heightened histone acetylation (Bardag-Gorce et al. 2007).

- Elevated ROS levels resulting from ethanol metabolism increase histone H3 acetylation in liver cells (i.e., hepatocytes) (Choudhury et al. 2010).

- Chronic alcohol exposure can mediate a shift in the ratio of reduced NAD^+ (NADH) to NAD^+, and this reduced redox state suppresses the activity of the redox-sensitive HDAC, SIRT1, thus augmenting histone acetylation in rats (Bardag-Gorce and French 2007; You et al. 2008).

- Ethanol metabolism dramatically increases production of acetyl-CoA, which is used in histone acetylation by HATs; consequently, ethanol exposure and metabolism amplifies the amount of substrate available for histone acetylation (Yamashita et al. 2001).

- Ethanol exposure causes dysregulated methionine metabolism, resulting in diminished production of a molecule called S-adenosylmethionine (SAMe), which serves as a methyl-group donor for both DNA and histone methylation (Lu and Mato 2005; Mason and Choi 2005; Shukla and Aroor 2006).

- Chronic ethanol exposure decreases the levels of the antioxidant glutathione, which serves as the predominant scavenger of ROS in the liver (Choudhury and Park 2010; Lu et al. 2000); this glutathione reduction leads to both regionally and globally reduced DNA methylation (i.e., hypomethylation) (Lee et al. 2004; Lertratanangkoon et al. 1997).

- Chronic ethanol exposure in rats leads to inhibition of a set of reactions called the ubiquitin–proteasome pathway, which helps eliminate molecules that are defective or no longer needed from the cell. This inhibition of the ubiquitin–proteasome pathway likely alters protein turnover of transcription factors and histone-modifying enzymes and is associated with epigenetic alteration at a specific lysine residue (K9) of histone H3 (i.e. increased H3K9-ac and reduced H3K9-me2) as well as DNA hypomethylation (Oliva et al. 2009).

- Acetylation of H3K9 also is associated with increased ADH1 expression in cultured rat hepatocytes treated with 100 mM ethanol for 24 hours, suggesting that ethanol (and its metabolites) may amplify ethanol metabolism (Park et al. 2005).

Through the various mechanisms discussed above, alcohol consumption can lead to multifactorial, dose-dependent, and tissue-specific epigenetic effects. For example, cultured primary rat hepatocytes demonstrated a dose- and time-dependent histone-acetylation response to ethanol exposure. Thus, cells treated with 5–100 mM ethanol for 24 hours exhibited a maximal, eightfold increase in H3K9-ac levels at 24 hours following treatment with the highest ethanol concentration (Park et al. 2003). Furthermore, histone acetylation seemed to be selective for the H3K9 residue, because acetylation of other H3 lysines (i.e., K14, K18, and K23) was unaffected by ethanol exposure (Park and Lim 2005; Park and Miller 2003). Similar findings were obtained in hepatic stellate cells (Kim and Shukla 2005). Finally, another study (Pal-Bhadra et al. 2007) found that hepatocytes treated for 24 hours with 50 mM and 100 mM ethanol also exhibited altered histone methylation status, resulting in increased H3K4 dimethylation (H3K4-me2) and decreased H3K9 dimethylation (H3K9-me2). However, unlike histone lysine acetylation, which was restored to baseline levels 24 hours after ethanol withdrawal in cultured hepatocytes, changes in histone lysine methylation status were not reversed and may provide a long-term epigenetic memory (Pal-Bhadra et al. 2007).

Ethanol metabolites, including acetaldehyde and acetate, also could cause H3K9-specific acetylation in rat hepatocytes. Interestingly, the signaling pathways activated by acetate and ethanol seemed to modulate H3K9-ac via different mechanisms. Thus, certain molecules (i.e., inhibitors of enzymes known as mitogen-activated protein kinases) prevented acetylation by ethanol but had no effect on the acetate-dependent formation of H3K9-ac (Park and Lim 2005). In addition to acetylation, ethanol and acetaldehyde exposure also promotes phosphorylation of histone H3 at serines 10 and 28 (Lee and Shukla 2007). Whereas ethanol exposure lead to similar phosphorylation levels at both serine 10 and serine 28, acetaldehyde generated greater phosphorylation at serine 28 than at serine 10 (Lee and Shukla 2007). These studies indicate that the complexity of ethanol-induced epigenetic changes increases even further when taking into account that ethanol metabolites also trigger epigenetic effects that may differ from those produced by ethanol exposure.

Rat models of acute/binge and chronic alcohol exposure have been utilized to examine the relationship between epigenetic gene regulation and alcohol exposure in vivo. In one of those models, a single dose of ethanol diluted in sterile water resulting in a concentration of 6 grams ethanol per kilogram bodyweight

(g/kg) was injected directly into the stomachs of 8-week-old male Sprague-Dawley rats. This high-dose binge-alcohol exposure model was used to compare H3K9 modification status across 11 different tissues at 1, 3, and 12 hours following ethanol exposure (Kim and Shukla 2006). The investigators found that in the testes, this alcohol exposure caused robust global increases in H3K9-ac at 1 hour but not at later time points. In contrast, in the lung and spleen robust increases in H3K9-ac were apparent at all three time points. In the liver, no marked elevation in H3K9-ac was observed at early (i.e., 1- or 3-hour) time points, but a profound elevation occurred at 12 hours. In addition, in the blood vessels, pancreas, colorectum, stomach, heart, brain, and kidney, no change in H3K9-ac was observed at any time-point tested. Finally, methylation of H3K9 was not altered in any tissue (Kim and Shukla 2006).

Other investigators evaluated changes in gene expression levels after chronic ethanol treatment using in vivo models. One of these models is the Tsukamoto-French rat model of alcoholic liver disease (Tsukamoto et al. 1985), in which male Wistar rats were fed a liquid diet containing a constant amount of alcohol (13 g/kg/day) for 30 days using an intragastric feeding tube. This treatment, which resulted in a 6- to 10-day cyclic pattern of urinary alcohol level (UAL) peaks (about 500 mg%) and troughs (about 100 mg%) (Bardag-Gorce and French 2002), allowed the investigators to compare gene expression profiles at high and low blood alcohol levels (BALs) by microarray analyses. These analyses identified dramatic changes in gene expression levels in the livers of the alcohol-treated rats. Overall, approximately 1,300 genes were dysregulated between BAL cycles (French et al. 2005), prompting additional studies aimed at elucidating the epigenetic contribution of alcohol-mediated transcriptional dysregulation in the liver and other tissues (Bardag-Gorce and French 2007; Kim and Shukla 2006; Park and Lim 2005). Furthermore, UAL peaks were associated with increased levels of the HAT, p300, which specifically transfers acetyl groups to H3K9 residues. This finding at least partially explains the selective H3K9 acetylation observed both in vitro and in vivo in correlation with ethanol exposure (Bardag-Gorce and French 2007). Finally, studies assessing the effects of changes in epigenetic mechanisms resulting from inhibition of the ubiquitin–proteasome pathway (using a drug called PS-341) or from chronic ethanol exposure in rats using the Tsukamoto-French model found increases in H3K9-ac levels, decreases in H3K9-me2 levels, and increased p300 levels in liver nuclear extracts (Oliva and Dedes 2009). These findings suggest that chronic ethanol exposure alters transcriptional regulation of a plethora of genes through many mechanisms that affect epigenetic modulators.

Understanding the role of nutrients in regulating epigenetic modifications will provide insight into potential dietary supplementation in chronic alcohol-abusing patients.

In summary, both acute/binge and chronic alcohol exposure can result in tissue- and cell-specific patterns of epigenetic responses. Future studies to determine the precise role of alcohol-mediated chromatin modifications hopefully will identify new epigenetic targets and pathophysiological mechanisms for regulating gene expression in diseases associated with alcohol consumption. The factors contributing to altered epigenetic modifications arising from acute versus chronic alcohol exposure may differ, because chronic alcohol exposure has been strongly correlated with nutrient deficiencies and a shift in the redox state. This implies that potential therapeutic interventions targeting epigenetic modifiers may need to differ depending on the degree of alcohol consumption. Furthermore, understanding the role of nutrients in regulating epigenetic modifications will provide insight into potential dietary supplementation in chronic alcohol-abusing patients.

Alcohol and the Immune System

A recent report from the Centers for Disease Control and Prevention (CDC) stated that alcohol abuse in the form of binge drinking (defined by the CDC as four or more drinks for women and five or more drinks for men in a short period of time) is the third-leading preventable cause of death in the United States, resulting in more than 80,000 deaths each year and enormous economic costs (i.e., more than $220 billion in 2006) (CDC 2012). A significant, positive correlation exists between the duration and amount of alcohol consumed and the risk for developing several serious medical conditions, including acute respiratory distress syndrome (ARDS) (Boe et al. 2009; Moss et al. 1999); liver cancer (i.e., hepatocellular carcinoma) (McKillop and Schrum 2009; Yamauchi et al. 1993); and alcoholic liver disease (ALD), which encompasses cirrhosis, hepatitis, and fibrosis (Gramenzi et al. 2006; Mann et al. 2003). Binge and chronic consumption (defined as more than eight drinks per day) renders patients more susceptible to various types of infection, such as hepatitis C virus infection in the liver and opportunistic infections in the respiratory system (e.g., ARDS and pneumonia), and advances the progression of HIV infection, largely through dysregulated immune responses (Baliunas et al. 2010; Bhatty et al. 2011; Prakash et al. 2002; Romeo et al. 2007*b*; Zhang et al. 2008) (figure 1).

The mammalian immune system is an elaborate network of molecules and cells that identify, combat, and eliminate harmful agents; it can be divided into two branches: innate and adaptive immunity. The innate immunity is

present from birth, whereas the adaptive immunity develops over the organism's life course with the continuous exposure to pathogens and other potentially harmful compounds.

The Innate Immune Response

Following pathogen or toxin exposure, the ancient innate immune response is responsible for immediate recognition, rapid attack, and destruction of foreign intruders and involves inflammatory reactions. Innate immune cells carry special molecules called Toll-like receptors (TLRs) on their surface that recognize and bind highly conserved structures on bacterial, fungal, or viral surfaces, including peptidoglycan, flagellin, zymosan, and lipopolysaccharide (LPS, also known as endotoxin) (Janeway and Medzhitov 2002). The innate-immune cells also activate the adaptive immune response by digesting the foreign intruders and then presenting certain molecules derived from these pathogens (i.e., antigens) on their surface for recognition by adaptive immune cells. This antigen presentation, which initiates the adaptive immune response and provides a "memory" of the initial recognition of the antigen, allows for a rapid immune response if the same infection occurs again in the future.

An important subset of innate immune cells are macrophages; they eliminate pathogens by a process called phagocytosis[2] and then present pathogen-derived molecules on their surface to activate

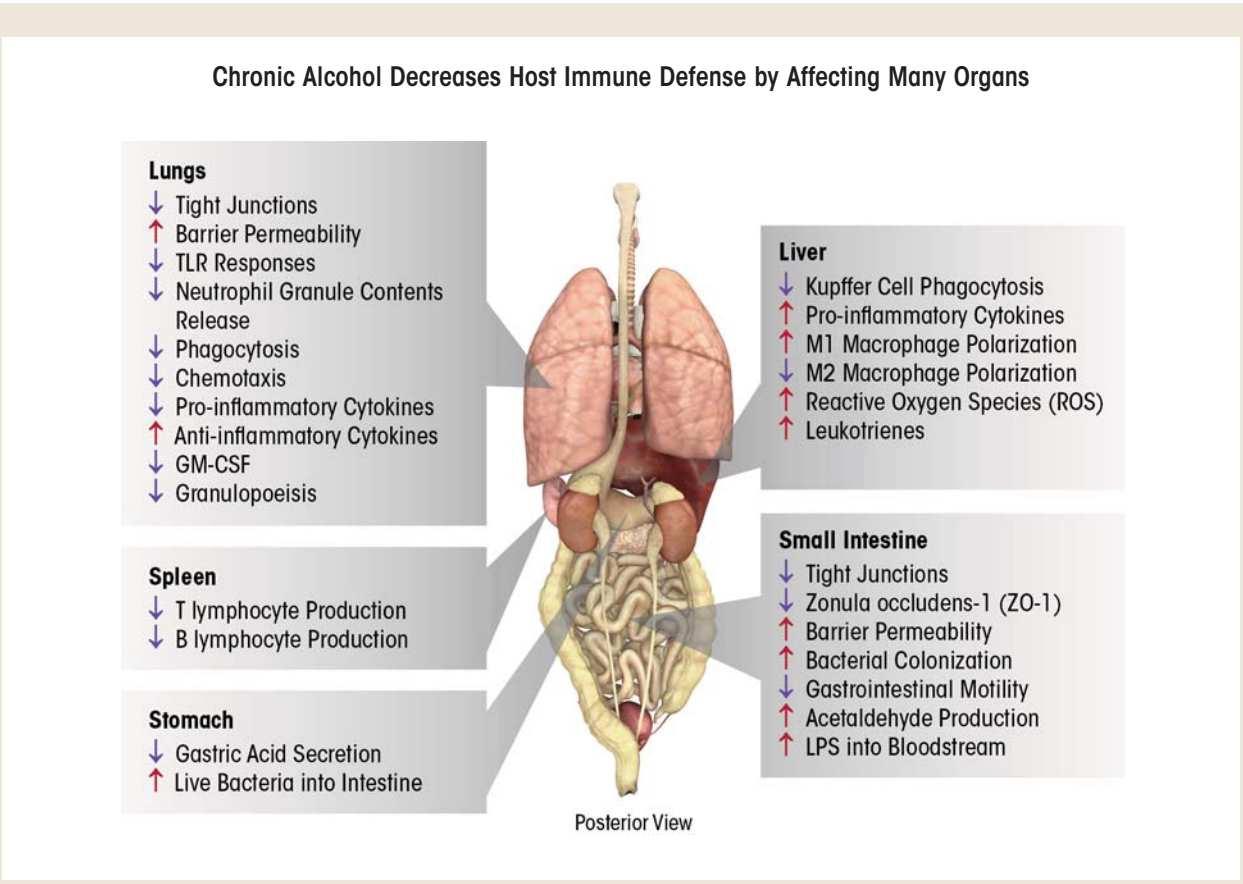


Figure 1 Chronic alcohol exposure causes immune dysfunction through effects on multiple organs. In the lungs, excessive inflammation causes tissue damage, increasing barrier permeability, and dampening many cellular immune responses, such as recognizing bacteria (through toll-like receptors [TLRs]), attacking pathogens (through phagocytosis), decreasing production of granulocytes (i.e., granulocytopenia) as well as their migration (i.e., chemotaxis), and altering important signaling and recruiting molecules (e.g., GM-CSF and chemokines). In the spleen, alcohol consumption affects immunity by decreasing T- and B-lymphocyte production. In the stomach, alcohol decreases gastric acid levels, allowing live bacteria to pass into the small intestine. Combined with decreased gastrointestinal motility, a byproduct of alcohol metabolism (i.e., acetaldehyde) increases intestinal barrier permeability by weakening cell–cell junctions, and allows bacterial toxins (i.e., lipopolysaccharide [LPS]) to pass into the bloodstream. LPS damages the liver, leading to excessive release of pro-inflammatory cytokines, leukotrienes, and ROS into the circulation. In addition, alcohol in the liver can alter macrophage (Kupffer cell) polarization and decrease phagocytosis.

adaptive immune cells. Macrophages can have alternate names based on their anatomical location; for example, macrophages residing in the liver are called Kupffer cells. Furthermore, macrophages can be subdivided into two groups based on their functional phenotype (Martinez et al. 2008) (see table 1):

- Classically activated (M1) macrophages, whose activation results in a proinflammatory response.
- Alternatively activated (M2) macrophages, whose activation results in an anti-inflammatory response.

After challenge to the immune system occurs (e.g., an infection), macrophages are generated by the maturation of precursor cells called monocytes. During this process, the macrophages can become either M1 or M2 macrophages; this is called macrophage polarization. The ratio of M1 to M2 macrophages changes depending on the presence of a variety of factors; this variability is known as macrophage plasticity and allows the organism to modulate the immune response. Accordingly, controlling macrophage plasticity is critical to first battle pathogens and then resolve the resulting inflammation to prevent tissue damage. Alcohol exposure skews macrophage polarization towards M1 (i.e., towards inflammation) in the liver (Louvet et al. 2011; Mandal et al. 2011), resulting in deleterious consequences (figure 2).

Dendritic cells (DCs) are an additional component of the innate immune response. They have an important role in linking the innate and adaptive branches of the immune system. To this end, the DCs exhibit proteins called major histocompatibility complex (MHCs) on their surface. With the MHC proteins, DCs present antigens to other cells that are part of the adaptive immune system—that is, B and T lymphocytes (also known as B and T-cells). DCs mature following stimulation by whole bacteria or LPS or after exposure to various signaling molecules, such as interleukin 1β (IL-1β), granulocyte macrophage colony–stimulating factor (GM-CSF), and tumor necrosis factor-α (TNFα) (Winzler et al. 1997). The mature DCs migrate to lymphoid organs to prime and activate naïve T-cells (Lee and Iwasaki 2007). Activated T-cells then complete the immune response by producing and releasing specific signaling molecules (i.e., cytokines) that will stimulate other innate immune cells or interact with B-cells, leading to the development of immune molecules (i.e., antibodies). Mature DCs also secrete high levels of IL-12 (Reis e Sousa et al. 1997), enhancing both innate and adaptive immune responses (summarized in table 2).

Alcohol consumption has a variety of effects on innate immune cells. For example, alcohol decreases the phagocytic activity of monocytes, macrophages, Kupffer cells, microglia, and DCs and diminishes their capacity to present antigens and produce the molecules necessary for microbe killing. In addi-

[2] During phagocytosis, the macrophage engulfs the foreign pathogen, thus ingesting it into the cell, where it is degraded and eliminated.

Table 1 Macrophages, Alcohol, and Potential Epigenetic Targets

	Subtype	Factors Contributing to Activation[1]	Major Roles Following Activation[1]	Defects Caused by Chronic Alcohol [2]	Potential Epigenetic Targets
Macrophages	M1 (Classical)	IFNγ Microbes	Engulf necrotic cells, toxic substances, and pathogens ↑ pro-inflammatory cytokines and reactive oxygen species (ROS) for direct pathogen killing and recruitment of other immune cells	Leads to predominant M1 polarization[3] Kupffer cells sensitized to endotoxin stimulation[3,4] ↑ Pro-inflammatory cytokines ↓ Phagocytic activity[5] ↓ Capacity to present antigen[6]	miR-155 promotes M2 polarization[7] Histone lysine demethylase, JmjD3, promotes transcription of M2-specific genes[8,9]
	M2 (Alternative)	Parasites Cytokines released by Th2, NK, basophils	↑anti-inflammatory cytokines Promote angiogenesis Promote wound healing	Macrophage polarization skewed towards M1 phenotype[3]	

SOURCES: [1] Gordon and Taylor, 2005 , [2] Goral et al., 2008 , [3] Thakur et al., 2007, [4] Mandrekar and Szabo, 2009, [5] Karavitis and Kovacs, 2011, [6] Szabo et al., 1993, [7] Ruggiero et al., 2009, [8] De Santa et al., 2007, [9] Satoh et al., 2010.

tion, alcohol alters expression of other proteins (i.e., pathogen pattern recognition receptors) on their cell surface that are required for cell–cell interactions among immune cells (for reviews, see (Goral et al. 2008; Karavitis and Kovacs 2011; Romeo and Warnberg 2007*b*). Furthermore, the levels of a type of immune cell called granulocytes often are very low in alcoholics with severe bacterial infections, which has been strongly correlated with increased mortality (Perlino and Rimland 1985). Finally, rodent models have demonstrated that following infection, alcohol significantly decreased both phagocytic activity and production of the signaling molecule granulocyte colony-stimulating factor (G-CSF) in a TNFα-dependent manner (Bagby et al. 1998) as well as blocked differentiation or maturation of granulocytes (i.e., granulopoiesis) (Zhang et al. 2009).

The Adaptive Immune Response

B-cells, T-cells, and antigen-presenting cells (APCs) are key players of the adaptive immune response. Like DCs, APCs present antigen to B and T-cells that have not yet been activated (i.e., naïve B and T-cells), contributing to their maturation and differentiation. Naïve T-cells are classified based on expression of specific proteins on their surface called cluster of differentiation (CD) proteins. Two of those proteins important in distinguishing different T-cell populations are CD4 and CD8. T-cells carrying the CD8 protein (i.e., $CD8^+$ cells) ultimately gain the ability to recognize and kill pathogens (i.e., become cytolytic T-cells). Conversely, $CD4^+$ T-cells give rise to several T helper (Th) cell subsets, including Th1, Th2, and Th17 cells, that will produce mutually exclusive groups of cytokines which help mount specific immune responses by stimulating other immune cells (Zygmunt and Veldhoen 2011) (table 3). Alcohol exposure can promote the development of Th2 cells over the other helper-cell populations. This shift in T helper differentiation towards Th2 is correlated strongly with defective immune responses as well as increased rates of infection, morbidity, and mortality (Cook et al. 2004; Romeo and Warnberg 2007*b*).

Epigenetic Modulation May Reverse M1 Polarization Caused by Chronic Alcohol Consumption

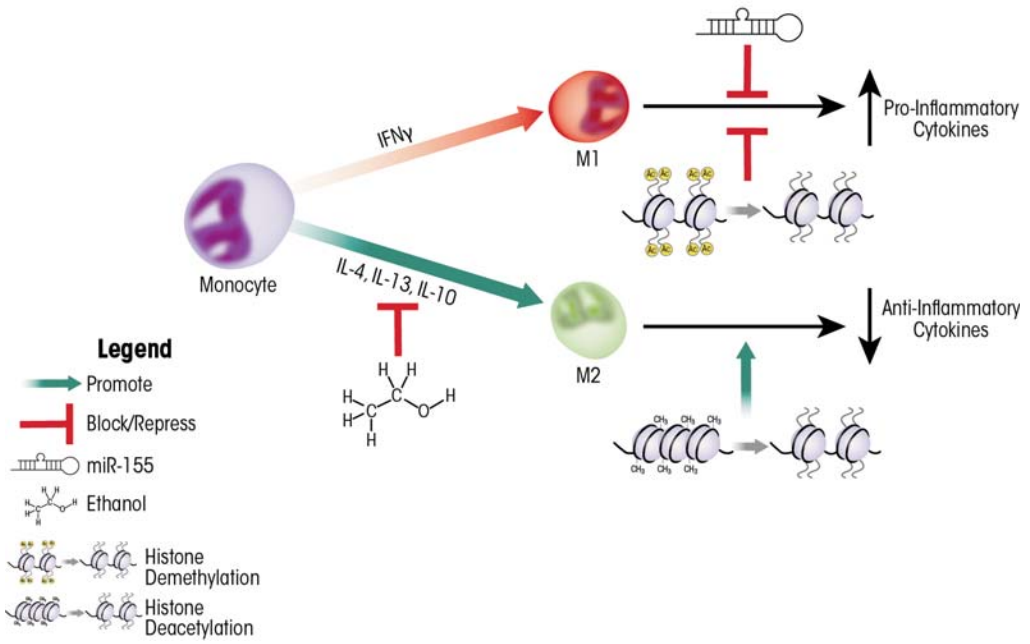


Figure 2 Chronic alcohol consumption skews macrophage polarization toward an M1 (i.e., pro-inflammatory) phenotype, leading to excessive or prolonged inflammation. Two approaches using epigenetic modulators—microRNA 155 (miR-155) and histone deacetylase inhibitors—can potentionally reverse protein translation or gene transcription of M1 pro-inflammatory cytokines. Another type of enzyme—histone lysine (H3K27) demethylases—increase transcription of M2 anti-inflammatory cytokines. Factors that increase protein levels or enhance activity of H3K27 demethylases therefore may potentially be utilized to promote M2 polarization.

The Effects of Alcohol Exposure on Innate Immune Cells and the Potential Role of Epigenetics

Epigenetics Play a Crucial Role in Innate Immune-Cell Differentiation and Maturation

During the early stages of blood cell formation (i.e., hematopoiesis), the developing cells fall into one of two developmental paths: the myeloid lineage, which includes granulocytes and monocytes (which then further differentiate into macrophages or DCs), and the lymphoid lineage, which includes B- and T-lymphocytes. This myeloid versus lymphoid lineage commitment corresponds with global and reduced DNA methylation, respectively (Ji et al. 2010). During infection, alcohol suppresses the development and maturation of granulocytes (i.e., granulopoiesis) (Zhang et al. 2009). Factors that increase DNA methylation, and therefore promote myeloid cell commitment, may serve as potential therapeutic targets for increasing granulocyte populations. Similarly, epigenetic factors play a crucial role in regulating monocyte terminal differentiation into DCs. Proper functioning of monocyte cells requires the expression of CD14, because it recognizes and binds LPS. DCs, however, do not utilize CD14, but instead require CD209 (DC-SIGN). Therefore, when monocytes differentiate into DCs, they lose expression of *CD14,* which is correlated with loss of epigenetic modifications associated with active transcription, including H3K9-Ac and H3K4me3. Concurrently, epigenetic changes occur within the *CD209* locus, leading to increased *CD209* transcription. The increase in *CD209* transcription is associated with loss of epigenetic modifications typically associated with transcriptional silencing, including DNA methylation and formation of H3K9me3 and H3K20me3 (Bullwinkel et al. 2011). In the future, therapeutics that specifically target epigenetic modifications within the *CD14* or *CD209* loci may be designed to direct monocyte terminal differentiation towards one particular cellular fate (Bullwinkel et al. 2011).

Epigenetic Regulation of Macrophage Polarization

Alcohol alters macrophage polarization in the liver—that is, it alters the normal ratio of M1 to M2 macrophages. Chronic alcohol exposure sensitizes Kupffer cells to LPS stimulation, leading to prolonged and predominant M1 polarization and the exacerbated release of pro-inflammatory cytokines (Mandrekar and Szabo 2009; Thakur et al. 2007). This shift in macrophage polarization is reversible, because recent studies demonstrated that a hormone produced by adipose cells (i.e., adiponectin), can shift Kupffer cells isolated from chronic alcohol-exposed rat livers towards M2 polarization (Mandal and Pratt 2011).

Another potential strategy for shifting Kupffer cell polarization is the use of therapeutic reagents that target epigenetic modifiers because epigenetic processes play central roles in the regulation of immune-system functions. For example, one critical mechanism to restore the internal balance (i.e., homeostasis) of the immune system in response to infection involves miRNA-dependent post-transcriptional regulation. Researchers found that expression of one specific miRNA called miR-155 was dramatically increased when macrophages derived from the bone marrow were stimulated by LPS. This enhanced miRNA expression served to fine-tune the expression of pro-inflammatory mediators and promote M2 polarization (Ruggiero et al. 2009). Similarly, ethanol exposure also can affect miR-155 expression. When a specific macrophage cell line (i.e., the RAW 264.7 macrophage cell line) was treated with 50 mM ethanol (corresponding to a BAL of 0.2 g/dl, which commonly is observed in chronic alcoholics), miR-155 expression was significantly enhanced (Bala et al. 2011). Ethanol treatment prior to stimulation with LPS further augmented miR-155 production, and a linear, significant correlation existed with increased TNFα production, likely because miR-155 increased TNFα mRNA stability (Bala and Marcos 2011). Finally, a murine model of ALD confirmed increased miR-155 and TNFα levels in Kupffer cells isolated from ethanol-treated animals compared with control animals, suggesting that miR-155 is an important regulator of TNFα in vivo and likely contributes to the elevated TNFα levels often observed in chronic alcoholics (Bala and Marcos 2011).

Besides ethanol-induced production of miR-155, histone modifications also can regulate macrophage polarization. As mentioned earlier, macrophages and other innate immune cells carry TLRs on their surface that can interact with LPS and other molecules, leading to the activation of the TLRs. Studies have demonstrated that when TLR4 was stimulated by LPS, histone acetylation and H3K4 tri-methylation (both of which are associated with active gene transcription) occurred in DNA regions encoding several pro-inflammatory cytokines (Foster et al. 2007; Takeuch and Akira 2011). Macrophage stimulation using the cytokine IL-4 and LPS also induced expression of an H3K27 histone lysine demethylase enzyme called Jumonji Domain Containing-3 (JmjD3/Kdm6b), causing transcription of specific M2-associated genes (De Santa et al. 2007; Satoh et al. 2010). The role of this demethylase is further supported by studies using cultured cells or mice in which specific genes were inactivated (i.e., knockout mice) that demonstrated that JmjD3/Kdm6b activity was not required for mounting antibacterial M1 responses, but was essential for M2 responses following exposure to a molecule (i.e., chitin) found in fungi and other parasites (Bowman and Free 2006; Satoh and Takeuchi 2010). Taken together, these findings suggest that epigenetic regulation of factors that specifically alter macrophage polarization may be able to shift and/or restore the normal M1/M2

physiological balance in alcohol-exposed patients (also see table 1 and figure 2).

The Effects of Alcohol Exposure on Adaptive Immunity and the Potential Role of Epigenetics

The Potential Role of Epigenetics in Reversing Th2 Polarization

Alcohol exposure impairs IL-12 production by DCs and IL-23 production by macrophages, thereby skewing T helper cell commitment towards a Th2 lineage (Happel et al. 2006; Mandrekar et al. 2004). Lysine methylation at histone H3K27 plays an important role in regulating transcription of the *IL-12* gene and thereby regulating DC activation (Wen et al. 2008). Accordingly, the development and use of drugs that target H3K27-specific histone methyltransferases or demethylases to treat diseases associated with alcoholism are a promising, future endeavor (see table 2).

T-cell production also is modulated by alcohol consumption, but at least some of the effects may be both gender- and dose-dependent. For example, moderate daily consumption of one beer by women or two beers by men for 30 days caused significantly higher abundance of $CD3^+$ T-cells in women, but not in men (Romeo et al. 2007*a*). Conversely, in male mice, chronic alcohol exposure was correlated with decreased $CD4^+$ and $CD8^+$ T-cells in the spleen and thymus (Saad and Jerrells 1991) and increased free (i.e., soluble) CD8 in the blood. This soluble CD8 can bind T-cell receptors, block activation by APCs, and thus impede viral clearance (Jerrells et al. 2002), indicating a way through which chronic alcoholism can impair the immune response. These findings indicate that drugs that can enhance cytokine production by the limited, inefficient T-cells found in alcoholics may restore the immune response. HDACis may be one such approach because histone deacetylation inhibits transcription of the gene encoding IL-4 (i.e., *Il4*) and inhibition of deacetylation accordingly could promote IL-4 production (Valapour et al. 2002). Drugs targeting DNA methylation also may be beneficial because DNA methylation plays an important role in regulating the transition of naïve T-cells to either Th1 or Th2 cell fates. Specifically, when naïve T-cells transition into Th2 cells, certain regions of the Il4 loci (specifically the 5' region) become hypomethylated. Conversely, when transitioning to Th1 cells, the 3' region of *Il4* becomes hypermethylated, demonstrating that a highly complex system of methylation/demethylation mediates T helper cell differentiation (Lee et al. 2002; Mullen et al. 2002). Treatment of T-cell lines with an agent called 5-azacytidine, which inhibits DNA methylation, leads to the production of cytokines not normally produced by these cells, including IL-2 and IFNγ (Ballas 1984; Young et al. 1994). This effect may help to restore the defective Th1 response in patients abusing alcohol (also see table 3 and figure 3).

The Effects of Chronic Alcohol and Inflammation and the Potential Role for Epigenetics

Chronic alcoholism is correlated with excessive or prolonged inflammation, caused in part through an overactive innate immune response and elevated oxidative stress (Khoruts et al. 1991). Studies have demonstrated that circulating levels of the pro-inflammatory cytokines TNFα, IL-1β, and IL-6 were much higher in alcoholics than in healthy nondrinkers (Khoruts and Stahnke 1991). The higher circulating levels of these cytokines resulted from increased production of pro-inflammatory cytokines by circulating monocytes and resident tissue macrophages, including Kupffer cells (for a review, see Cook 1998). These cells were also more sensitive to stimulation by LPS, which further exacerbated TNFα secretion and contributed to cytotoxicity (Schafer et al. 1995). The increased sensitivity to LPS stimulation partially was caused by decreased production of the anti-inflammatory cytokine, IL-10, which negatively regulates TNFα

Table 2 Dendritic Cells, Alcohol, and Potential Epigenetic Targets

Factors Contributing to Activation[1]	Major Roles Following Activation[2]	Defects Caused by Chronic Alcohol	Potential Epigenetic Targets
Whole bacteria LPS IL-1β GM-CSF, TNFα	Migrate to lymphoid organs and present antigens to naïve T and B lymphocytes ↑IL-12 to enhance innate and adaptive immunity[5]	↓ IL-12 production[3]	Histone lysine methylation (H3K27) controls transcription of the IL-12 gene[4]

SOURCES: [1] Winzler et al., 1997 , [2] Lee and Iwasaki, 2007, [3] Reis e Sousa et al, 1997, [4] Mandrekar et al., 2004, [5] Wen et al., 2008.

secretion by monocytes (Le Moine et al. 1995). Thus, chronic alcohol exposure disrupts the delicate and precise regulation of inflammatory regulators.

To assess alcohol's effects on the inflammatory responses of macrophages, researchers have used a human monoblastic cell line, MonoMac6, which has many features of mature macrophages and has been used to model Kupffer cell responses (Zhang et al. 2001). Preliminary studies demonstrated that prolonged (i.e., 7 day) exposure of these cells to high-dose (86 mM) ethanol dramatically enhanced pro-inflammatory cytokine responses following LPS stimulation and was correlated with increased histone H3 and H4 global acetylation, as well as elevated acetylation of specific cytokine gene promoters, including those encoding *IL-6* and *TNF* (Kendrick et al. 2010). This increased acetylation was dependent upon conversion of ethanol to its metabolites, acetate and acetyl-coA, by two enzymes called acetyl-coenzyme A synthetase short-chain family members 1 and 2 (ACSS1 and ACSS2) and also was associated with a significant decrease in HDAC activity (Kendrick and O'Boyle 2010). Interestingly, unlike with rat hepatocytes and hepatic stellate cells, no global modulation of histone acetylation was observed with acute ethanol treatment (Kendrick and O'Boyle 2010).

ACSS1 and ACSS2 only are activated for acetate and acetyl-CoA formation during ethanol metabolism but not during normal sugar metabolism that also results in acetyl-CoA generation. Therefore, they represent an exciting potential therapeutic target for reducing the exacerbated inflammatory response observed with chronic alcohol exposure because their depletion should not alter normal cellular metabolism and energy generation. Another potential approach to restoring cytokine homeostasis may be to reduce proinflammatory cytokine transcription by administering drugs that increase HDAC recruitment to actively transcribed chromatin (e.g., theophylline), thereby counteracting the decreased HDAC activity induced by chronic ethanol exposure (Kendrick and O'Boyle 2010).

Although drugs that modulate epigenetic targets have not yet been used specifically to treat alcohol-induced inflammation, research of other inflammatory and autoimmune diseases suggest that epigenetic modulation plays a critical role in regulating the inflammatory cytokine network (Ballestar 2011; Halili et al. 2009; Rodriguez-Cortez et al. 2011). Accordingly, agents that normalize this epigenetic modulation (e.g., HDACis) are a promising therapy for the treatment

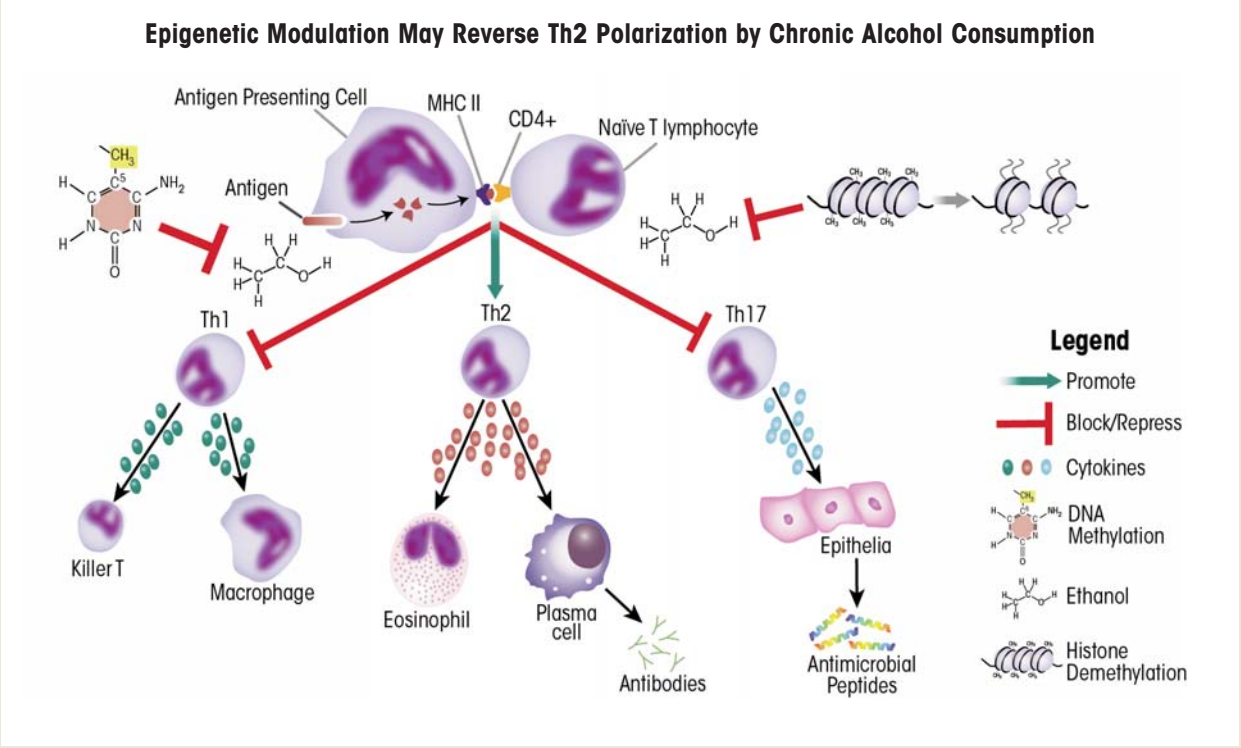


Figure 3 Alcohol-induced T helper cell polarization towards a Th2 phenotype suppresses immune responses. Alcohol decreases IL-12 production by antigen presenting cells, resulting in fewer naïve T-cell differentiating into Th1 cells, and blocks the release of IL-23 from macrophages, thereby preventing Th17 differentiation. Methylation of DNA or histones (H3K27) may reverse Th2 polarization.

of inflammatory and autoimmune diseases, including the exacerbated inflammation observed with chronic alcohol exposure. HDACis are efficacious in animal models of inflammatory bowel disease, septic shock, graft-versus-host disease, and rheumatoid arthritis (Bodar et al. 2011; Halili and Andrews 2009; Joosten et al. 2011; Reddy et al. 2004, 2008). Furthermore, the HDACi vorinostat has been used in clinical trials for reducing the severity of graft-versus-host disease in patients with bone marrow transplants (Choi and Reddy 2011), and the HDACi givinostat has been studied for the treatment of several other inflammatory conditions. These HDACis originally were developed to increase transcription of genes that induce cell death (i.e., apoptosis) of malignant cells. The doses of HDACi required to diminish inflammatory processes, however, are dramatically lower than the doses required for cancer treatment, and minimal side effects have been reported (Dinarello 2010; Vojinovic and Damjanov 2011). The importance of lysine acetylation as a regulatory mechanism has been supported by a study characterizing the entirety of all proteins that are acetylated in the human body (i.e., the human lysine acetylome). This study identified 1,750 proteins that could be acetylated on lysine side chains, including proteins involved in diverse biological processes, such as the processing of mRNAs (i.e., splicing), cell-cycle regulation, chromatin remodeling, and nuclear transport (Choudhary et al. 2009). In fact, protein acetylation may be as important as phosphorylation in governing cellular processes (Choudhary and Kumar 2009; Kouzarides 2000). For example, acetylation of proteins in the fluid filling the cell (i.e., the cytosol) can either activate or block essential signaling cascades and may partially explain how low-dose HDACi treatment decreases the production of pro-inflammatory cytokines (Dinarello et al. 2011).

It is important to note that the development of selective HDACis may be complicated by the fact that most HDACs are components of multi-protein complexes, which often include other HDACs (Downes et al. 2000; Fischle et al. 2001). Therefore, it is possible that inhibition of one HDAC inadvertently may alter the activity of other HDACs present in the complex. It also is likely that some functional redundancy exists among HDACs as well as within the biological inflammatory pathways they regulate. Moreover, the role of individual HDACs is tissue and cell-type specific; accordingly, development of specific HDACi molecules for treatment of each particular inflammatory disease will require cell- or tissue-targeting components.

Alcohol Abuse and Leaky Barriers

Another important component of the innate immune system are the epithelial cells that line the outer surfaces of exposed tissues, such as the skin, respiratory, gastrointestinal (GI), and urogenital tracts. These cells provide a physical barrier that impedes pathogen invasion by forming strong intercellular associations (Tam et al. 2011; Turner 2009). Another critical function of epithelial cells in the innate immune system is their production of cytokines and chemokines in response to pathogen

Table 3 T-Cells, Alcohol, and Potential Epigenetic Targets

T-Cells Subtype	Major Roles Following Activation by Specific Antigen-Presenting–Cell Interaction	Defects Caused by Chronic Alcohol	Potential Epigenetic Targets
$CD8^+$Cytolytic T-cells	Direct pathogen killing	↓ $CD8^+$ production in spleen and thymus[1] ↑ soluble CD8→ blocks APC activation[2]	
$CD4^+$T helper 1 (Th1)	↑ IFNγ → activates macrophages and cytolytic T-cells	↓ $CD4^+$ production in spleen and thymus[1] ↓IL-12 production by DC→ ↓Th1 lineage specification[3]	↓ DNA methylation →↑transcription of the gene coding for IFNγ (*Ifng*)[4]
$CD4^+$T helper 2 (Th2)	↑ IL-4, IL-5, IL-13 → activates eosinophils ↑antibody production by plasma cells Important for humoral immunity and allergic response	↓ $CD4^+$ production in spleen and thymus[1] ↓$Th1^+$ and ↓Th17→ Th2 predominates	↑ DNA methylation → ↓ transcription of gene coding for IL-4 (Il4)[5] ↑ histone acetylation → ↓ Il4 transcription[6]
$CD4^+$T helper 17 (Th17)	↑ IL-17, IL-17F, IL-21, IL-22, IL-23, IL-26→ ↑Antimicrobial peptides Important for mucosal barrier maintenance and immunity	↓ CD4+ production in spleen and thymus[1] ↓IL-23 production by macrophages→ ↓Th17 lineage specification[7]	

SOURCES: [1] Saad and Jerrells, 1991, [2] Jerrells et al., 2002, [3] Mandrekar et al., 2004, [4] Young et al., 1994, [5] Lee et al., 2002, [6] Valapouret al., 2002, [7] Happel et al., 2006.

detection. (Elias 2007; Izcue et al. 2009; Parker and Prince 2011; Quayle 2002; Schleimer et al. 2007; Tracey 2002). Alcohol abuse is strongly correlated with defective, leaky barriers, particularly in the GI and respiratory tracts (Bhatty and Pruett 2011; Purohit et al. 2008).

The Effect of Alcohol on the Gut and the Potential Role of Epigenetics

Chronic alcohol consumption increases microbial colonization and LPS accumulation in the small intestine by decreasing gastric acid secretion in the stomach and delaying GI motility (Bienia et al. 2002; Bode and Bode 1997; Bode et al. 1984). The intestinal epithelial barrier must allow water and nutrients to pass freely, yet prevent transfer of larger macromolecules. Whereas the epithelial cells themselves are impermeable to substances dissolved in water (i.e., hydrophilic solutes), the space between the cells (i.e., paracellular space) must be sealed to maintain this barrier function. A leaky intestinal barrier is deleterious because it allows transfer of potentially harmful macromolecules and bacterial products (e.g., LPS) into the blood and lymph (Rao 2009). If it reaches the liver, LPS can target multiple cell types there, including Kupffer cells, neutrophils, hepatocytes, sinusoidal endothelial cells, and stellate cells (Brun et al. 2005; Duryee et al. 2004; Hoek and Pastorino 2002; Paik et al. 2003). Activation of these cells results in the release of pro-inflammatory mediators, such as ROS, leukotrienes, chemokines, and cytokines (e.g., TNFα and IL-1β), thereby directly contributing to liver damage and prolonged inflammation in chronic alcohol-abusing patients (Albano 2008; Brun and Castagliuolo 2005; Khoruts and Stahnke 1991; McClain et al. 2004).

The multifactorial contributions of chronic alcohol consumption to the development of ALD largely have been deciphered using rodent models. For example, investigators demonstrated a direct translocation of LPS across the gut mucosa in rats continuously administered alcohol directly into the stomach for 9 weeks (Mathurin et al. 2000). Other studies using mice in which the TNF-receptor 1 (TNF-R1) was removed (i.e., TNF-R1 knockout mice) and that were treated continuously with alcohol for 4 weeks determined that the alcohol-induced presence of LPS in the blood (i.e., endotoxemia) led to the release of TNFα from Kupffer cells, that in turn played a direct role in ALD (Yin et al. 1999). TNFα production is negatively regulated by H3K9 methylation (Gazzar et al. 2007), indicating that histone methylation can play a role in regulating inflammatory processes. This observation suggests that the prolonged inflammatory state associated with chronic alcohol exposure partially may be controlled by drugs targeting H3K9-specific demethylase enzymes.

Although alcohol itself does not alter intestinal permeability, one of the products of alcohol metabolism (i.e., acetaldehyde) increases barrier permeability in a dose-dependent manner (Basuroy et al. 2005) by disrupting intercellular connections, including both tight and adherens junctions (Atkinson and Rao 2001). One of the critical proteins ensuring the functionality of tight junctions is called zonula occludens 1 (ZO-1), and disrupted ZO-1 complexes are strongly correlated with increased intestinal barrier permeability (Walker and Porvaznik 1978). Interestingly, studies using a human intestinal cell line called Caco-2 found that ZO-1 production is regulated by microRNA-212 (miR-212). When these cells were cultured in the presence of 1 percent alcohol for 3 hours, they contained 71 percent less ZO-1 compared with cells not treated with alcohol. Moreover, the expression of miR-212 increased with alcohol treatment in a concentration-dependent manner; thus, cells treated with 1 percent alcohol for 3 hours had 2-fold higher expression of miR-212. These changes corresponded with defective tight junction morphology. Importantly, studies of colon samples taken from patients with ALD found significantly increased miR-212 expression compared with healthy control subject, and this increase paralleled a decrease in ZO-1. These findings demonstrate that miR-212 may play an important role in leaky intestinal barriers in ALD patients (Tang et al. 2008).

The Effect of Alcohol on the Respiratory System and the Potential Role of Epigenetics

Mucosal organ leakiness also contributes to respiratory infections, partially by altering tight junctions between epithelial cells lining the air sacs in lungs where gas exchange occurs (i.e., the alveoli) (Simet et al. 2012). This leaky barrier provides the ideal opportunity for bacteria normally found in the body (i.e., commensal bacteria), such as *Streptococcus pneumoniae,* to invade the tissues and become pathogenic (Bhatty and Pruett 2011). In fact, alcohol consumption is correlated with increased incidence of community-acquired pneumonia, with approximately 50 percent of adult pneumonia patients reporting a history of alcohol abuse (Goss et al. 2003). Furthermore, alcohol abuse worsens complications from pneumonia (Saitz et al. 1997) and increases mortality (Harboe et al. 2009) in a dose-dependent manner (Samokhvalov et al. 2010). Alcohol also shifts the cytokine balance in the lung, contributing to the development of ARDS (Boe and Vandivier 2009; Crews et al. 2006; Moss and Steinberg 1999).

When an infection occurs, neutrophils and monocytes are recruited to the lungs (Goto et al. 2004). Upon activation, monocytes differentiate into alveolar macrophages, which play a crucial role in the clearance of *S. pneumoniae* (Goto and Hogg 2004). Rodent models have demonstrated that chronic alcohol exposure contributed to increased infection susceptibility by causing mucosal organ leakiness, as well as defective leukocyte recruitment and decreased neutrophil maturation, adhesion, chemotaxis, and phagocytosis.

These changes partly resulted from faulty production of important signaling molecules, including G-CSF, GM-CSF, IL-8, IL-6, macrophage inflammatory protein (MIP-2), and CXC chemokine cytokine-induced neutrophil chemoattractant (CINC) (Boe et al. 2001). Alcohol also affected anti-inflammatory mediators by increasing the production of IL-10 and TGF-β (Boe and Vandivier 2009). Furthermore, chronic alcohol exposure inhibited the responses of $CD8^+$ T-cells , which increased the morbidity and mortality associated with influenza virus infection (Meyerholz et al. 2008), and decreased IFNγ production following infection with *Klebsiella pneumoniae* (Zisman et al. 1998) in murine models.

Several strategies targeting epigenetic regulatory mechanisms may be effective in the treatment of alcohol-induced lung infections. For example, therapies that restore neutrophil recruitment to infected lungs through regulation of cytokine production would be beneficial. In support of this notion, it was demonstrated that pretreatment with G-CSF prior to alcohol exposure and *K. pneumoniae* infection was protective in mouse models (Nelson et al. 1991). Targeting miRNAs for treatment of inflammatory lung diseases, such as ARDS, offers an additional, novel therapeutic approach because the production of several miRNAs, including miR-9, miR-146a, miR-147, miR-148, and miR-152, was induced by LPS stimulation in mouse lungs (Bazzoni et al. 2009; Liu et al. 2009, 2010; Nahid et al. 2009; Taganov et al. 2006; Tili et al. 2007; Zhou et al. 2011). Several of these upregulated miRNAs created a negative feedback loop to prevent excessive production of pro-inflammatory cytokines, therefore contributing to immune regulation and homeostasis (Bazzoni et al. 2009; Liu et al. 2009, 2010). Although most research focused on understanding the role of miRNAs in inflammatory lung disease has been performed using animal models, future studies using human cell lines, tissues, and eventually patient samples clearly are warranted.

Summary

The relationship between alcohol exposure and altered immune responses is complex. Chronic alcohol abuse is correlated with increased susceptibility to infection and causes tissue damage from an overactive innate immune response, excessive oxidative stress, and exacerbated or prolonged inflammation. Alcohol exposure has tissue- and immune cell-type–specific effects, such as influencing cell recruitment to infected or inflamed tissue, altering cytokine and chemokine production and secretion, skewing differentiation towards a particular cell fate or preventing cell replication, impairing antigen presentation, interfering with phagocytosis and granulopoiesis, or inducing apoptosis. Although the specific role of epigenetic modulation in this alcohol-induced immune dysregulation has not yet been determined, research in related fields strongly suggests that experimental and clinical studies are warranted. ■

Acknowledgments

Funding provided by the National Institute on Alcohol Abuse and Alcoholism of the National Institutes of Health under award numbers R01–AA–012034 and T32–AA–013527 to Elizabeth J. Kovacs, NIH grant F31–AA–019913 to Anita Zahs, NIH grant F32- AA-021636 to Brenda J. Curtis, and by the Dr. Ralph and Marian C. Falk Medical Research Trust to Elizabeth J. Kovacs.

Financial Disclosure

The authors declare that they have no competing financial interests.

References

Albano, E. Oxidative mechanisms in the pathogenesis of alcoholic liver disease. *Molecular Aspects of Medicine* 29(1-2):9–16, 2008. PMID: 18045675

Atkinson, K.J., and Rao, R.K. Role of protein tyrosine phosphorylation in acetaldehyde-induced disruption of epithelial tight junctions. American Journal of Physiology. *Gastrointestinal and Liver Physiology* 280(6):G1280–G1288, 2001. PMID: 11352822

Bagby, G.J.; Zhang, P.; Stoltz, D.A.; and Nelson, S. Suppression of the granulocyte colony-stimulating factor response to Escherichia coli challenge by alcohol intoxication. *Alcoholism: Clinical and Experimental Research* 22(8):1740–1745, 1998. PMID: 9835289

Bala, S.; Marcos, M.; Kodys, K.; et al. Up-regulation of microRNA-155 in macrophages contributes to increased tumor necrosis factor {alpha} (TNF{alpha}) production via increased mRNA half-life in alcoholic liver disease. *Journal of Biological Chemistry* 286(2):1436–1444, 2011. PMID: 21062749

Baliunas, D.; Rehm, J.; Irving, H.; and Shuper, P. Alcohol consumption and risk of incident human immunodeficiency virus infection: A meta-analysis. *International Journal of Public Health* 55(3):159–166, 2010. PMID: 19949966

Ballas, Z.K. The use of 5-azacytidine to establish constitutive interleukin 2-producing clones of the EL4 thymoma. *Journal of Immunology* 133(1):7–9, 1984. PMID: 6202793

Ballestar, E. Epigenetic alterations in autoimmune rheumatic diseases. *Nature Reviews. Rheumatology* 7(5):263–271, 2011. PMID: 21343899

Bannister, A.J., and Kouzarides, T. Reversing histone methylation. *Nature* 436(7054):1103–1106, 2005. PMID: 16121170

Bardag-Gorce, F.; French, B.A.; Joyce, M.; et al. Histone acetyltransferase p300 modulates gene expression in an epigenetic manner at high blood alcohol levels. *Experimental and Molecular Pathology* 82(2):197–202, 2007. PMID: 17208223

Bardag-Gorce, F.; French, B.A.; Li, J.; al. The importance of cycling of blood alcohol levels in the pathogenesis of experimental alcoholic liver disease in rats. *Gastroenterology* 123(1):325–335, 2002. PMID: 12105860

Basuroy, S.; Sheth, P.; Mansbach, C.M.; and Rao, R.K. Acetaldehyde disrupts tight junctions and adherens junctions in human colonic mucosa: Protection by EGF and L-glutamine. *American Journal of Physiology. Gastrointestinal and Liver Physiology* 289(2): G367–G375, 2005. PMID: 15718285

Bazzoni, F.; Rossato, M.; Fabbri, M.; et al. Induction and regulatory function of miR-9 in human monocytes and neutrophils exposed to proinflammatory signals. *Proceedings of the National Academy of Sciences of the United States of America* 106(13):5282–5287, 2009. PMID: 19289835

Berger, S.L.; Kouzarides, T.; Shiekhattar, R.; and Shilatifard, A. An operational definition of epigenetics. *Genes & Development* 23(7):781–783, 2009. PMID: 19339683

Best, C.A., and Laposata, M. Fatty acid ethyl esters: Toxic non-oxidative metabolites of ethanol and markers of ethanol intake. *Frontiers in Bioscience* 8:e202–e217, 2003. PMID: 12456329

Bhatty, M.; Pruett, S.B.; Swiatlo, E.; and Nanduri, B. Alcohol abuse and Streptococcus pneumoniae infections: Consideration of virulence factors and impaired

immune responses. *Alcohol* 45(6):523–539, 2011. PMID: 21827928

Bienia, A.; Sodolski, W.; and Luchowska, E. The effect of chronic alcohol abuse on gastric and duodenal mucosa. *Annales Universitatis Mariae Curie-Sklodowska Sectio D: Medicina* 57(2):570–582, 2002. PMID: 12898897

Bodar, E.J.; Simon, A.; and van der Meer, J.W. Effects of the histone deacetylase inhibitor ITF2357 in autoinflammatory syndromes. *Molecular Medicine* 17(56):363–368, 2011. PMID: 21274502

Bode, C., and Bode, J.C. Alcohol's role in gastrointestinal tract disorders. *Alcohol Health & Research World* 21(1):76–83, 1997. PMID: 15706765

Bode, J.C.; Bode, C.; Heidelbach, R.; et al. Jejunal microflora in patients with chronic alcohol abuse. *Hepato-Gastroenterology* 31(1):30–34, 1984. PMID: 6698486

Boe, D.M.; Nelson, S.; Zhang, P.; and Bagby, G.J. Acute ethanol intoxication suppresses lung chemokine production following infection with Streptococcus pneumoniae. *Journal of Infectious Diseases* 184(9):1134–1142, 2001. PMID: 11598836

Boe, D.M.; Vandivier, R.W.; Burnham, E.L.; and Moss, M. Alcohol abuse and pulmonary disease. *Journal of Leukocyte Biology* 86(5):1097–1104, 2009. PMID: 19602670

Bowman, S.M., and Free, S.J. The structure and synthesis of the fungal cell wall. *BioEssays* 28(8):799–808, 2006. PMID: 16927300

Brun, P.; Castagliuolo, I.; Pinzani, M.; et al. Exposure to bacterial cell wall products triggers an inflammatory phenotype in hepatic stellate cells. American Journal of Physiology. *Gastrointestinal and Liver Physiology* 289(3):G571–G578, 2005. PMID: 15860640

Bullwinkel, J.; Ludemann, A.; Debarry, J.; and Singh, P.B. Epigenotype switching at the CD14 and CD209 genes during differentiation of human monocytes to dendritic cells. *Epigenetics* 6(1):45–51, 2011. PMID: 20818162

Carthew, R.W., and Sontheimer, E.J. Origins and mechanisms of miRNAs and siRNAs. *Cell* 136(4):642–655, 2009. PMID: 19239886

Choi, S., and Reddy, P. HDAC inhibition and graft versus host disease. *Molecular Medicine* 17(5-6):404–416, 2011. PMID: 21298214

Choudhary, C.; Kumar, C.; Gnad, F.; et al. Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science* 325(5942):834–840, 2009. PMID: 19608861

Choudhury, M.; Park, P.H.; Jackson, D.; and Shukla S.D. Evidence for the role of oxidative stress in the acetylation of histone H3 by ethanol in rat hepatocytes. *Alcohol* 44(6):531–540, 2010. PMID: 20705415

Choudhury, M.; and Shukla, S.D. Surrogate alcohols and their metabolites modify histone H3 acetylation: Involvement of histone acetyl transferase and histone deacetylase. *Alcoholism: Clinical and Experimental Research* 32(5):829–839, 2008. PMID: 18336638

Cook, R.T. Alcohol abuse, alcoholism, and damage to the immune system: A review. *Alcoholism: Clinical and Experimental Research* 22(9):1927–1942, 1998. PMID: 9884135

Cook, R.T.; Zhu, X.; Coleman, R.A.; et al. T-cell activation after chronic ethanol ingestion in mice. *Alcohol* 33(3):175–181, 2004. PMID: 15596085

Crews, F.T.; Bechara, R.; Brown, L.A.; et al. Cytokines and alcohol. *Alcoholism: Clinical and Experimental Research* 30(4):720–730, 2006. PMID: 16573591

De Santa, F.; Totaro, M.G.; Prosperini, E.; et al. The histone H3 lysine-27 demethylase Jmjd3 links inflammation to inhibition of polycomb-mediated gene silencing. *Cell* 130(6):1083–1094, 2007. PMID: 17825402

Dey, A., and Cederbaum, A.I. Alcohol and oxidative liver injury. *Hepatology* 43(2 Suppl 1):S63–S74, 2006. PMID: 16447273

Dinarello, C.A. Anti-inflammatory agents: Present and future. *Cell* 140(6):935–950, 2010. PMID: 20303881

Dinarello, C.A.; Fossati, G.; and Mascagni, P. Histone deacetylase inhibitors for treating a spectrum of diseases not related to cancer. *Molecular Medicine* 17(5-6):333–352, 2011. PMID: 21556484

Downes, M.; Ordentlich, P.; Kao, H.Y.; et al. Identification of a nuclear domain with deacetylase activity. *Proceedings of the National Academy of Sciences of the United States of America* 97(19):10330–10335, 2000. PMID: 10984530

Duryee, M.J.; Klassen, L.W.; Freeman, T.L.; et al. Lipopolysaccharide is a cofactor for malondialdehyde-acetaldehyde adduct-mediated cytokine/chemokine release by rat sinusoidal liver endothelial and Kupffer cells. *Alcoholism: Clinical and Experimental Research* 28(12):1931–1938, 2004. PMID: 15608611

Elias, P.M. The skin barrier as an innate immune element. *Seminars in Immunopathology* 29(1):3–14, 2007. PMID: 17621950

Feil, R., and Fraga, M.F. Epigenetics and the environment: Emerging patterns and implications. *Nature Reviews. Genetics* 13(2):97–109, 2012. PMID: 22215131

Fischle, W.; Dequiedt, F.; Fillion, M.; et al. Human HDAC7 histone deacetylase activity is associated with HDAC3 in vivo. *Journal of Biological Chemistry* 276(38):35826–35835, 2001. PMID: 11466315

Foster, S.L.; Hargreaves, D.C.; and Medzhitov, R. Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature* 447(7147):972–978, 2007. PMID: 17538624

French, B.A.; Dedes, J.; Bardag-Gorce, F.; et al. Microarray analysis of gene expression in the liver during the urinary ethanol cycle in rats fed ethanol intragastrically at a constant rate. *Experimental and Molecular Pathology* 79(2):87–94, 2005. PMID: 16098508

Goral, J.; Karavitis, J.; and Kovacs, E.J. Exposure-dependent effects of ethanol on the innate immune system. *Alcohol* 42(4):237–247, 2008. PMID: 18411007

Goss, C.H.; Rubenfeld, G.D.; Park, D.R.; et al. Cost and incidence of social comorbidities in low-risk patients with community-acquired pneumonia admitted to a public hospital. *Chest* 124(6):2148–2155, 2003. PMID: 14665494

Goto, Y.; Hogg, J.C.; Whalen, B.; et al. Monocyte recruitment into the lungs in pneumococcal pneumonia. *American Journal of Respiratory Cell and Molecular Biology* 30(5):620–626, 2004. PMID: 14578212

Gramenzi, A.; Caputo, F.; Biselli, M.; et al. Review article: Alcoholic liver disease—Pathophysiological aspects and risk factors. *Alimentary Pharmacology & Therapeutics* 24(8):1151–1161, 2006. PMID: 17014574

Halili, M.A.; Andrews, M.R.; Sweet, M.J.; and Fairlie, D.P. Histone deacetylase inhibitors in inflammatory disease. *Current Topics in Medicinal Chemistry* 9(3):309–319, 2009. PMID: 19355993

Happel, K.I.; Odden, A.R.; Zhang, P.; et al. Acute alcohol intoxication suppresses the interleukin 23 response to Klebsiella pneumoniae infection. *Alcoholism: Clinical and Experimental Research* 30(7):1200–1207, 2006. PMID: 16792568

Harboe, Z.B.; Thomsen, R.W.; Riis, A.; et al. Pneumococcal serotypes and mortality following invasive pneumococcal disease: A population-based cohort study. *PLoS Medicine* 6(5):e1000081, 2009. PMID: 19468297

Heintzman, N.D.; Stuart, R.K.; Hon, G.; et al. Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. *Nature* Genetics 39(3):311–318, 2007. PMID: 17277777

Hoek, J.B., and Pastorino, J.G. Ethanol, oxidative stress, and cytokine-induced liver cell injury. *Alcohol* 27(1):63–68, 2002. PMID: 12062639

Izcue, A.; Coombes, J.L.; and Powrie, F. Regulatory lymphocytes and intestinal inflammation. *Annual Review of Immunology* 27:313–38, 2009. PMID: 19302043

Janeway, C.A., Jr., and Medzhitov, R. Innate immune recognition. *Annual Review of Immunology* 20:197–216, 2002. PMID: 11861602

Jenuwein, T., and Allis, C.D. Translating the histone code. *Science* 293(5532):1074–1080, 2001. PMID: 11498575

Jerrells, T.R.; Mitchell, K.; Pavlik, J.; et al. Influence of ethanol consumption on experimental viral hepatitis. *Alcoholism: Clinical and Experimental Research* 26(11):1734–1746, 2002. 12436064

Ji, H.; Ehrlich, L.I.; Seita, J.; et al. Comprehensive methylome map of lineage commitment from haematopoietic progenitors. *Nature* 467(7313):338–342, 2010. PMID: 20720541

Joosten, L.A.; Leoni, F.; Meghji, S.; and Mascagni, P. Inhibition of HDAC activity by ITF2357 ameliorates joint inflammation and prevents cartilage and bone destruction in experimental arthritis. *Molecular Medicine* 17(5-6):391–396, 2011. PMID: 21327299

Karavitis, J., and Kovacs, E.J. Macrophage phagocytosis: Effects of environmental pollutants, alcohol, cigarette smoke, and other external factors. *Journal of Leukocyte Biology* 90(6):1065–1078, 2011. PMID: 21878544

Kendrick, S.F.; O'Boyle, G.; Mann, J.; et al. Acetate, the key modulator of inflammatory responses in acute alcoholic hepatitis. *Hepatology* 51(6):1988–1997, 2010. PMID: 20232292

Khoruts, A.; Stahnke, L.; McClain, C.J.; et al. Circulating tumor necrosis factor, interleukin-1 and interleukin-6

concentrations in chronic alcoholic patients. *Hepatology* 13(2):267–276, 1991. PMID: 1995437

Kim, J.S., and Shukla, S.D. Histone h3 modifications in rat hepatic stellate cells by ethanol. *Alcohol and Alcoholism* 40(5):367–372, 2005. PMID: 15939707

Kim, J.S., and Shukla, S.D. Acute in vivo effect of ethanol (binge drinking) on histone H3 modifications in rat tissues. *Alcohol and Alcoholism* 41(2):126–132, 2006. PMID: 16314425

Kouzarides, T. Acetylation: A regulatory modification to rival phosphorylation? *EMBO Journal* 19(6):1176–1179, 2000. PMID : 10716917

Le Moine, O.; Marchant, A.; De Groote, D.; et al. Role of defective monocyte interleukin-10 release in tumor necrosis factor-alpha overproduction in alcoholic cirrhosis. *Hepatology* 22(5):1436–1439, 1995. PMID: 7590660

Lee, D.U.; Agarwal, S.; and Rao, A. Th2 lineage commitment and efficient IL-4 production involves extended demethylation of the IL-4 gene. *Immunity* 16(5):649–660, 2002. PMID: 12049717

Lee, H.K., and Iwasaki, A. Innate control of adaptive immunity: Dendritic cells and beyond. *Seminars in Immunology* 19(1):48–55, 2007. PMID: 17276695

Lee, T.D.; Sadda, M.R.; Mendler, M.H.; et al. Abnormal hepatic methionine and glutathione metabolism in patients with alcoholic hepatitis. *Alcoholism: Clinical and Experimental Research* 28(1):173–181, 2004. PMID: 14745316

Lee, Y.J., and Shukla, S.D. Histone H3 phosphorylation at serine 10 and serine 28 is mediated by p38 MAPK in rat hepatocytes exposed to ethanol and acetaldehyde. *European Journal of Pharmacology* 573(1-3):29–38, 2007. PMID: 17643407

Lertratanangkoon, K.; Wu, C.J.; Savaraj, N.; and Thomas, M.L. Alterations of DNA methylation by glutathione depletion. *Cancer Letters* 120(2):149–156, 1997. PMID: 9461031

Liu, G.; Friggeri, A.; Yang, Y.; et al. miR-147, a microRNA that is induced upon Toll-like receptor stimulation, regulates murine macrophage inflammatory responses. *Proceedings of the National Academy of Sciences of the United States of America* 106(37):15819–15824, 2009. PMID: 19721002

Liu, X.; Zhan, Z.; Xu, L.; et al. MicroRNA-148/152 impair innate response and antigen presentation of TLR-triggered dendritic cells by targeting CaMKIIα. *Journal of Immunology* 185(12):7244–7251, 2010. PMID: 21068402

Louvet, A.; Teixeira-Clerc, F.; Chobert, M.N.; et al. Cannabinoid CB2 receptors protect against alcoholic liver disease by regulating Kupffer cell polarization in mice. *Hepatology* 54(4):1217–1226, 2011. PMID: 21735467

Lu, S.C.; Huang, Z.Z.; Yang, H.; et al. Changes in methionine adenosyltransferase and S-adenosylmethionine homeostasis in alcoholic rat liver. American Journal of Physiology. *Gastrointestinal and Liver Physiology* 279(1):G178–G185, 2000. PMID: 10898761

Lu, S.C., and Mato, J.M. Role of methionine adenosyltransferase and S-adenosylmethionine in alcohol-associated liver cancer. *Alcohol* 35(3):227–234, 2005. PMID: 16054984

Mandal, P.; Pratt, B.T.; Barnes, M.; et al. Molecular mechanism for adiponectin-dependent M2 macrophage polarization: Link between the metabolic and innate immune activity of full-length adiponectin. *Journal of Biological Chemistry* 286(15):13460–13469, 2011. PMID: 21357416

Mandrekar, P.; Catalano, D.; Dolganiuc, A.; et al. Inhibition of myeloid dendritic cell accessory cell function and induction of T-cell anergy by alcohol correlates with decreased IL-12 production. *Journal of Immunology* 173(5):3398-3407, 2004. PMID: 15322204

Mandrekar, P., and Szabo, G. Signalling pathways in alcohol-induced liver inflammation. *Journal of Hepatology* 50(6):1258–1266, 2009. PMID: 19398236

Mann, R.E.; Smart, R.G.; and Govoni, R. The epidemiology of alcoholic liver disease. *Alcohol Research & Health* 27(3):209–219, 2003. PMID: 15535449

Martin, C., and Zhang, Y. The diverse functions of histone lysine methylation. *Nature Reviews. Molecular Cell Biology* 6(11):838–849, 2005. PMID: 16261189

Martinez, F.O.; Sica, A.; Mantovani, A.; and Locati, M. Macrophage activation and polarization. *Frontiers in Bioscience* 13:453–461, 2008. PMID: 17981560

Mason, J.B., and Choi, S.W. Effects of alcohol on folate metabolism: Implications for carcinogenesis. *Alcohol* 35(3):235–241, 2005. PMID: 16054985

Mathurin, P.; Deng, Q.G.; Keshavarzian, A.; et al. Exacerbation of alcoholic liver injury by enteral endotoxin in rats. *Hepatology* 32(5):1008–1017, 2000. PMID: 11050051

McClain, C.J.; Song, Z.; Barve, S.S.; et al. Recent advances in alcoholic liver disease. IV. Dysregulated cytokine metabolism in alcoholic liver disease. *American Journal of Physiology. Gastrointestinal and Liver Physiology* 287(3):G497–G502, 2004. PMID: 15331349

McKillop, I.H., and Schrum, L.W. Role of alcohol in liver carcinogenesis. *Seminars in Liver Disease* 29(2):222–232, 2009. PMID: 19387921

Messingham, K.A.; Faunce, D.E.; and Kovacs, E.J. Alcohol, injury, and cellular immunity. *Alcohol* 28(3):137–149, 2002. PMID: 12551755

Meyerholz, D.K.; Edsen-Moore, M.; McGill, J.; et al. Chronic alcohol consumption increases the severity of murine influenza virus infections. *Journal of Immunology* 181(1):641–648, 2008. PMID: 18566431

Moss, M.; Steinberg, K.P.; Guidot, D.M.; et al. The effect of chronic alcohol abuse on the incidence of ARDS and the severity of the multiple organ dysfunction syndrome in adults with septic shock: An interim and multivariate analysis. *Chest* 116(1 Suppl):97S–98S, 1999. PMID: 10424617

Mullen, A.C.; Hutchins, A.S.; High, F.A.; et al. Hlx is induced by and genetically interacts with T-bet to promote heritable T(H)1 gene induction. *Nature* Immunology 3(7):652–658, 2002. PMID: 12055627

Nahid, M.A.; Pauley, K.M.; Satoh, M.; and Chan, E.K. miR-146a is critical for endotoxin-induced tolerance: Implications in innate immunity. *Journal of Biological Chemistry* 284(50):34590–34599, 2009. PMID: 19840932

Nelson, S.; Summer, W.; Bagby, G.; et al. Granulocyte colony-stimulating factor enhances pulmonary host defenses in normal and ethanol-treated rats. *Journal of Infectious Diseases* 164(5):901-906, 1991. PMID: 1719103

Oliva, J.; Dedes, J.; Li, J.; et al. Epigenetics of proteasome inhibition in the liver of rats fed ethanol chronically. *World Journal of Gastroenterology* 15(6):705–712, 2009. PMID: 19222094

Paik, Y.H.; Schwabe, R.F.; Bataller, R.; et al. Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells. *Hepatology* 37(5):1043–1055, 2003. PMID: 12717385

Pal-Bhadra, M.; Bhadra, U.; Jackson, D.E.; et al. Distinct methylation patterns in histone H3 at Lys-4 and Lys-9 correlate with up- & down-regulation of genes by ethanol in hepatocytes. *Life Sciences* 81(12):979–987, 2007. PMID: 17826801

Park, P.H.; Lim, R.W.; and Shukla, S.D. Involvement of histone acetyltransferase (HAT) in ethanol-induced acetylation of histone H3 in hepatocytes: Potential mechanism for gene expression. *American Journal of Physiology. Gastrointestinal and Liver Physiology* 289(6):G1124–G1136, 2005. PMID: 16081763

Park, P.H.; Miller, R.; and Shukla, S.D. Acetylation of histone H3 at lysine 9 by ethanol in rat hepatocytes. *Biochemical and Biophysical Research Communications* 306(2):501–504, 2003. PMID: 12804592

Parker, D., and Prince, A. Innate immunity in the respiratory epithelium. *American Journal of Respiratory Cell and Molecular Biology* 45(2):189–201, 2011. PMID: 21330463

Perlino, C.A., and Rimland, D. Alcoholism, leukopenia, and pneumococcal sepsis. *American Review of Respiratory Disease* 132(4):757–760, 1985. PMID: 4051312

Prakash, O.; Mason, A.; Luftig, R.B.; and Bautista, A.P. Hepatitis C virus (HCV) and human immunodeficiency virus type 1 (HIV-1) infections in alcoholics. *Frontiers in Bioscience* 7:e286–e300, 2002. PMID: 12086918

Purohit, V.; Bode, J.C.; Bode, C.; et al. Alcohol, intestinal bacterial growth, intestinal permeability to endotoxin, and medical consequences: Summary of a symposium. *Alcohol* 42(5):349–361, 2008. PMID: 18504085

Quayle, A.J. The innate and early immune response to pathogen challenge in the female genital tract and the pivotal role of epithelial cells. *Journal of Reproductive Immunology* 57(1-2):61–79, 2002. PMID: 12385834

Rambaldi, A.; Dellacasa, C.M.; Finazzi, G.; et al. A pilot study of the histone-deacetylase inhibitor Givinostat in patients with JAK2V617F positive chronic myeloproliferative neoplasms. *British Journal of Haematology* 150(4):446–455, 2010. PMID: 20560970

Rao, R. Endotoxemia and gut barrier dysfunction in alcoholic liver disease. *Hepatology* 50(2):638–44, 2009. PMID: 19575462

Reddy, P.; Maeda, Y.; Hotary, K.; et al. Histone deacetylase inhibitor suberoylanilide hydroxamic acid reduces acute graft-versus-host disease and preserves graft-versus-leukemia effect. *Proceedings of the National Academy of Sciences of the United States of America* 101(11):3 921–3926, 2004. PMID: 15001702

Reddy, P.; Sun, Y.; Toubai, T.; et al. Histone deacetylase inhibition modulates indoleamine 2,3-dioxygenase-dependent DC functions and regulates experimental graft-versus-host disease in mice. *Journal of Clinical Investigation* 118(7):2562–2573, 2008. PMID: 18568076

Reis e Sousa, C.; Hieny, S.; Scharton-Kersten, T.; et al. In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T-cell areas. *Journal of Experimental Medicine* 186(11):1819–1829, 1997. PMID: 9382881

Richardson, B.; Scheinbart, L.; Strahler, J.; et al. Evidence for impaired T-cell DNA methylation in systemic lupus erythematosus and rheumatoid arthritis. *Arthritis and Rheumatism* 33(11):1665–1673, 1990. PMID: 2242063

Richardson, B.C.; Strahler, J.R.; Pivirotto, T.S.; et al. Phenotypic and functional similarities between 5-azacytidine-treated T-cells and a T-cell subset in patients with active systemic lupus erythematosus. *Arthritis and Rheumatism* 35(6):647–662, 1992. PMID : 1376122

Rodriguez-Cortez, V.C.; Hernando, H.; de la Rica, L.; et al. Epigenomic deregulation in the immune system. *Epigenomics* 3(6):697–713, 2011. PMID: 22126290

Romeo, J.; Warnberg, J.; Diaz, L.E.; et al. Effects of moderate beer consumption on first-line immunity of healthy adults. *Journal of Physiology and Biochemistry* 63(2): 153–159, 2007a. PMID: 17933389

Romeo, J.; Warnberg, J.; Nova, E.; et al. Moderate alcohol consumption and the immune system: A review. *British Journal of Nutrition* 98(Suppl. 1):S111–S115, 2007*b*. PMID: 17922947

Ruggiero, T.; Trabucchi, M.; De Santa, F.; et al. LPS induces KH-type splicing regulatory protein-dependent processing of microRNA-155 precursors in macrophages. *FASEB Journal* 23(9):2898–2908, 2009. PMID: 19423639

Saad, A.J., and Jerrells, T.R. Flow cytometric and immunohistochemical evaluation of ethanol-induced changes in splenic and thymic lymphoid cell populations. *Alcoholism: Clinical and Experimental Research* 15(5):796–803, 1991. PMID: 1755511

Saitz, R.; Ghali, W.A.; and Moskowitz, M.A. The impact of *alcohol-related diagnoses on pneumonia outcomes. Archives of Internal Medicine* 157(13):1446–1452, 1997. PMID: 9224223

Samokhvalov, A.V.; Irving, H.M.; Rehm, J. Alcohol consumption as a risk factor for atrial fibrillation: A systematic review and meta-analysis. *European Journal of Cardiovascular Prevention and Rehabilitation* 17(6): 706–712, 2010. PMID: 21461366

Satoh, T.; Takeuchi, O.; Vandenbon, A.; et al. The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. *Nature Immunology* 11(10):936–944, 2010. PMID: 20729857

Schafer, C.; Schips, I.; Landig, J.; et al. Tumor-necrosis-factor and interleukin-6 response of peripheral blood monocytes to low concentrations of lipopolysaccharide in patients with alcoholic liver disease. *Zeitschrift fur Gastroenterologie* 33(9):503–508, 1995. PMID: 8525652

Schleimer, R.P.; Kato, A.; Kern, R.; et al. Epithelium: At the interface of innate and adaptive immune responses. *Journal of Allergy and Clinical Immunology* 120(6): 1279–1284, 2007. PMID: 17949801

Shakespear, M.R.; Halili, M.A.; Irvine, K.M.; et al. Histone deacetylases as regulators of inflammation and immunity. *Trends in Immunology* 32(7):335–343, 2011. PMID: 21570914

Shilatifard, A. Chromatin modifications by methylation and ubiquitination: Implications in the regulation of gene expression. *Annual Review of Biochemistry* 75:243–269, 2006. PMID: 16756492

Shukla, S.D., and Aroor, A.R. Epigenetic effects of ethanol on liver and gastrointestinal injury. *World Journal of Gastroenterology* 12(33):5265–5271, 2006. PMID: 16981253

Shukla, S.D.; Sun, G.Y.; Gibson Wood, W.; et al. Ethanol and lipid metabolic signaling. *Alcoholism: Clinical and Experimental Research* 25(5 Suppl ISBRA):33S–39S, 2001. PMID: 11391046

Simet, S.M.; Wyatt, T.A.; DeVasure, J.; et al. Alcohol increases the permeability of airway epithelial tight junctions in Beas-2B and NHBE cells. *Alcoholism: Clinical and Experimental Research* 36(3):432–442, 2012. PMID: 21950588

Strahl, B.D., and Allis, C.D. The language of covalent histone modifications. *Nature* 403(6765):41–45, 2000. PMID: 10638745

Taganov, K.D.; Boldin, M.P.; Chang, K.J.; and Baltimore, D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proceedings of the National Academy of Sciences of the United States of America* 103(33):12481–12486, 2006. PMID: 16885212

Takeuch, O., and Akira, S. Epigenetic control of macrophage polarization. *European Journal of Immunology* 41(9): 2490–2493, 2011. PMID: 21952803

Tam, A.; Wadsworth, S.; Dorscheid, D.; et al. The airway epithelium: More than just a structural barrier. *Therapeutic Advances in Respiratory Disease* 5(4):255–273, 2011. PMID: 21372121

Tang, Y.; Banan, A.; Forsyth, C.B.; et al. Effect of alcohol on miR-212 expression in intestinal epithelial cells and its potential role in alcoholic liver disease. *Alcoholism: Clinical and Experimental Research* 32(2):355–364, 2008. PMID: 18162065

Thakur, V.; McMullen, M.R.; Pritchard, M.T.; and Nagy, L.E. Regulation of macrophage activation in alcoholic liver disease. *Journal of Gastroenterology and Hepatology* 22(Suppl. 1):S53–S56, 2007. PMID: 17567466

Tili, E.; Michaille, J.J.; Cimino, A.; et al. Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. *Journal of Immunology* 179(8):5082–5089, 2007. PMID: 17911593

Tracey, K.J. The inflammatory reflex. *Nature* 420(6917): 853–859, 2002. PMID: 15656871

Tsukamoto, H.; French, S.W.; Benson, N.; et al. Severe and progressive steatosis and focal necrosis in rat liver induced by continuous intragastric infusion of ethanol and low fat diet. *Hepatology* 5(2):224–232, 1985. PMID: 3979954

Turner, B.M. Histone acetylation and an epigenetic code. *Bioessays* 22(9):836–845, 2000. PMID: 10944586

Turner, J.R. Intestinal mucosal barrier function in health and disease. *Nature Reviews. Immunology* 9(11):799–809, 2009. PMID: 19855405

Valapour, M.; Guo, J.; Schroeder, J.T.; et al. Histone deacetylation inhibits IL4 gene expression in T-cells. *Journal of Allergy and Clinical Immunology* 109(2): 238–245, 2002. PMID: 11842291

Vojinovic, J., and Damjanov, N. HDAC inhibition in rheumatoid arthritis and juvenile idiopathic arthritis. *Molecular Medicine* 17(5-6):397–403, 2011. PMID: 21308151

Vojinovic, J.; Damjanov, N.; D'Urzo, C.; et al. Safety and efficacy of an oral histone deacetylase inhibitor in systemic-onset juvenile idiopathic arthritis. *Arthritis and Rheumatism* 63(5):1452–1458, 2011. PMID: 21538322

Walker, R.I., and Porvaznik, M.J. Disruption of the permeability barrier (zonula occludens) between intestinal epithelial cells by lethal doses of endotoxin. *Infection and Immunity* 21(2):655–658, 1978. PMID: 689739

Wen, H.; Schaller, M.A.; Dou, Y.; et al. Dendritic cells at the interface of innate and acquired immunity: The role for epigenetic changes. *Journal of Leukocyte Biology* 83(3):439–446, 2008. PMID: 17991763

Winzler, C.; Rovere, P.; Rescigno, M.; et al. Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. *Journal of Experimental Medicine* 185(2):317–328, 1997. PMID: 9016880

Yamashita, H.; Kaneyuki, T.; and Tagawa, K. Production of acetate in the liver and its utilization in peripheral tissues. *Biochimica et Biophysica Acta* 1532(1–2):79–87, 2001. PMID: 11420176

Yamauchi, M.; Nakahara, M.; Maezawa, Y.; et al. Prevalence of hepatocellular carcinoma in patients with alcoholic cirrhosis and prior exposure to hepatitis C. *American Journal of Gastroenterology* 88(1):39–43, 1993. PMID: 7678368

Yin, M.; Wheeler, M.D.; Kono, H.; et al. Essential role of tumor necrosis factor alpha in alcohol-induced liver injury in mice. *Gastroenterology* 117(4):942–952, 1999. PMID: 10500078

You, M.; Liang, X.; Ajmo, J.M.; and Ness, G.C. Involvement of mammalian sirtuin 1 in the action of ethanol in the liver. *American Journal of Physiology. Gastrointestinal and Liver Physiology* 294(4):G892–G898, 2008. PMID: 18239056

Young, H.A.; Ghosh, P.; Ye, J.; et al. Differentiation of the T helper phenotypes by analysis of the methylation state of the IFN-gamma gene. *Journal of Immunology* 153(8): 3603–3610, 1994. PMID: 7523497

Zhang, P.; Bagbyc, G.J.; Happel, K.I.; et al. Alcohol abuse, immunosuppression, and pulmonary infection. *Current Drug Abuse Reviews* 1(1):56–67, 2008. PMID: 19630706

Zhang, P.; Welsh, D.A.; Siggins, R.W., 2nd.; et al. Acute alcohol intoxication inhibits the lineage- c-kit+ Sca-1+ cell response to Escherichia coli bacteremia. *Journal of Immunology* 182(3):1568–1576, 2009. PMID: 19155505

Zhang, Z.; Bagby, G.J.; Stoltz, D.; et al. Prolonged ethanol treatment enhances lipopolysaccharide/phorbol myristate acetate-induced tumor necrosis factor-alpha production in human monocytic cells. *Alcoholism: Clinical and Experimental Research* 25(3):444–449, 2001. PMID: 11290857

Zhou, T.; Garcia, J.G.; and Zhang, W. Integrating microRNAs into a system biology approach to acute lung injury. *Translational Research* 157(4):180–190, 2011. PMID: 21420028

Zisman, D.A.; Strieter, R.M.; Kunkel, S.L.; et al. Ethanol feeding impairs innate immunity and alters the expression of Th1- and Th2- phenotype cytokines in murine Klebsiella pneumonia. *Alcoholism: Clinical and Experimental Research* 22(3):621–627, 1998. PMID: 9622442

Zygmunt, B., and Veldhoen, M. T helper cell differentiation more than just cytokines. *Advances in Immunology* 109:159–196, 2011. PMID: 21569915G

Circadian Disruption

Potential Implications in Inflammatory and Metabolic Diseases Associated With Alcohol

Robin M. Voigt, Ph.D.; Christopher B. Forsyth, Ph.D.; and Ali Keshavarzian, M.D.

Robin M. Voigt, Ph.D., *is an assistant professor;* **Christopher B. Forsyth, Ph.D.,** *is an assistant professor; and* **Ali Keshavarzian, M.D.** *is a professor and Josephine M. Dyrenforth Chair of Gastroenterology; all at Rush University Medical Center, Chicago, Illinois.*

Circadian rhythms are a prominent and critical feature of cells, tissues, organs, and behavior that help an organism function most efficiently and anticipate things such as food availability. Therefore, it is not surprising that disrupted circadian rhythmicity, a prominent feature of modern-day society, promotes the development and/or progression of a wide variety of diseases, including inflammatory, metabolic, and alcohol-associated disorders. This article will discuss the influence of interplay between alcohol consumption and circadian rhythmicity and how circadian rhythm disruption affects immune function and metabolism as well as potential epigenetic mechanisms that may be contributing to this phenomenon. **KEY WORDS: Alcohol consumption; alcohol-related disorders; disease factors; risk factors; circadian disruption; circadian rhythm; circadian clock; immune function; metabolism; inflammatory diseases; metabolic diseases; epigenetic mechanisms**

Circadian Disruption and Society

The circadian clock is a sophisticated mechanism that functions to synchronize (i.e., entrain) endogenous systems with the 24-hour day in a wide variety of organisms, from simple organisms such as fungi up to the complex mammalian systems. Circadian rhythms control a variety of biological processes, including sleep/wake cycles, body temperature, hormone secretion, intestinal function, metabolic glucose homeostasis, and immune function. Functional consequences of modern-day society, such as late-night activity, work schedules that include long-term night shifts and those in which employees change or rotate shifts (i.e., shift work), and jet lag are substantial environmental disruptors of normal circadian rhythms. Fifteen percent of American workers perform shift work (Bureau of Labor Statistics 2005), indicating the pervasiveness of circadian disruption as a normal part of modern-day society. This change from the diurnal lifestyle of our ancestors to one that is more prominently nocturnal results in misalignment between natural rhythms based on the 24-hour day and behavioral activity patterns (i.e, circadian misalignment). Circadian misalignment has a significant detrimental effect on cell, tissue, and whole-organism function. These alterations can manifest in humans as chronic health conditions, such as metabolic syndrome,[1] diabetes, cardiovascular disease, cancer, and intestinal disorders (Karlsson et al. 2001; Morikawa et al. 2005; Schernhammer et al. 2003; Penev et al. 1998; Caruso et al. 2004). The increased prevalence of diseases associated with circadian disruption underscores the need to better understand how circadian disruption can wreak havoc in so many different ways throughout the body.

[1] Metabolic syndrom is a combination of disorders that can lead to diabetes and cardiovascular disease, characterized by abnormal levels of fat and/or cholesterol in the blood and insulin resistance.

Central and Peripheral Circadian Rhythms

The master or central circadian clock (i.e., "pacemaker") is located in the suprachiasmatic nucleus (SCN) in the anterior hypothalamus in the brain (Turek 1981) (see figure 1). The SCN is regulated by light stimulating retinal ganglion cells in the eye (Berson et al. 2002), and it is by this mechanism that light directs central circadian rhythms. Circadian rhythms are found in nearly every cell in the body, including the periphery, encompassing the immune system, heart, adipose tissue, pancreas, and liver (Allaman-Pillet et al. 2004; Boivin et al. 2003; Storch et al. 2002;

Yoo et al. 2004; Zvonic et al. 2006). The SCN synchronizes circadian rhythms found in the periphery (figure 2A) via several mechanisms, including communication with nerve cells that influence visceral functions such as digestion, heart rate, etc., via direct release of the hormones oxytocin and vasopression into the general vasculature or indirectly via release of local signals that affect the release of hormones from the anterior pituitary gland (i.e., neuroendocrine and autonomic neurons) (Buijs et al. 2003). In addition, peripheral circadian rhythms can be regulated by external factors other than central light-entrained rhythms. For instance, abnormal feeding patterns can cause peripheral circadian rhythms (i.e., in the intestine and liver) to become misaligned with central rhythms if feeding is out of synch with the normal 24-hour pattern, a phenomenon that can be observed in both animals and humans (see figure 2B). Peripheral tissues express self-sustained rhythms that are able to function independent of the central clock in the SCN. For example, following SCN lesion that terminates central circadian rhythmicity, peripheral circadian clocks continue to demonstrate rhythmicity; however, peripheral rhythms become desynchronized from each other over time (Yoo et al. 2004) (see figure 2C). This internal misalignment is particularly detrimental because peripheral circadian clocks directly regulate up to 5 to 20 percent of the genome (i.e., so-called clock-controlled genes) (Bozek et al. 2009). Furthermore, reports indicate that 3 to 20 percent of the entire genome demonstrates 24-hour oscillations in gene expression, including genes critical for metabolic processes. This observation suggests that although not directly controlled by the circadian clock, genes are influenced as a consequence of rhythmic changes in transcription factors and transcriptional (i.e., the process of creating a complementary RNA copy of a sequence of DNA) and translational (i.e., when RNA is used to produce a specific protein) modifiers (i.e., proteins controlling the levels and activity of various processes including lipid metabolism and glucose synthesis) (Panda et al. 2002).

At the cellular level, circadian rhythms originate from self-sustained, autoregulated, cyclic expressions of clock genes, which constitute the molecular clock. The molecular circadian clock consists of transcriptional activators and repressors—that is, proteins that stimulate and repress the production of proteins, respectively, in a cyclic process that is approximately 24-hours in duration (Reppert and Weaver 2002). The molecular circadian cycle is initiated when the transcriptional activators Clock and Bmal1 (Bunger et al. 2000) combine (i.e., heterodimerize) to stimulate the transcription of target circadian genes including period (*Per*) and cryptochrome (*Cry*) (i.e., *Per1* to *Per3* and *Cry1* and *Cry2*) as well as a host of other clock-controlled genes. When PER and CRY proteins accumulate in the cytosol, they heterodimerize and translocate to the nucleus where they act as transcriptional repressors to terminate CLOCK-BMAL1–mediated transcription, thus ending the molecular circadian cycle (van der Horst et al. 1999) (see figure 3). The cycle is further regulated by additional proteins, including the enzyme sirtuin 1 (SIRT1), a histone deacetylase that modifies circadian proteins or DNA by removing acetyl groups to alter gene expression. SIRT1 is sensitive to levels of the coenzyme nicotinomide adenine dinucleotide (NAD^+), making NAD availability a potential regulator of the molecular circadian clock (Grimaldi et al. 2009). The details of this oscillating cycle are found elsewhere (Reppert and Weaver 2002).

Demonstrating the importance of the molecular circadian clock, mutations of the core circadian clock components can have a devastating effect on the function of the circadian clock. This is true for both *Bmal1* (Bunger et al.

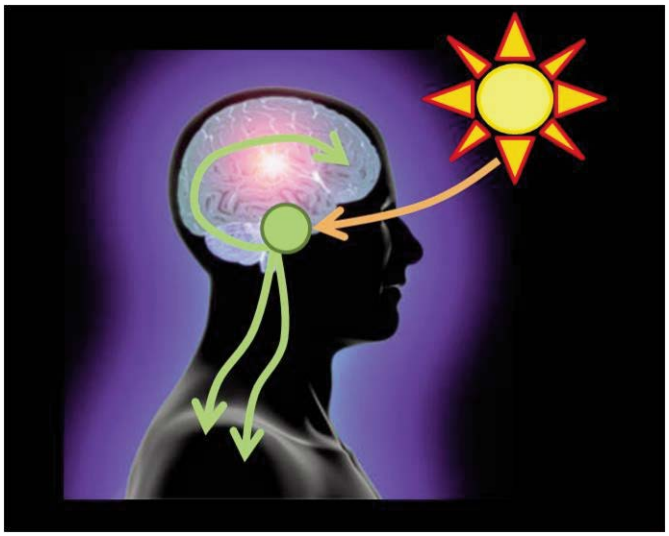


Figure 1 The suprachiasmatic nucleus (SCN) is the central circadian pacemaker. The SCN is located in the hypothalamus and is regulated by light signals from the eye. The SCN then affects a wide variety of physiological and behavioral outcomes.

2000) and *Clock* (Oishi et al. 2006). Likewise, molecular perturbation of the circadian clock (i.e., altering the *Clock, Bmal1, Per1, Per2, Cry1,* or *Cry2* expression via genetic manipulations including deleting or mutating the gene of interest to affect the levels of functional protein produced) disrupts normal circadian behavioral rhythms (Antoch et al. 1997; Bunger et al. 2000; van der Horst et al. 1999; Zheng et al. 2001). This article will discuss the influence of alcohol on circadian rhythms and how circadian-rhythm disruption affects immune function and metabolism, significant factors for alcohol-associated poor health outcomes. It also will discuss potential epigenetic mechanisms by which circadian disruption and alcohol may establish long-term changes in gene expression, resulting in adverse health outcomes.

Alcohol and Circadian Rhythmicity

Circadian organization and stable circadian rhythms are vital for optimal health as numerous diseases are associated with circadian-rhythm disruption. Environmental factors such as shift work or jet lag are obvious disrupters of circadian rhythmicity. However, other environmental factors, such as alcohol consumption and the timing of food intake, can profoundly disrupt and disorganize circadian rhythmicity, which can be observed on behavioral, cellular, and molecular levels.

Alcohol Disrupts Behavioral and Biological Circadian Rhythms

Alcohol has a dramatic effect on circadian rhythms. These circadian abnormalities include disrupted sleep/wake

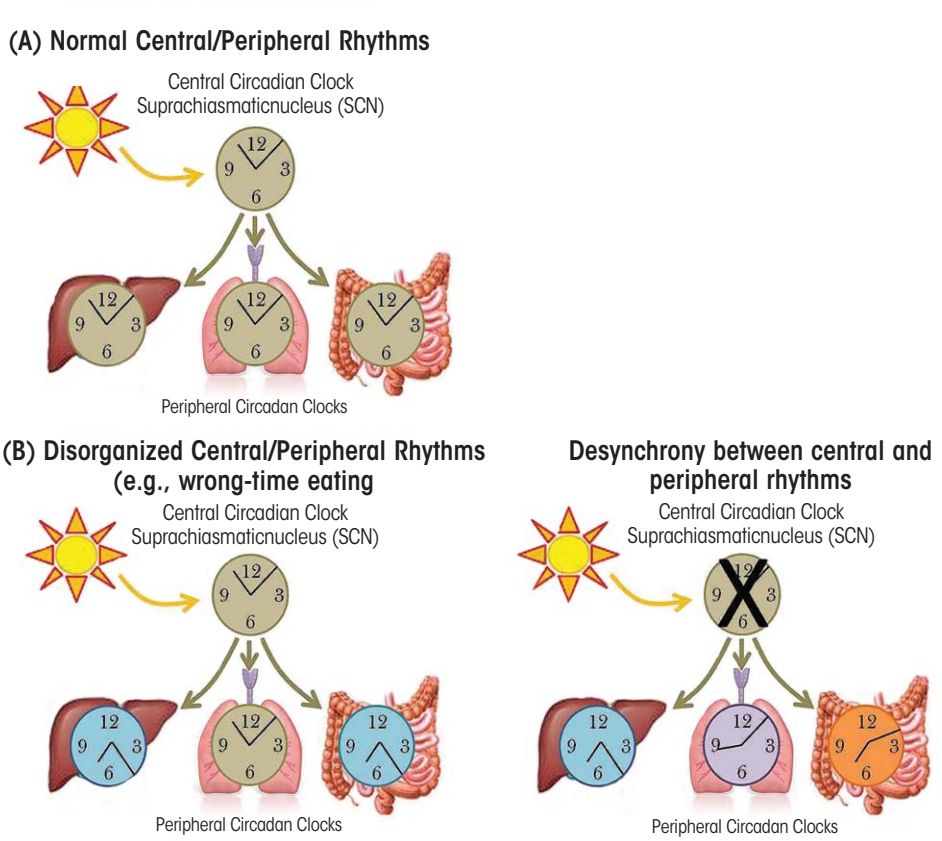


Figure 2 Central and peripheral circadian rhythms. **(A)** Under normal conditions, the central circadian clock in the suprachiasmatic nucleus which is entrained by light, then regulates peripheral circadian clocks. **(B)** Wrong-time eating can cause misalignment between the central circadian clock (entrained by light) and the peripheral circadian clocks entrained by food (illustrated here are intestine and liver). **(C)** When the central circadian clock is disrupted (e.g., due to lesion) peripheral circadian clocks will continue to cycle but will gradually become more misaligned with each other.

cycles in humans (Brower 2001; Imatoh et al. 1986) as well as disrupted circadian responses to light and abnormal activity patterns in rodents (Brager et al. 2010; Rosenwasser et al. 2005). The changes observed in behavioral patterns and responses to light may be the consequence of alcohol-induced disruption of normal tissue/organ function and neuroendocrine function. For example, normal cyclic patterns associated with body temperature (i.e., thermoregulation) (Crawshaw et al. 1998), blood pressure (Kawano et al. 2002), and characteristics of biochemical circadian rhythms including glucose and cholesterol rhythms (Rajakrishnan et al. 1999) are significantly affected by alcohol consumption. In addition, the circadian-driven production of hormones including melatonin (i.e., an endocrine hormone that is important in circadian entrainment) in rats (Peres et al. 2011) and humans (Conroy et al. 2012), corticosterone (i.e., a steroid hormone produced by the adrenal gland that responds to stress and regulates metabolism) (Kakihana and Moore 1976), and pro-opiomelanocortin (i.e., a polypeptide hormone that is a precursor to several hormones) (Chen et al. 2004) are disrupted by alcohol consumption. Alcohol-induced changes such as these have a profound impact on the functioning of a wide variety of peripheral organs and biological processes, which are dependent upon central circadian synchronization for proper function.

Alcohol Disrupts the Molecular Circadian Clock

Not surprisingly, the changes observed in the behavioral and biological systems also are observed on the molecular level as a disrupted molecular circadian clock, an effect that is evident both in vitro and in vivo. Exposure of intestinal epithelial cells (i.e., Caco-2 cells, a widely used model of the human intestinal barrier) to alcohol increases the levels of circadian clock proteins CLOCK and PER2 (Swanson et al. 2011). Likewise, alcohol-fed mice have disrupted expression of *Per1–Per3* in the hypothalamus (Chen et al. 2004), human alcoholics demonstrate markedly lower expression of *Clock, BMAL1, Per1, Per2, Cry1,* and *Cry2* in peripheral blood mononuclear cells (i.e., immune cells) compared with nonalcoholics (Huang et al. 2010), and in humans alcohol consumption is inversely correlated to BMAL1 expression in peripheral blood cells (Ando et al. 2010). The alcohol-induced changes seem to have long-lasting effects on the circadian clock, particularly when the exposure occurs early in life, which may be the consequence of epigenetic modifications (discussed below). For example, neonatal alcohol exposure in rats disrupts normal circadian-clock expression levels and expression patterns over a 24-hour period (i.e., rhythmicity) (Chen et al. 2006; Farnell et al. 2008). These examples illustrate the ability of alcohol to have profound and long-lasting effects on clock-gene expression in multiple organs and tissues.

Feed-Forward Cycle: Alcohol Promotes Circadian Disruption and Circadian Disruption Promotes Alcohol Consumption

Interestingly, circadian-clock disruption can promote alcohol consumption, which can further exacerbate this cycle. For example, *Per2* mutant mice exhibit increased alcohol consumption compared with wild-type counterparts (Spanagel et al. 2005), an effect attributed to altered reinforcement systems leading to enhanced motivation to consume alcohol. This may explain why humans with circadian disruption are more prone to substance abuse disorders (Trinkoff and Storr 1998). This phenomenon also sets up a potentially devastating cycle in which circadian disruption drives alcohol consumption, which further exacerbates circadian disruption.

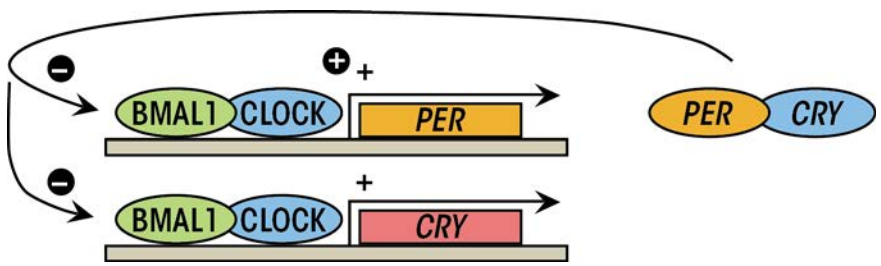


Figure 3 The molecular circadian clock. Transcription of the clock-controlled genes, including *Per* and *Cry* is initiated by the heterodimerization and binding of BMAL1 and CLOCK (the positive limb of the molecular circadian clock). Once sufficient amounts of PER and CRY have been produced, they dimerize and inhibit further BMAL1/CLOCK-mediated transcription (the negative limb of the molecular circadian clock).

Mechanisms of Alcohol-Induced Circadian Disruption

The mechanisms by which alcohol disrupts circadian rhythmicity are likely a consequence of alcohol metabolism and alcohol-induced changes in intestinal barrier integrity.

Consequences of Alcohol Metabolism

Alcohol is metabolized via several mechanisms, including the enzymes catalase, alcohol dehydrogenase (ADH), and cytochrome P450 (CYP2E1) (Lu and Cederbaum 2008). Although alcohol metabolism most prominently occurs in the liver, other tissues such as the stomach, intestine, and brain also play a role in this process. One consequence of alcohol metabolism that is particularly relevant for alcohol-induced disruption of circadian rhythmicity is a shift in the cellular NAD^+/NADH ratio. SIRT1, which regulates the molecular circadian clock, is highly sensitive to the cellular NAD^+/NADH ratio. Therefore, a perturbation in the availability of NAD^+ (e.g., as a consequence of alcohol metabolism by ADH or as a consequence of aldehyde metabolism by acetaldehyde) would be one mechanism by which alcohol could disrupt the molecular circadian clock and resulting circadian rhythms.

Alcohol, the Intestine, and Inflammation

Another mechanism by which alcohol can exert a negative influence on circadian rhythmicity is by promoting intestinal hyperpermeability. Alcohol disrupts intestinal barrier integrity in vitro (Swanson et al. 2011), in rodents (Keshavarzian et al. 2009), and humans (Keshavarzian et al. 1994, 1999). Intestinal hyperpermeability allows luminal bacterial contents such as endotoxin (e.g., lipopolysaccharide (LPS) to translocate through the intestinal epithelium into the systemic circulation. Endotoxin can disrupt circadian rhythms. LPS administered to rodents impairs the expression of *Per* in the heart, liver, SCN, and hypothalamus (Okada et al. 2008; Yamamura et al. 2010) and suppresses clock gene expression in human peripheral blood leukocytes (Haimovich et al. 2010). Thus, intestinal-derived LPS may be one mechanism by which alcohol disrupts circadian rhythmicity. In addition, LPS elicits a robust immune response in the periphery (Andreasen et al. 2008), and systemic inflammation disrupts normal circadian rhythmicity (Coogan and Wyse 2008). For example, tumor necrosis factor α (TNFα), a cytokine produced in response to endotoxins, disrupts normal locomotor behavior and sleep/wake cycles and alters expression of the molecular circadian clock in the liver (Cavadini et al. 2007). Thus, there are several plausible mechanisms by which alcohol-induced effects on the intestine may disrupt central and peripheral circadian rhythms.

Intestinal-derived LPS may be one mechanism by which alcohol disrupts circadian rhythmicity.

It is clear that alcohol-induced effects on the intestine are highly detrimental to circadian rhythmicity. Interestingly, the reverse also is true in that the molecular circadian clock in the intestine influences alcohol-induced effects. Intestinal circadian rhythms are largely driven by feeding patterns (Hoogerwerf et al. 2007; Scheving 2000) and even the apical junctional complex (AJC) proteins, which regulate tight junctions (and thus intestinal permeability), are clock controlled in the kidney (Yamato et al. 2010). Alcohol exposure increases intestinal circadian gene expression, and knocking out *Clock* or *Per2* in intestinal epithelial cells (i.e., Caco-2 cells) prevents alcohol-induced intestinal hyperpermeability (Swanson et al. 2011). Taken together, alcohol—via metabolism products or intestine effects including endotoxemia and systemic inflammation—disrupts intestinal circadian rhythms, an effect that can further exacerbate internal misalignment.

Circadian Rhythms and Immune Function

The immune system demonstrates robust circadian rhythmicity with daily variations in immune parameters, including lymphocyte proliferation, antigen presentation, and cytokine gene expression (Fortier et al. 2011; Levi et al. 1991). These rhythms seem to be sensitive to perturbations in circadian homeostasis, with differential effects depending on the cell type, model system, and outcome measure. For example, inhibition of *Per2* in natural killer (NK) cells (part of the innate immune system) decreases the expression of the immune effectors granzyme-B and porforin (i.e., critical cytotoxic components) (Arjona and Sarkar 2006*a*). Despite these changes, selective reduction of *Per2* in NK cells does not effect NK rhythmic production of the cytokine interferon-γ (IFNγ), which is important for the formation and release of reactive oxygen species. In contrast, whole-animal *Per2*-deficient mice have drastically disrupted IFNγ rhythms (Arjona and Sarkar 2006*b*). The IFNγ rhythmic disruption in *Per2*-deficient mice but not after selective reduction of *Per2* in isolated NK cells would be expected if IFNγ is dependent upon other circadian parameters, such as circadian fluctuations in hormones or temperature. Indeed, rhythmic hormones such as glucocorticoids and melatonin, which are significantly affected by circadian disruption, modulate immune function (Dimitrov et al. 2004; Srinivasan et al. 2005). *Per2*-deficient mice also demonstrate blunted LPS-induced septic shock compared with wild-type mice (Liu et al. 2006), indicating a

functional change that has important biological implications. These studies demonstrate the significant disturbances that can occur as a consequence of a disrupted molecular circadian clock.

In addition to genetically manipulating circadian homeostasis, environmentally disrupting circadian rhythms also negatively affects immune function. For example, loss of regular sleep/wake cycles alters the normal circadian rhythmicity observed in immune cells (Bryant et al. 2004; Vgontzas et al. 2004) and increases the susceptibility to infections (Everson 1993; Mohren et al. 2002). Indeed, chronically shifting light/dark cycles in mice augments LPS-induced immune response, resulting in greater mortality compared with non–circadian-disrupted mice (Castanon-Cervantes et al. 2010).

Taken together, these studies provide evidence that circadian disruption can significantly, and typically negatively, influence immune function. Therefore, alcohol-induced circadian disruption may be a susceptibility factor for immune dysregulation, which may promote alcohol-associated inflammatory processes. Furthermore, the altered response to LPS has particular relevance in light of the alcohol-induced effects on intestinal permeability.

Circadian Rhythms and Metabolic Syndrome

Although only a few metabolic genes are direct targets of circadian genes (Noshiro et al. 2007; Panda et al. 2002), the direct targets do include many transcription factors and other modulators of transcription and translation. These clock-controlled genes include factors regulating lipid and cholesterol biosynthesis, carbohydrate metabolism, oxidative phosphorylation, and glucose levels (Oishi et al. 2003; Panda et al. 2002).

Eating is an environmental factor that selectively affects peripheral circadian rhythmicity in the intestine and liver. Feeding at the incorrect time (e.g., late-night eating for humans) can result in internal circadian misalignment. For example, restricted feeding paradigms in which animals only have access to food during inappropriate times (i.e., during the light cycle for nocturnal rodents) results in misalignment between central light-entrained circadian rhythms (i.e., in the SCN) and peripheral food-entrained circadian rhythms, including those in the liver (Damiola et al. 2000). Recent studies suggest that this internal misalignment scenario is linked to weight gain, obesity, and metabolic syndrome. Indeed, mice fed during the inappropriate time gain more weight (Arble et al. 2009; Salgado-Delgado et al. 2010) than mice fed during appropriate time, despite similar activity levels and caloric intake (Arble et al. 2009). This phenomenon also is observed in humans; people who skip breakfast and have eating patterns shifted toward late-night eating tend to be more overweight than those who consume food during more appropriate time periods (Berkey et al. 2003; Ma et al. 2003).

Feeding at the incorrect time (e.g., late-night eating for humans) can result in internal circadian misalignment.

Genetic abnormalities in the molecular circadian clock also are associated with metabolic disorders, including obesity, metabolic syndrome, and diabetes (Scott et al. 2008; Woon et al. 2007). For example, *Clock* mutant mice, which have disrupted circadian rhythms (Vitaterna et al. 1994), are obese and demonstrate characteristics of metabolic syndrome such as high cholesterol levels and high blood glucose (Turek et al. 2005). *Bmal1* mutant mice also have disrupted circadian rhythmicity (Bunger et al. 2000), disrupted adipogenesis (Shimba et al. 2005), and demonstrate markers of metabolic syndrome (e.g., higher levels of triglycerides and glucose) (Marcheva et al. 2010; Rudic et al. 2004). Similarly, mutations in *Cry* genes disrupt hormonal rhythms (Fu et al. 2005; Yang et al. 2009) and *Cry* mutants show markers of metabolic syndrome (Okano et al. 2009). It should be noted that although some of these mutant mice demonstrate disrupted locomotion and feeding behaviors (i.e., wrong-time feeding), the abnormalities seem to be attributable to mutations in the circadian clock machinery rather than to appropriate feeding times because mice (e.g., *Bmal1* mutant mice) that do exhibit normal activity/feeding patterns still exhibit markers of metabolic syndrome (Lamia et al. 2008; Marcheva et al. 2010).

In addition to these effects of circadian rhythms on indices of metabolism, it is also important to consider the effect of circadian disruption on the immune system because chronic inflammation is a prominent feature associated with metabolic syndrome. Thus, the immune dysfunction that occurs upon circadian rhythm disruption may be a predisposing or exacerbating factor for metabolic syndrome.

Epigenetic Alterations: Circadian Rhythm Disruption and Alcohol

Epigenetics is the study of stable changes in gene expression that do not involve DNA sequence modifications but rather are the consequence of processes such as DNA methylation, histone modification (i.e., acetylation, methylation, phosphorylation, ubiquitinylation, ADP-ribosylation, and sumoylation), and noncoding microRNAs (miRNAs). These changes in gene expression are critical to optimize cellular function and for cellular development and differentiation. However, epigenetic changes also occur in response to environmental changes,

including circadian rhythm disruption and alcohol use.

Shift work (i.e., chronic circadian disruption) is associated with an increased incidence of cancer. Potential mechanisms for this relationship include changes in melatonin levels and levels of circadian clock genes (Straif et al. 2007). However, epigenetics also may influence circadian rhythm disruption and thereby affect cellular function. Indeed, long-term shift work affects promoter methylation of the circadian genes *Clock* and *Cry2* (Zhu et al. 2011) with increased methylation of *Clock* (Hoffman et al. 2010*a*) and decreased methylation of *Cry* (Hoffman et al. 2010*b*) observed in cancer patients. Epigenetic changes also occur as a consequence of chronic circadian disruption in the promoter regions of genes encoding glucocorticoid receptors (important for hypothalamic–pituitary–adrenal axis function), TNFα (a cytokine critical for cell functioning and inflammation), and IFNγ (Bollati et al. 2010). Changes such as these may play a critical role in how chronic circadian disruption promotes cancer, inflammation, and metabolic disorders.

In addition to circadian-disruption–induced epigenetic changes, alcohol consumption is also associated with epigenetic modifications. Alcohol-induced DNA acetylation is observed in vitro in rat hepatocytes (Park et al. 2003), in vivo in rat hepatic stellate cells (Kim and Shukla 2005, 2006), lung, spleen, and testes (Kim and Shukla 2006). Similar to the increased cancer risk associated with chronic circadian disruption, alcohol-induced epigenetic changes are associated with the development of cancer. Indeed, colorectal cancer in high-alcohol– consuming humans is associated with high levels of promoter hypermethylation of several relevant genes when compared with low- or no-alcohol– consuming counterparts with colorectal cancer (van Engeland et al. 2003; Giovannucci et al. 1995). Similarly, alcohol-consuming individuals with head and neck cancer have hypermethylated gene promoters for specific genes of interest compared with non-alcohol–drinking individuals (Puri et al. 2005) and alcohol-dependent humans have hypermethylation of liver and peripheral blood cell DNA. Thus, it seems that both circadian disruption and alcohol consumption can affect long-term changes in gene expression via epigenetic modifications that may impact a wide variety of health outcomes.

Summary and Future Directions

Circadian rhythms are a prominent and critical feature of cells, tissues, organs, and behavior that help an organism function most efficiently and anticipate things such as food availability. Therefore, it is not surprising that disrupted circadian rhythms or misalignment between central and peripheral circadian rhythms predispose and/or exacerbate a wide variety of diseases, including alcohol-associated disorders. One environmental factor that has been shown to have a disruptive effect on circadian rhythms is alcohol consumption. This disruption occurs at the molecular levels (i.e., changes in the expression levels of the circadian clock genes), also affects tissues and organs (e.g., changes in the cyclic pattern of hormones), and leads to overt behavioral changes. Thus, in the context of alcoholism, disrupted circadian rhythms may create a positive feedback loop that markedly exaggerates alcohol-induced immune/inflammatory-mediated diseases by (1) negatively influencing immune function and (2) promoting alcohol consumption that leads to further circadian-rhythm disruption. These changes are highly relevant because circadian-rhythm disruption has a substantial impact on immune function, which in turn has important implications for a wide variety of pathological conditions, including metabolic syndrome. A better understanding of how circadian rhythms influence such a wide variety of systems and bodily functions and how environmental factors such as alcohol use influence these processes is vital to our ever more circadian-disrupted society.

A better understanding of the mechanisms by which circadian disruption affects health outcomes such as cancer, inflammation, metabolic disease, and alcohol-induced pathology is critical. This information may lead to the development of chronotherapeutic approaches to prevent and/or treat a wide variety of conditions that are promoted or exacerbated by circadian-rhythm disruption and may lead to better risk stratification for individuals who are at risk for developing chronic conditions. Going forward, characterizing the epigenetic modifications that occur during chronic circadian disruption may be critical for understanding not only how disruption affects an individual but also how these modifications are passed on to offspring, which may influence the health of future generations. Thus, the issue of circadian disruption is vitally important for the health and well-being of current and future generations. ■

Acknowledgments

This work was supported in part by National Institutes of Health grants RC2–AA–019405 (to Dr. Keshavarzian) and AA–020216 (to Drs. Keshavarzian and Forsyth).

Financial Disclosure

The authors declare that they have no competing financial interests.

References

Allaman-Pillet, N.; Roduit, R.; Oberson, A.; et al. Circadian regulation of islet genes involved in insulin production and secretion. *Molecular and Cellular Endocrinology* 226:59–66, 2004. PMID: 15489006

Ando, H.; Ushijima, K.; Kumazaki, M.; et al. Associations of metabolic parameters and ethanol consumption with messenger RNA expression of clock genes in healthy men. *Chronobiology International* 27:194–203, 2010. PMID: 20205566

Andreasen, A.S.; Krabbe, K.S.; Krogh-Madsen, R.; et al. Human endotoxemia as a model of systemic inflamma-

tion. *Current Medicinal Chemistry* 15:1697–1705, 2008. PMID: 18673219

Antoch, M.P.; Song, E.J.; Chang, A.M.; et al. Functional identification of the mouse circadian Clock gene by transgenic BAC rescue. *Cell* 89:655–667, 1997. PMID: 9160756

Arble, D.M.; Bass, J.; Laposky, A.D.; et al. Circadian timing of food intake contributes to weight gain. *Obesity (Silver Spring)* 17:2100–2102, 2009. PMID: 19730426

Arjona, A, and Sarkar, D.K. Evidence supporting a circadian control of natural killer cell function. *Brain, Behavior, and Immunity* 20:469–476, 2006a. PMID: 16309885

Arjona, A., and Sarkar, D.K. The circadian gene mPer2 regulates the daily rhythm of IFN-gamma. *Journal of Interferon & Cytokine Research* 26:645-649, 2006b. PMID: 16978068

Berkey, C.S.; Rockett, H.R.; Gillman, M.W.; et al. Longitudinal study of skipping breakfast and weight change in adolescents. *International Journal of Obesity Related Metabolic Disorders* 27:1258–1266, 2003. PMID: 14513075

Berson, D.M.; Dunn, F.A.; and Takao, M. Phototransduction by retinal ganglion cells that set the circadian clock. *Science* 295:1070–1073, 2002. PMID: 11834835

Boivin, D.B.; James, F.O.; Wu, A.; et al. Circadian clock genes oscillate in human peripheral blood mononuclear cells. *Blood* 102:4143–4145, 2003. PMID: 12893774

Bollati, V.; Baccarelli, A.; Sartori, S.; et al. Epigenetic effects of shiftwork on blood DNA methylation. *Chronobiology International 27:1093–1104, 2010. PMID: 20636218*

Bozek, K.; Relogio, A.; Kielbasa, S.M.; et al. Regulation of clock-controlled genes in mammals. *PLoS One* 4:e4882, 2009. PMID: 19287494

Brager, A.J.; Ruby, C.L.; Prosser, R.A.; and Glass, J.D. Chronic ethanol disrupts circadian photic entrainment and daily locomotor activity in the mouse. *Alcoholism: Clinical and Experimental Research* 34:1266–1273, 2010. PMID: 20477766

Brower, K.J. Alcohol's effects on sleep in alcoholics. *Alcohol Research & Health* 25:110–125, 2001. PMID: 11584550

Bryant, P.A.; Trinder, J.; and Curtis, N. Sick and tired: Does sleep have a vital role in the immune system? *Nature Reviews. Immunology* 4:457–467, 2004. PMID: 15173834

Buijs, R.M.; van Eden, C.G.; Goncharuk, V.D.; and Kalsbeek, A. The biological clock tunes the organs of the body: Timing by hormones and the autonomic nervous system. *Journal of Endocrinology* 177:17–26, 2003. PMID: 12697033

Bunger, M.K.; Wilsbacher, L.D.; Moran, S.M.; et al. Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell* 103:1009–1017, 2000. PMID: 11163176

Bureau of Labor Statistics. *Workers on Flexible Work Schedules in May 2004.* Washington, DC, U.S. Department of Labor, 2005.

Caruso, C.C.; Lusk, S.L.; and Gillespie, B.W. Relationship of work schedules to gastrointestinal diagnoses, symptoms, and medication use in auto factory workers. *American Journal of Industrial Medicine* 46:586–598, 2004. PMID: 15551368

Castanon-Cervantes, O.; Wu, M.; Ehlen, J.C.; et al. Dysregulation of inflammatory responses by chronic circadian disruption. *Journal of Immunology* 185:5796–5805, 2010. PMID: 20944004

Cavadini, G.; Petrzilka, S.; Kohler, P.; et al. TNF-alpha suppresses the expression of clock genes by interfering with E-box-mediated transcription. *Proceedings of the National Academy of Sciences of the United States of America* 104:12843–12848, 2007. PMID: 17646651

Chen, C.P.; Kuhn, P.; Advis, J.P.; and Sarkar, D.K. Chronic ethanol consumption impairs the circadian rhythm of pro-opiomelanocortin and period genes mRNA expression in the hypothalamus of the male rat. *Journal of Neurochemistry* 88:1547–1554, 2004. PMID: 15009656

Chen, C.P.; Kuhn, P.; Advis, J.P.; and Sarkar, D.K. Prenatal ethanol exposure alters the expression of period genes governing the circadian function of beta-endorphin neurons in the hypothalamus. *Journal of Neurochemisty* 97:1026–1033, 2006. PMID: 16686691

Conroy, D.A.; Hairston, I.S.; Arnedt, J.T.; et al. Dim light melatonin onset in alcohol-dependent men and women compared with healthy controls. *Chronobiology International* 29:35–42, 2012. PMID: 22217099

Coogan, A.N., and Wyse, C.A. Neuroimmunology of the circadian clock. *Brain Research* 1232:104–112, 2008. PMID: 18703032

Crawshaw, L.I.; Wallace, H.; and Crabbe, J. Ethanol, body temperature and thermoregulation. *Clinical and Experimental Pharmacology & Physiology* 25:150–154, 1998. PMID: 9493506

Damiola, F.; Le Minh,N.; Preitner, N.; et al. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes & Development* 14:2950–2961, 2000. PMID: 11114885

Dimitrov, S.; Lange, T.; Fehm, H.L.; and Born, J. A regulatory role of prolactin, growth hormone, and corticosteroids for human T-cell production of cytokines. *Brain, Behavior, and Immunity* 18:368–374, 2004. PMID: 15157954

Everson, C.A. Sustained sleep deprivation impairs host defense. *American Journal of Physiology* 265(5 Pt. 2):R1148–R1154, 1993. PMID: 8238617

Farnell, Y.Z.; Allen, G.C.; Nahm, S.S.; et al. Neonatal alcohol exposure differentially alters clock gene oscillations within the suprachiasmatic nucleus, cerebellum, and liver of adult rats. *Alcoholism: Clinical and Experimental Research* 32:544–552, 2008. PMID: 18215209

Fortier, E.E.; Rooney, J.; Dardente, H.; et al. Circadian variation of the response of T-cells to antigen. *Journal of Immunology* 187:6291–6300, 2011. PMID: 22075697

Fu, L.; Patel, M.S.; Bradley, A.; et al. The molecular clock mediates leptin-regulated bone formation. *Cell* 122: 803–815, 2005. PMID: 16143109

Giovannucci, E.; Rimm, E.B.; Ascherio, A.; et al. Alcohol, low-methionine—low-folate diets, and risk of colon cancer in men. *Journal of the National Cancer Institute* 87:265–273, 1995. PMID: 7707417

Grimaldi, B.; Nakahata, Y.; Kaluzova, M.; et al. Chromatin remodeling, metabolism and circadian clocks: The interplay of CLOCK and SIRT1. *International Journal of Biochemistry & Cell Biology* 41:81–86, 2009. PMID: 18817890

Haimovich, B.; Calvano, J.; Haimovich, A.D.; et al. In vivo endotoxin synchronizes and suppresses clock gene expression in human peripheral blood leukocytes. *Critical Care Medicine* 38:751–758, 2010. PMID: 20081528

Hoffman, A.E.; Yi, C.H.; Zheng, T.; et al. CLOCK in breast tumorigenesis: Genetic, epigenetic, and transcriptional profiling analyses. *Cancer Research* 70:1459–1468, 2010a. PMID: 20124474

Hoffman, A.E.; Zheng, T.; Yi, C.H.; et al. The core circadi an gene Cryptochrome 2 influences breast cancer risk, possibly by mediating hormone signaling. *Cancer Prevention Research* (Philadelphia) 3:539–548, 2010b. PMID: 20233903

Hoogerwerf, W.A.; Hellmich, H.L.; Cornelissen, G.; et al. Clock gene expression in the murine gastrointestinal tract: Endogenous rhythmicity and effects of a feeding regimen. *Gastroenterology* 133:1250–1260, 2007. PMID: 17919497

Huang, M.C.; Ho, C.W.; Chen, C.H.; et al. Reduced expression of circadian clock genes in male alcoholic patients. *Alcoholism: Clinical and Experimental Research* 34:1899–1904, 2010. PMID: 20735373

Imatoh, N.; Nakazawa, Y.; Ohshima, H.; et al. Circadian rhythm of REM sleep of chronic alcoholics during alcohol withdrawal. *Drug and Alcohol Dependence* 18:77–85, 1986. PMID: 3780412

Kakihana, R., and Moore, J.A. Circadian rhythm of corticosterone in mice: The effect of chronic consumption of alcohol. *Psychopharmacologia* 46:301–305, 1976. PMID: 986057

Karlsson, B.; Knutsson, A.; and Lindahl, B. Is there an association between shift work and having a metabolic syndrome? Results from a population based study of 27,485 people. *Occupational and Environmental Medicine* 58:747–752, 2001. PMID: 11600731

Kawano, Y.; Pontes, C.S.; Abe, H.; et al. Effects of alcohol consumption and restriction on home blood pressure in hypertensive patients: Serial changes in the morning and evening records. *Clinical and Experimental Hypertension* 24:33–39, 2002. PMID: 11848167

Keshavarzian, A.; Farhadi, A.; Forsyth, C.B.; et al. Evidence that chronic alcohol exposure promotes intestinal oxidative stress, intestinal hyperpermeability and endotoxemia prior to development of alcoholic steatohepatitis in rats. *Journal of Hepatology* 50:538–547, 2009. PMID: 19155080

Keshavarzian, A.; Fields, J.Z.; Vaeth, J.; and Holmes, E.W. The differing effects of acute and chronic alcohol on gastric and intestinal permeability. *American Journal of Gastroenterology* 89:2205–2211, 1994. PMID: 7977243

Keshavarzian, A.; Holmes, E.W.; Patel, M.; et al. Leaky gut in alcoholic cirrhosis: A possible mechanism for alcohol-induced liver damage. *American Journal of Gastroenterology* 94:200–207, 1999. PMID: 9934756

Kim, J.S., and Shukla, S.D. Histone h3 modifications in rat hepatic stellate cells by ethanol. *Alcohol and Alcoholism* 40:367–372, 2005. PMID: 15939707

Kim, J.S., and Shukla, S.D. Acute in vivo effect of ethanol (binge drinking) on histone H3 modifications in rat tissues. *Alcohol and Alcoholism* 41:126–132, 2006. PMID: 16314425

Lamia, K.A.; Storch, K.F.; and Weitz, C.J. Physiological significance of a peripheral tissue circadian clock. *Proceedings of the National Academy of Sciences of the United States of America* 105:15172–15177, 2008. PMID: 18779586

Levi, F.; Canon, C.; Dipalma, M.; et al. When should the immune clock be reset? From circadian pharmacodynamics to temporally optimized drug delivery. *Annals of the New York Academy of Sciences* 618:312–329, 1991. PMID: 2006792

Liu, J.; Malkani, G.; Shi, X.; et al. The circadian clock Period 2 gene regulates gamma interferon production of NK cells in host response to lipopolysaccharide-induced endotoxic shock. *Infections and Immunity* 74:4750–4756, 2006. PMID: 16861663

Lu, Y., and Cederbaum, A.I. CYP2E1 and oxidative liver injury by alcohol. *Free Radical Biology & Medicine* 44:723–738, 2008. PMID: 18078827

Ma, Y.; Bertone, E.R.; Stanek, E.J., 3rd; et al. Association between eating patterns and obesity in a free-living US adult population. *American Journal of Epidemiology* 158:85–92, 2003. PMID: 12835290

Marcheva, B.; Ramsey, K.M.; Buhr, E.D.; et al. Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes. *Nature* 466:627–631, 2010. PMID: 20562852

Mohren, D.C.; Jansen, N.W.; Kant, I.J.; et al. Prevalence of common infections among employees in different work schedules. *Journal of Occupational Environmental Medicine* 44:1003–1011, 2002. PMID: 12449906

Morikawa, Y.; Nakagawa, H.; Miura, K.; et al. Shift work and the risk of diabetes mellitus among Japanese male factory workers. *Scandinavian Journal of Work, Environment & Health* 31:179–183, 2005. PMID: 15999569

Noshiro, M.; Usui, E.; Kawamoto, T.; et al. Multiple mechanisms regulate circadian expression of the gene for cholesterol 7alpha-hydroxylase (Cyp7a), a key enzyme in hepatic bile acid biosynthesis. *Journal of Biological Rhythms* 22:299–311, 2007. PMID: 17660447

Oishi, K.; Miyazaki, K.; Kadota, K.; et al. Genome-wide expression analysis of mouse liver reveals CLOCK-regulated circadian output genes. *Journal of Biological Chemistry* 278:41519–41527, 2003. PMID: 12865428

Oishi, K.; Ohkura, N.; Kadota, K.; et al. Clock mutation affects circadian regulation of circulating blood cells. *Journal of Circadian Rhythms* 4:13, 2006. PMID: 17014730

Okada, K.; Yano, M.; Doki, Y.; et al. Injection of LPS causes transient suppression of biological clock genes in rats. *Journal of Surgical Research* 145:5–12, 2008. PMID: 18279697

Okano, S.; Akashi, M.; Hayasaka, K.; and Nakajima, O. Unusual circadian locomotor activity and pathophysiology in mutant CRY1 transgenic mice. *Neuroscience Letters* 451:246–251, 2009. PMID: 19159659

Panda, S.; Antoch, M.P.; Miller, B.H.; et al. Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell* 109:307–320, 2002. PMID: 12015981

Park, P.H.; Miller, R.; and Shukla, S.D. Acetylation of histone H3 at lysine 9 by ethanol in rat hepatocytes. *Biochemical and Biophysical Research Communications* 306:501–504, 2003. PMID: 12804592

Penev, P.D.; Kolker, D.E.; Zee, P.C.; and Turek, F.W. Chronic circadian desynchronization decreases the survival of animals with cardiomyopathic heart disease. *American Journal of Physiology* 275(6 Pt 2):H2334–H2337, 1998. PMID: 9843836

Peres, R.; do Amaral, F.G.; Madrigrano, T.C.; et al. Ethanol consumption and pineal melatonin daily profile in rats. *Addiction Biology* 16:580–590, 2011. PMID: 21635669

Puri, S.K.; Si, L.; Fan, C.Y.; and Hanna, E. Aberrant promoter hypermethylation of multiple genes in head and neck squamous cell carcinoma. *American Journal of Otolaryngology* 26:12–17, 2005. PMID: 15635575

Rajakrishnan, V.; Subramanian, P.; Viswanathan, P.; and Menon, V.P. Effect of chronic ethanol ingestion on biochemical circadian rhythms in Wistar rats. *Alcohol* 18:147–152, 1999. PMID: 10456565

Reppert, S.M., and Weaver, D.R. Coordination of circadian timing in mammals. *Nature* 418:935–941, 2002. PMID: 12198538

Rosenwasser, A.M.; Logan, R.W.; and Fecteau, M.E. Chronic ethanol intake alters circadian period-responses to brief light pulses in rats. *Chronobiology International* 22:227–236, 2005. PMID: 16021840

Rudic, R.D.; McNamara, P.; Curtis, A.M.; et al. BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. *PLoS Biology* 2:e377, 2004. PMID: 15523558

Salgado-Delgado, R.; Angeles-Castellanos, M.; Saderi, N.; et al. Food intake during the normal activity phase prevents obesity and circadian desynchrony in a rat model of night work. *Endocrinology* 151:1019–1029, 2010. PMID: 20080873

Schernhammer, E.S.; Laden, F.; Speizer, F.E.; et al. Night-shift work and risk of colorectal cancer in the Nurses' Health Study. *Journal of the National Cancer Institute* 95:825–828, 2003. PMID: 12783938

Scheving, L.A. Biological clocks and the digestive system. *Gastroenterology* 119:536–549, 2000. PMID: 10930389

Scott, E.M.; Carter, A.M.; and Grant, P.J. Association between polymorphisms in the Clock gene, obesity and the metabolic syndrome in man. *International Journal of Obesity (London)* 32:658–662, 2008. PMID: 18071340

Shimba, S.; Ishii, N.; Ohta, Y.; et al. Brain and muscle Arnt-like protein-1 (BMAL1), a component of the molecular clock, regulates adipogenesis. *Proceedings of the National Academy of Sciences of the United States of America* 102:12071–12076, 2005. PMID: 16093318

Spanagel, R.; Pendyala, G.; Abarca, C.; et al. The clock gene Per2 influences the glutamatergic system and modulates alcohol consumption. *Nature Medicine* 11:35–42, 2005. PMID: 15608650

Srinivasan, V.; Maestroni, G.J.; Cardinali, D.P.; et al. Melatonin, immune function and aging. *Immunity & Ageing* 2:17, 2005. PMID: 16316470

Storch, K.F.; Lipan, O.; Leykin, I.; et al. Extensive and divergent circadian gene expression in liver and heart. *Nature* 417:78–83, 2002. PMID: 11967526

Straif, K.; Baan, R.; Grosse, Y.; et al. Carcinogenicity of shift-work, painting, and fire-fighting. *Lancet Oncology* 8:1065–1066, 2007. PMID: 19271347

Swanson, G.; Forsyth, C.B.; Tang, Y.; et al. Role of intestinal circadian genes in alcohol-induced gut leakiness. *Alcoholism: Clinical and Experimental Research* 35:1305–1314, 2011. PMID: 21463335

Trinkoff, A.M., and Storr, C.L. Work schedule characteristics and substance use in nurses. *American Journal of Industrial Medicine* 34:266–271, 1998. PMID: 9698996

Turek, F.W. Are the suprachiasmatic nuclei the location of the biological clock in mammals? *Nature* 292:289–290, 1981. PMID: 7254326

Turek, F.W.; Joshu, C.; Kohsaka, A.; et al. Obesity and metabolic syndrome in circadian Clock mutant mice. *Science* 308:1043–1045, 2005. PMID: 15845877

van der Horst, G.T.; Muijtjens, M.; Kobayashi, K.; et al. Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature* 398:627–630, 1999. PMID: 10217146

Van Engeland, M.; Weijenberg, M.P.; Roemen, G.M.; et al. Effects of dietary folate and alcohol intake on promoter methylation in sporadic colorectal cancer: The Netherlands Cohort Study on Diet and Cancer. *Cancer Research* 63:3133–3137, 2003. PMID: 12810640

Vgontzas, A.N.; Zoumakis, E.; Bixler, E.O.; et al. Adverse effects of modest sleep restriction on sleepiness, performance, and inflammatory cytokines. *Journal of Clinical Endocrinology and Metabolism* 89:2119–2126, 2004. PMID: 15126529

Vitaterna, M.H.; King, D.P.; Chang, A.M.; et al. Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. *Science* 264:719–725, 1994. PMID: 8171325

Woon, P.Y.; Kaisaki, P.J.; Braganca, J.; et al. Aryl hydrocarbon receptor nuclear translocator-like (BMAL1) is associated with susceptibility to hypertension and type 2 diabetes. *Proceedings of the National Academy of Sciences of the United States of America* 104:14412–14417, 2007. PMID: 17728404

Yamamura, Y.; Yano, I.; Kudo, T.; and Shibata, S. Time-dependent inhibitory effect of lipopolysaccharide injection on Per1 and Per2 gene expression in the mouse heart and liver. *Chronobiology International* 27:213–232, 2010. PMID: 20370466

Yamato, M.; Ito, T.; Iwatani, H.; et al. E-cadherin and claudin-4 expression has circadian rhythm in adult rat

kidney. *Journal of Nephrology* 23:102–110, 2010. PMID: 20091493

Yang, S.; Liu, A.; Weidenhammer, A.; et al. The role of mPer2 clock gene in glucocorticoid and feeding rhythms. *Endocrinology* 150:2153–2160, 2009. PMID: 19179447

Yoo, S.H.; Yamazaki, S.; Lowrey, P.L.; et al. PERIOD2:LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proceedings of the National Academy of Sciences of the United States of America* 101:5339–5346, 2004. PMID: 14963227

Zheng, B.; Albrecht, U.; Kaasik, K.; et al. Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. *Cell* 105:683–694, 2001. PMID: 11389837

Zhu, Y.; Stevens, R.G.; Hoffman, A.E.; et al. Epigenetic impact of long-term shiftwork: Pilot evidence from circadian genes and whole-genome methylation analysis. *Chronobiology International* 28:852–861, 2011. PMID: 22080730

Zvonic, S.; Ptitsyn, A.A.; Conrad, S.A.; et al. Characterization of peripheral circadian clocks in adipose tissues. *Diabetes* 55:962–970, 2006. PMID: 16567517

Prenatal Alcohol Exposure and Cellular Differentiation

A Role for Polycomb and Trithorax Group Proteins in FAS Phenotypes?

Kylee J. Veazey; Daria Muller; and Michael C. Golding, Ph.D.

Kylee J. Veazey *and* **Daria Muller** *are graduate assistants, and* **Michael C. Golding, Ph.D.,** *is an assistant professor, all in the Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, Texas.*

Exposure to alcohol significantly alters the developmental trajectory of progenitor cells and fundamentally compromises tissue formation (i.e., histogenesis). Emerging research suggests that ethanol can impair mammalian development by interfering with the execution of molecular programs governing differentiation. For example, ethanol exposure disrupts cellular migration, changes cell–cell interactions, and alters growth factor signaling pathways. Additionally, ethanol can alter epigenetic mechanisms controlling gene expression. Normally, lineage-specific regulatory factors (i.e., transcription factors) establish the transcriptional networks of each new cell type; the cell's identity then is maintained through epigenetic alterations in the way in which the DNA encoding each gene becomes packaged within the chromatin. Ethanol exposure can induce epigenetic changes that do not induce genetic mutations but nonetheless alter the course of fetal development and result in a large array of patterning defects. Two crucial enzyme complexes—the Polycomb and Trithorax proteins—are central to the epigenetic programs controlling the intricate balance between self-renewal and the execution of cellular differentiation, with diametrically opposed functions. Prenatal ethanol exposure may disrupt the functions of these two enzyme complexes, altering a crucial aspect of mammalian differentiation. Characterizing the involvement of Polycomb and Trithorax group complexes in the etiology of fetal alcohol spectrum disorders will undoubtedly enhance understanding of the role that epigenetic programming plays in this complex disorder. **KEY WORDS: Alcohol exposure; ethanol exposure; prenatal alcohol exposure; prenatal alcohol exposure; fetal alcohol spectrum disorders; fetal alcohol syndrome (FAS); FAS phenotypes; fetal development; epigenetics; epigenetic mechanisms; epigenetic changes; gene expression; developmental programming; transcription; cellular differentiation; Polycomb group proteins; Trithorax group proteins**

Exposure of the developing embryo and fetus to alcohol can have profound adverse effects on physical, behavioral, and cognitive development. The resulting deficits collectively have been termed fetal alcohol spectrum disorders (FASD). They range in severity from mild cognitive deficits to a well-defined syndrome (i.e., fetal alcohol syndrome [FAS]), which is broadly characterized by low birth weight, distinctive craniofacial malformations, smaller-than-normal head size (i.e., microcephaly), and central nervous system dysfunction (Riley et al. 2011). The mechanisms underlying ethanol's harmful effects on development are not yet fully understood. Studies in recent years have indicated that epigenetic mechanisms may play a role in the etiology of FASD. This article describes the proposed roles of epigenetic mechanisms in FASD and cell differentiation in general and introduces two protein complexes that are hypothesized to play central roles in these events.

Role of Epigenetics in Developmental Programming and FASD

Mammalian development consists of a series of carefully orchestrated changes in gene expression that occur as stem or progenitor cells differentiate to form

the tissues and organs making up the growing fetus.[1] Once the identity of each new cell type has been established by lineage-specific transcription factors, this identity is maintained through unique alterations in the way in which the DNA encoding each gene becomes packaged around certain proteins (i.e., the histones) within the chromatin structure of the nucleus (Hemberger et al. 2009). Much like a closed book cannot be read whereas an open book can, the DNA can either be tightly wound up into a structure that silences the encoded genes, or the DNA can be in a relaxed, open, and active state. As development proceeds, the DNA of each cell becomes packaged in a way that is unique to that cell type and thus is programmed to express only a specific set of genes that confer the cell's individual identity and physiological function (Barrero et al. 2010). Three enzymatic mechanisms control the assembly and regulation of chromatin structure: DNA methylation, modification of the histone proteins (i.e., posttranslational histone modification), and ATP-dependent chromatin remodeling (Barrero et al. 2010). These fundamental modifications, which control gene packaging, are passed on to the daughter cells when a cell divides. They are referred to as epigenetic changes because they impart a level of regulation that is above ("epi") the direct genetic modifications of the DNA (Hemberger et al. 2009).

Studies using a diverse range of model organisms have led to the conclusion that epigenetic modifications to the chromatin structure provide a plausible link between exposure to environmental substances that can harm the developing fetus (i.e., teratogens) and lasting alterations in gene expression leading to disease phenotypes. Numerous studies have demonstrated that exposure to ethanol is associated with both genome-wide and gene-specific changes in DNA methylation (Bielawski et al. 2002; Downing et al. 2011; Garro et al. 1991; Haycock and Ramsey 2009; Hicks et al. 2010; Liu et al. 2009; Ouko et al. 2009; Zhou et al. 2011), alterations in posttranslational histone modifications (Kim and Shukla 2005; Pal-Bhadra et al. 2007; Park et al. 2005), and a profound shift in epigenetically sensitive phenotypes (Kaminen-Ahola et al. 2010). Collectively, all of these observations indicate that ethanol can act as a powerful epigenetic disruptor and alter chromatin structure.

Although the mechanisms by which alcohol impacts chromatin structure are not completely understood, recent work suggests that some epigenetic changes result from altered cellular metabolism. For example, Choudhury and colleagues (2010) observed an increase in reactive oxygen species (ROS) within primary rat liver cells (i.e., hepatocytes) treated with ethanol. This increase in ROS was correlated with an increase in a specific modification of histone 3 (i.e., acetylation of histone 3 at lysine 9); moreover, when the cells were treated with cellular antioxidants to eliminate the ROS, these alcohol-induced chromatin modifications were abated (Choudhury et al. 2010). In addition, ethanol exposure has well-documented effects on one-carbon metabolism and the bioavailability of the crucial methyl donor, s-adenosylmethionine (SAMe). Impaired levels of SAMe disrupt the cells' ability to methylate DNA and histones, resulting in compromised epigenetic programming (Zeisel 2011). Interestingly, many of the birth defects observed in FASD also have been noted in studies examining deficiencies in one-carbon metabolism (summarized in Zeisel 2011).

Although alcohol induces several global changes in chromatin structure, many of the associated developmental defects seem to be rooted in gene-specific alterations. A study by Hashimoto-Torii and colleagues (2011) examining global changes in gene transcription within ethanol-exposed samples of brain tissue (i.e., cerebral cortex) reported that of 39,000 candidate messenger RNAs (mRNAs) assessed, only 636 transcripts were differentially expressed. Other researchers have identified alcohol-induced alterations in the expression of only a small number of key developmental regulators, including several transcription factors known as *HOX* factors, which play crucial roles in directing organ patterning and morphogenesis (Godin et al. 2011; Mo et al. 2012; Rifas et al. 1997; Vangipuram and Lyman 2012). In rodent models, these alterations have been associated with neural patterning defects and the development of abnormalities in structures of the head and face (i.e., craniofacial dysmorphogenesis), reminiscent of those observed in clinical studies of FASD (Parnell et al. 2009; Rifas et al. 1997). However, these alcohol-induced alterations in gene expression often are limited to a specific tissue type and arise only when ethanol exposure occurs during select developmental windows (Godin et al. 2011; Kim et al. 2010; Mo et al. 2012; Parnell et al. 2009). These observations suggest that the molecular machinery involved in epigenetic programming also may be disrupted by ethanol exposure and, as a consequence, key epigenetic cues regulating development are not properly established.

Epigenetic Control and Developmental Programming of Differentiation

Of the three classes of epigenetic modifications, posttranslational modification of histone proteins undoubtedly is the most complex. Posttranslational enzymatic modifications, such as acetylation, methylation, phosphorylation, and ubiquitination (which have been studied most extensively), work together to produce a combinatorial "histone code" that serves to regulate cell-lineage–specific patterns of chromatin structure throughout development (Fisher and Fisher 2011). Within the unique transcriptional environment of embryonic stem cells, several developmentally

[1] Stem cells still have the ability to differentiate into any type of specialized cell (e.g., nerve, blood, or muscle cells); therefore, they are called pluripotent. Progenitor cells already are more specialized and therefore committed to a certain type of tissue; for example, neural progenitor cells can develop into different types of nerve cells or supporting brain cells (i.e., glial cells) but can no longer differentiate into muscle or blood cells. Thus, these progenitor cells are multipotent but not pluripotent.

crucial genes are marked in a coordinated fashion with both activating and repressive histone modifications (Bernstein et al. 2006; Jiang et al. 2011; Lim et al. 2009). Specifically, histone 3 (around which DNA sequences are wrapped) is modified by the addition of three methyl groups to the fourth lysine residue (i.e., histone 3 lysine 4 trimethylation), which typically is associated with gene activation, as well as by trimethylation of lysine 27, which has repressive effects (see figure 1A). The DNA sequences wrapped around these uniquely marked histones are termed bivalent domains and generally encode transcription factors directing tissue-specific programs of differentiation (Fisher and Fischer 2011). This same distinctive signature is found, albeit less frequently, in placental, neuronal, and other tissue-specific progenitor cell types (Lim et al. 2009; Rugg-Gunn et al. 2010). These bivalently marked genes generally are not expressed but are thought to be "primed" for either rapid activation or silencing during differentiation. Once a progenitor cell's fate has been established by lineage-specific transcription factor networks, the cell's transcriptional memory is maintained by removing one of the coexisting modifications and leaving only the modification indicative of the active or silent state in place. Importantly, many bivalently marked genes are disrupted in prenatal models of alcohol exposure, which potentially may explain the constellation of effects observed in FASD. For example, in a neural stem cell model ethanol exposure alters both histone 3 lysine 4 and lysine 27 trimethylation (Veazey et al. 2013). Understanding the mechanistic basis of these epigenetic defects is crucial to deciphering the developmental origins of FASD.

Seminal studies using the fruit fly *Drosophila melanogaster* in the late 1970s to early 1980s revealed the existence of two large multiprotein complexes with diametrically opposite roles in the regulation of gene expression: the Polycomb group (PcG) and Trithorax group (TrxG) (Lewis 1978; Poux et al. 2002; Schuettengruber et al. 2007). These two developmentally crucial enzyme complexes function at the hub of mammalian development; by binding to the regulatory regions of bivalent genes, they regulate the intricate balance between self-renewal of stem and progenitor cells and the execution of cellular differentiation. As differentiation progresses, these regulatory regions "commit" to one of these two protein complexes and become occupied exclusively by either the PcG or TrxG proteins. This commitment occurs in a cell-lineage–dependent manner, and

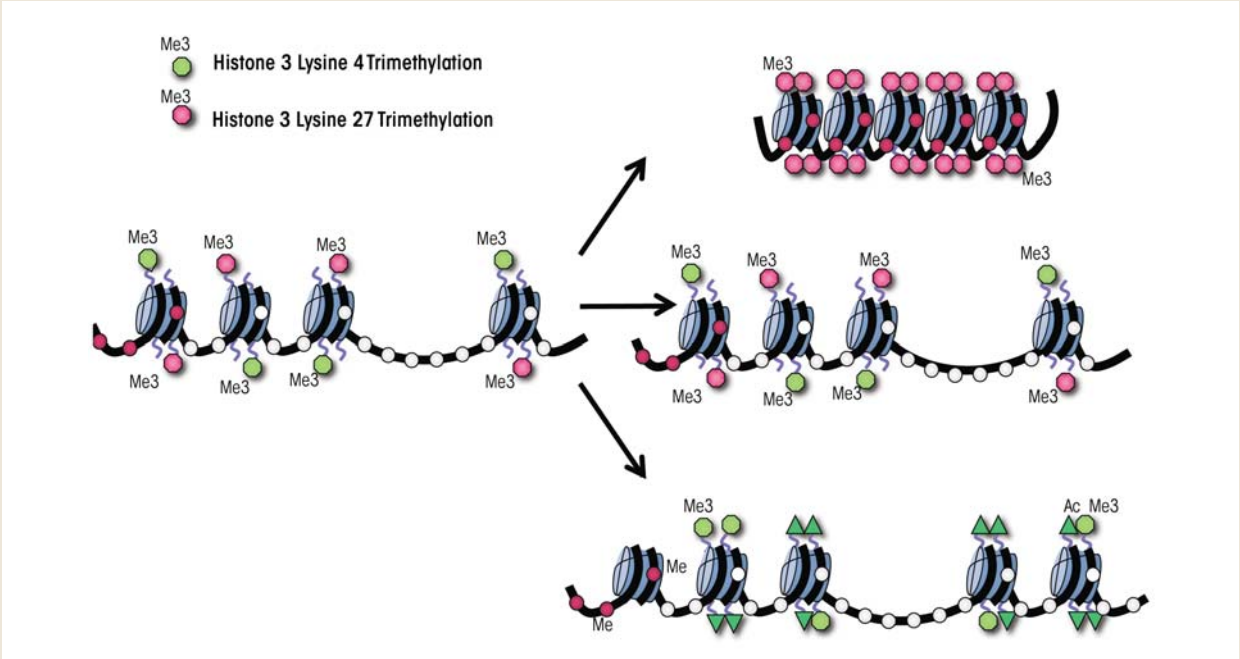


Figure 1A Bivalent state of the DNA in mammalian cells and its resolution during differentiation. In stem or progenitor cells, numerous developmentally relevant genes encoding factors that drive lineage-specific patterning are simultaneously marked with both activating and repressive histone modifications. This bivalent chromatin signature is thought to silence lineage-specifying genes through histone 3 lysine 27 trimethylation (H3K27me3) while at the same time poising them for activation during differentiation through the presence of histone 3 lysine 4 trimethylation (H3K4me3). As differentiation progresses, these domains can either adopt a silent conformation (top), become transcriptionally active (bottom), or persist into the next progenitor cell type (middle).

as a result the chromatin structure of these bivalent genes becomes fixed in either an active or a silent state. Any defects in this delicate balancing act, particularly during the differentiation towards a neural lineage, results in developmental defects and causes disease. Despite their fundamental importance to the processes of epigenetic programming and mammalian development, however, the roles of PcG and TrxG proteins in the etiology of FASD to date have not been examined.

PcG Proteins

The PcG proteins and the genes encoding them originally were discovered over 30 years ago as key regulators of the processes that specify which end of the embryo forms the head and which the rear during the development of Drosophila (Lewis 1978). Since then, researchers have found that these gene families encode essential regulators governing mammalian processes of cellular determination and lineage-specific patterns of differentiation. In mammals, two major PcG complexes have been characterized that modify chromatin structure; these are called Polycomb Repressive Complexes 1 and 2 (PRC1 and PRC2). Each complex is composed of several proteins with different biochemical functions, many of which are not well understood (see figure 2). PRC1 acts by mediating the ubiquitination of the 119th lysine residue of histone H2A; this is achieved by two of the PRC1 proteins called ring finger protein 1A and 1B (RING1A and RING1B) (Wang et al. 2004). This posttranslational modification pushes the local chromatin structure towards a transcriptionally repressive state and its proper establishment is essential to the coordinated silencing of genes throughout mammalian development (Boyer et al. 2006; Wang et al. 2004). In embryonic stem cells, histone ubiquitination stabilizes the presence of an enzyme called RNA polymerase II (which is required for gene expression) at bivalent chromatin domains and is crucial for maintaining the pluripotent state of undifferentiated cells (Ku et al. 2008).

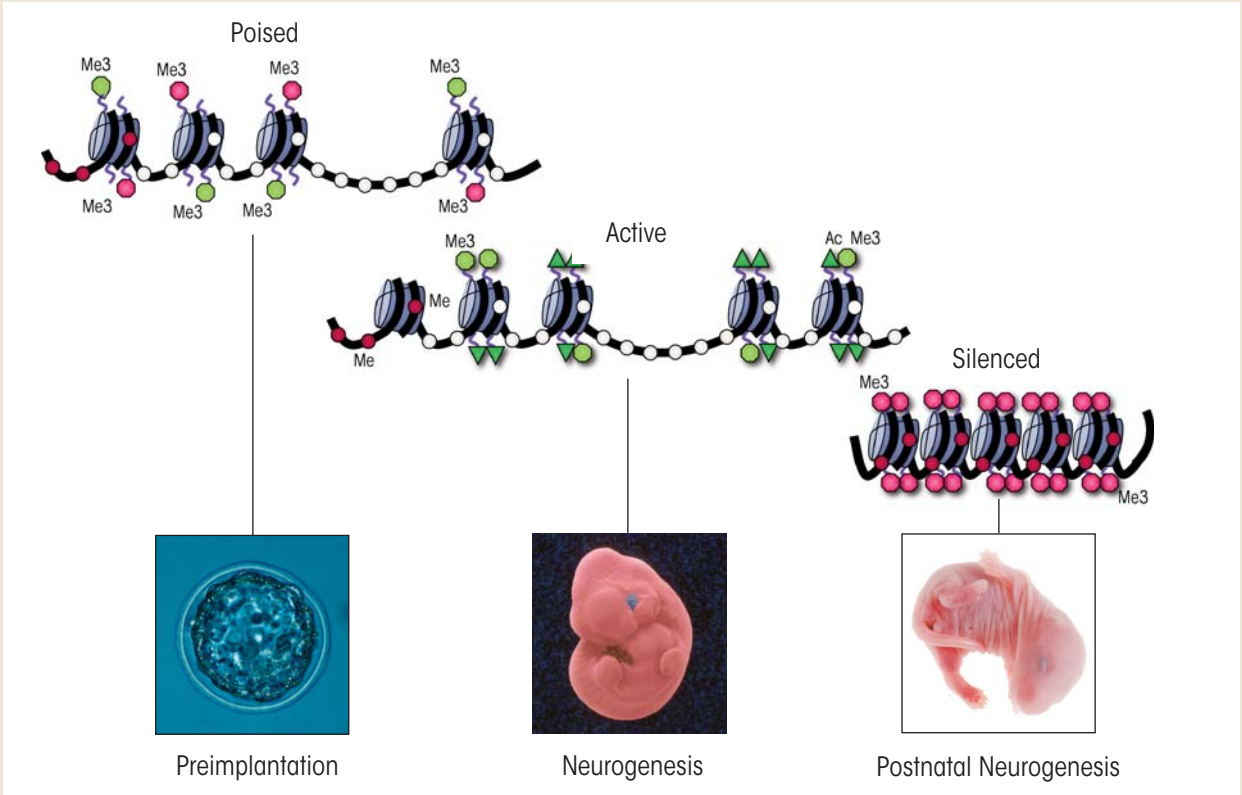


Figure 1B During development of the nervous system, many genes controlling neural patterning are held in a poised or bivalent conformation during early embryogenesis, resolve towards the active conformation during neural patterning, and are silenced during postnatal life. Repression (i.e., trimethylation of histone 3 lysine 27 [H3K27me3]) is imposed by the polycomb group proteins (PcG) (small red circles), whereas activation H3K4me3 is imparted by the mammalian homologues of the trithorax group proteins (TrxG) (green triangles). Correct biochemical function of these proteins and the coordination of the marks they impart are essential to mammalian neurogenesis.

PRC2 has similar repressive properties to PRC1 and also is an essential regulator of cellular differentiation. It facilitates the silencing of developmentally crucial genes through mono-, di-, and trimethylation of the histone 3 lysine 27 and trimethylation of histone 3 lysine 9 (Cao et al. 2002; Czermin et al. 2002), both of which repress gene expression. Together, the methylation of these two lysine residues promotes the generation of facultative heterochromatin[2] and mediates a transcriptionally silent state.

Adding an additional layer of complexity, PRC2 associates with the mammalian enzymes responsible for DNA methylation (i.e., DNA methyltransferase complexes); this association aids in the ability of PRC2 complexes to repress their target loci (Viré et al. 2006). This physical interaction suggests that the PcG complexes and the DNA methyltransferases act together to maintain the epigenetic memory of chromatin states throughout differentiation. Proper functioning of this gene family and their interacting proteins is essential for the execution of cell-specific differentiation programs and proper lineage specification (Pasini et al. 2007).

[2] The term "facultative heterochromatin" refers to gene-rich regions of the genome that are silent, but which can dynamically cycle into periods of transcriptional activity. For example, a chromosome region containing a gene that is active only during late development will be silent during early development, become transcriptionally active during late development, and then return to a silent state for the adult stage of life. Conversely, other genomic regions are held perpetually in a tightly compact, silent state; these regions are known as constitutive heterochromatin.

TrxG Proteins

In fruit flies, maternal transcription factors that were included in the egg cells and which are distributed unevenly throughout the developing embryo shape gene expression in the early embryo. The levels of these transcription factors diminish over time, and once they disappear from the developing embryo, the memory of which genes were active in a given cell is propagated through the action of the TrxG proteins (Lewis 1978; Poux et al. 2002). These proteins also have been identified in mammals, where they have been implicated in fundamental epigenetic and cellular processes, including X-chromosome inactivation, genomic imprinting, stress response, programmed cell death (i.e., apoptosis), development

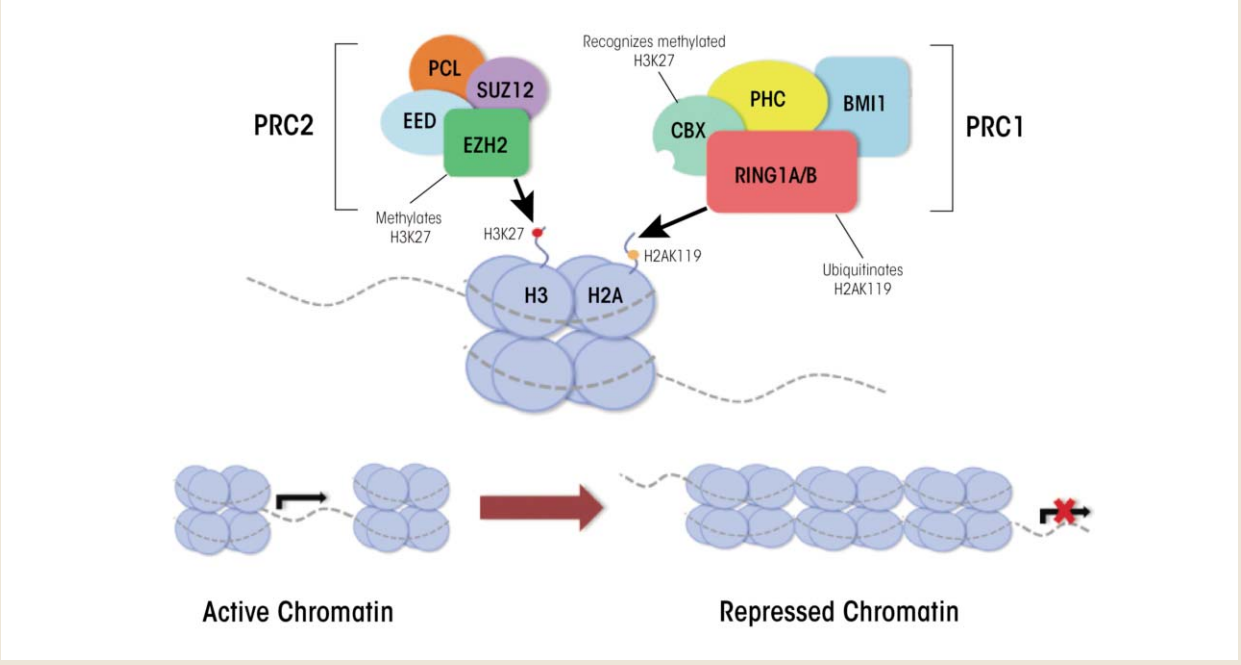


Figure 2A Transcriptional regulation by the Polycomb and Trithorax complexes. Polycomb repressive complex 1 (PRC1) consists of four core proteins including: polyhomeotic homolog (PHC), ring finger protein 1A or 1B (RING1A or RING1B), B-lymphoma Mo-MLV insertion region 1 homolog (BMI1), and chromobox homolog (CBX). The RING1A/RING1B subunits are the catalytic engine of the PRC1 complex and carry out ubiquitination of histone 2A at lysine 119 (H2AK119ub). PRC2 consists of four core proteins including: embryonic ectoderm development (EED), enhancer of zeste 2 (EZH2), suppressor of zeste 12 (SUZ12), and polycomb like (PCL). EZH2 serves as the catalytic subunit of PRC2 and trimethylates lysine 27 on histone 3 (H3K27me3). Current models suggest that H3K27me3 generated by PRC2 facilitates compaction of chromatin leading to the repression of gene expression. Subsequently, the CBX subunit of the PRC1 complex recognizes H3K27me3, and the RING1A/RING1B subunits of PRC1 ubiquitinate H2AK119 to facilitate the maintenance of the repressed state.

of tumors (i.e., tumorigenesis), cell proliferation, and embryonic stem cell renewal. However, compared with the PRC1 and PRC2 complexes, very little information exists on individual TrxG proteins or their biochemical functions (Schuettengruber et al. 2007). It is known that TrxG proteins function as multiprotein complexes that mediate the trimethylation of histone 3 lysine 4 (H3K4me3) and which have been conserved across different species (Jiang et al. 2011). In mammalian cells the TrxG complex is formed by a core group of structural proteins that combine with at least one of six interchangeable histone methyltransferases.

The main core of TrxG complexes is composed of four proteins called WD40 repeat domain 5 (WDR5), retinoblastoma binding protein 5 (RbBP5), dosage compensation–related protein 30 (Dpy30), and absent, small, or homeotic-like protein (Ash2L) (see figure 3). WDR5 recognizes histone 3 molecules that are methylated at lysine 4 and allows the methyltransferase in the TrxG complex to bind to this region and add another methyl group; thus, WDR5 is an essential regulator of global H3K4 trimethylation (Wysocka et al. 2005). RbB5 is necessary for proper differentiation of embryonic stem cells into neural progenitor cells and, together with Dpy30, also is essential for regulating global levels of H3K4 trimethylation (Jiang et al. 2011).

The TrxG core interacts with a group of interchangeable H3K4 methyltransferases, including some called mixed lineage leukemia (MLL) proteins (i.e., MLL1, MLL2, MLL3, and MLL4) and proteins called SET1A and SET1B (Jiang et al. 2011; Steward et al. 2006). MLL1 initially was discovered in cells of patients with different types of leukemia (i.e., acute lymphoid and acute myeloid leukemia). It is thought to promote cell-specific patterns of gene expression by regulating global and gene-specific H3K4 methylation during early embryonic development (Yu et al. 1995), because mice in which the corresponding mouse gene (*MLL1*) has been eliminated, or knocked out, show alterations in H3K4 methylation. In contrast, knockout of the *MLL2* gene in mouse embryonic stem cells leads to skewed differentiation but no concrete alterations to H3K4 methylation (Lubitz et al. 2007). For the remaining methyltransferases (i.e., MLL3, MLL4, and SET1A/1B), little is known except that they are involved in H3K4 methylation. Deletion of any one of these other methyltransferases seems to have

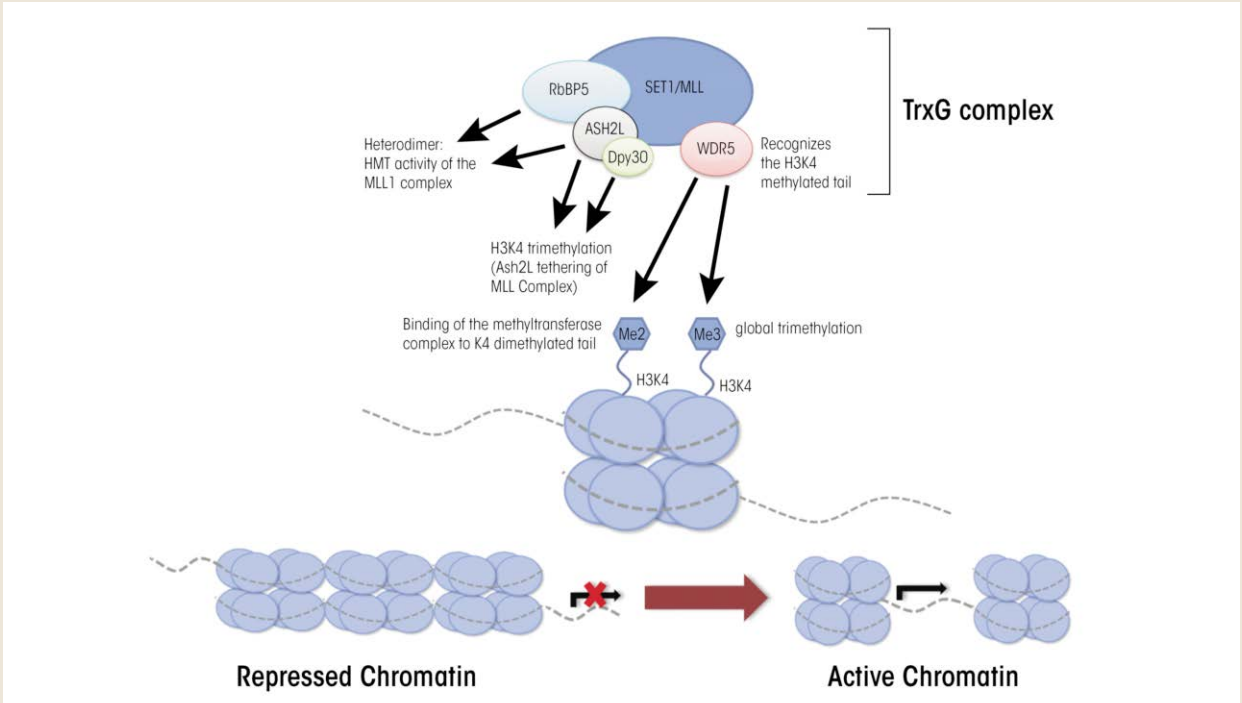


Figure 3 Trithorax (TrxG) proteins function as a conserved multi-component complex that regulates the trimethylation of histone 3 lysine 4 (H3K4me3). The four core structural components of the TrxG complex are: WD40 repeat domain 5 (WDR5), retinoblastoma binding protein 5 (RbBP5), dosage compensation-related protein 30 (Dpy30), and absent, small, or homeotic-like (Ash2L). These proteins serve as a scaffold to regulate the biological activity of the H3K4 methyltransferase family of enzymes, which include mixed-lineage leukemia (MLL) proteins MLL1, MLL2, MLL3, and MLL4, as well as SET1A and SET1B.

only minimal effects on global levels of H3K4 methylation, likely because the remaining MLL family members can substitute for the deleted ones (Jiang et al. 2011). Thus, although researchers have made progress in clarifying the roles of TrxG proteins, much remains unknown regarding the temporal and tissue-specific regulatory events these proteins promote.

Role of PcG and TrxG in the Etiology of FASD

Postmortem studies of children that succumbed to FAS revealed groups of poorly differentiated neuronal and glial cells at abnormal sites within the brain, suggesting large-scale problems with cellular proliferation and differentiation resulting from prenatal alcohol exposure (Swayze et al. 1997). Furthermore, studies using animal models have demonstrated reduced brain size and abnormal migration of neural cells in mice exposed to ethanol in utero (Godin et al. 2010; Parnell et al. 2009). Collectively, these observations indicate that alcohol impairs the cellular processes of neuronal differentiation and migration during fetal development. In support of this conclusion, studies using human and rodent neurosphere cultures have demonstrated that treatment with ethanol increases neurosphere size, skews the developmental potential of neural progenitor cells, and fundamentally alters the neuronal differentiation program (Roitbak et al. 2011; Vangipuram and Lyman 2012). However, the specific molecular mechanisms by which ethanol disrupts the cellular processes governing differentiation remain poorly defined. Recent studies examining the consequences of ethanol exposure during embryonic stem cell differentiation demonstrate a delay in the ability of exposed cells to silence regulatory factors promoting pluripotency, including the transcription factors OCT4, NANOG, and SOX2 (Arzumnayan et al. 2009). These studies strongly suggest that ethanol interferes with the ability of differentiating cells to recruit epigenetic modifiers to genes playing key roles in development and to execute the molecular programs governing cellular differentiation.

During early mammalian development, approximately 2,000 genes are bivalently marked as described earlier, and these marks progressively resolve towards the lineage-specific patterns of chromatin organization characterizing each unique cell type (Rugg-Gunn et al. 2010). As development proceeds, many precursor cell types maintain a subset of developmentally critical genes in this conformation as well push new groups of cellular factors into a bivalent state. For example, in pluripotent embryonic stem cells (which can differentiate into any cell type) the neural precursor genes *Dlx2, Hand1, Msx2, Nestin, Nkx2.1, Nkx2.2, Olig2, Pax6,* and *Sox1* all are bivalently marked, whereas in multipotent, neural precursor cells (which only can develop further into different types of neurons) only *Dlx2* and *Pax6* maintain this conformation. Interestingly, two genes encoding marker proteins that are found only in a type of glial cell called astrocyte (i.e., myelin basic protein [MBP] and glial fibrillary acidic protein [GFAP]) establish novel bivalent domains so that these genes can be kept in an active or inactive state, depending on whether they will become nerve cells or astrocytes (Golebiewska et al. 2009). Proper functioning of the TrxG complexes is indispensable to converting these bivalent loci into the actively transcribed state required for the induction of nerve cell formation (i.e., neurogenesis) (Huang et al. 2007; Jiang et al. 2011; Lim et al. 2009). Similarly, PcG complexes are necessary to silence the myriad of developmental regulators that would be required if the cells would differentiate into other cell types; thus, these complexes also help ensure that lineage-specific patterns of gene expression arise (Pereira et al. 2010). By propagating the transcriptional memory established by lineage-specific transcription factor networks, the TrxG and PcG complexes cooperatively regulate the balance between stem cell renewal and lineage differentiation.

Importantly, the expression of many of these factors that are bivalently marked and regulated by PcG and TrxG is disrupted in various models of prenatal alcohol exposure; moreover, this disruption is associated with profound errors in neuronal patterning. For example, alcohol suppresses the activation of two neural precursor genes—*Msx2* and *Pax6*—leading to craniofacial abnormalities and excessive differentiation of glutamatergic neurons, respectively (Kim et al. 2010; Mo et al. 2012; Rifas et al. 1997). Similarly, both the expression and localization of *Nkx2.1* and *Olig2* are diminished by alcohol, potentially disrupting the balance between excitation and inhibition in the cerebral cortex after birth (Godin et al. 2011). Finally, recent studies by Taléns-Visconti and colleagues (2011) have demonstrated that ethanol affects the proliferation of neural progenitor cells and markedly reduces their potential to differentiate into mature neurons, astrocytes, and another type of glial cell called oligodendrocytes. Given this broad-spectrum impediment to nearly every neuronal developmental fate, it is possible that the observed impact of ethanol on the overall architecture and size of the brain in FAS children stems from effects on some aspect of PcG/TrxG regulation of neural precursor differentiation. Using a neurosphere model of differentiation, Mo and colleagues (2012) recently demonstrated that expression of the *Pax6* gene at a site other than where it usually is expressed could ameliorate the impact of ethanol on cell proliferation and neurogenesis. These results suggest that within a limited scope it may be possible to reverse alcohol's effects on developmental programs.

Conclusions

One of the most difficult aspects in the study of FASDs has been trying to explain the wide range of severity and enormous variation in FASD-associated

birth defects. The process of organ formation is initiated during the early stages of embryonic development, and different rudimentary organ systems are formed and grow during unique developmental windows (Zorn and Wells 2009). Each organ system cycles between periods of intense growth and steady-state maintenance. The periods of growth are characterized by carefully orchestrated changes in DNA methylation and chromatin structure as differentiating cells are programmed with their epigenetic identity (Zhou et al. 2011). Studies using animal models analyzing the correlation of ethanol exposure at varying developmental time points with major periods of tissue growth strongly indicate that different tissues primarily are susceptible to ethanol-induced teratogenesis during specific developmental windows (Becker et al. 1996). Given the demonstrated ability of alcohol to alter DNA methylation and chromatin structure, it is likely that in organ systems which enter or are in a period of active epigenetic programming, ethanol exposure induces lasting epigenetic lesions that persist throughout organogenesis, whereas non-developing systems remain largely refractory to alcohol's effects. Thus, the epigenetic errors resulting from alcohol exposure can vary greatly depending on the specific timing and dose of alcohol exposure, which can explain the wide diversity in severity and range of birth defects that characterize FASD (Becker et al. 1996).

Since their discovery, the PcG and TrxG protein complexes have been identified in numerous disease contexts, including cellular transformation of normal cells into tumor cells as well as structural defects and mental illness (Huang et al. 2007; Varambally et al. 2002; Yu et al. 1995). These studies have demonstrated that a molecular event or teratogen (e.g., ethanol) that alters PcG/ TrxG programming within even a few neural progenitor stem cells during fetal growth can disproportionately influence subsequent brain development and potentially impart severe neurological birth defects (Boyer et al. 2006, Hirabayashi and Gotch. 2010). A complete characterization of the involvement of PcG and TrxG complexes in the etiology of FASD will undoubtedly aid in understanding the role of epigenetic programming in this complex disorder. ■

Acknowledgements

K.J. Veazey was supported through the Texas A&M University College of Veterinary Medicine & Biomedical Sciences Graduate Student Research Trainee Award. This work was supported by the NIH Grants NIAAA 1–R03–AA–020129, NICHD 5–R01–HD–058969 and NCRR 1–R24–RR–032683.

Financial Disclosure

The authors declare that they have no competing financial interests.

References

Arzumnayan, A.; Anni, H.; Rubin, R.; and Rubin, E. Effects of ethanol on mouse embryonic stem cells. *Alcoholism: Clinical and Experimental Research* 33(12):2172–2179, 2009. PMID: 19764938

Barrero, M.J.; Boué, S.; and Izpisúa Belmonte, J.C. Epigenetic mechanisms that regulate cell identity. *Cell Stem Cell* 7(5):565–570, 2010. PMID: 21040898

Becker, H.C.; Diaz-Granados, J.L.; and Randall, C.L. Teratogenic actions of ethanol in the mouse: A minireview. *Pharmacology, Biochemistry, and Behavior* 55(4):501–513, 1996. PMID: 8981580

Bernstein, B.E.; Mikkelsen, T.S.; Xie, X.; et al. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* 125(2):315–326, 2006. PMID: 16630819

Bielawski, D.M.; Zaher, F.M.; Svinarich, D.M.; and Abel, E.L. Paternal alcohol exposure affects sperm cytosine methyltransferase messenger RNA levels. *Alcoholism: Clinical and Experimental Research* 26(3):347–351, 2002. PMID: 11923587

Boyer, L.A.; Plath, K.; Zeitlinger, J.; et al. Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* 441 (7091):349–353, 2006. PMID: 16625203

Cao R., Wang L., Wang H., et al. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* 298(5595):1039–1043, 2002. PMID: 12351676

Choudhury, M.; Park, P.H.; Jackson, D.; and Shukla, S.D. Evidence for the role of oxidative stress in the acetylation of histone H3 by ethanol in rat hepatocytes. *Alcohol* 44(6):531–540, 2010. PMID: 20705415

Czermin, B.; Melfi, R.; McCabe, D.; et al. Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. *Cell* 111(2):185–196, 2002. PMID: 12408863

Downing, C.; Johnson, T.E.; Larson, C.; et al. Subtle decreases in DNA methylation and gene expression at the mouse Igf2 locus following prenatal alcohol exposure: Effects of a methyl-supplemented diet. *Alcohol* 45(1):65–71, 2011. PMID: 20705422

Fisher, C.L., and Fisher A.G. Chromatin states in pluripotent, differentiated, and reprogrammed cells. *Current Opinion in Genetics & Development* 21(2):140–146, 2011. 21316216

Garro, A.J.; McBeth, D.L.; Lima, V.; and Lieber, C.S. Ethanol consumption inhibits fetal DNA methylation in mice: Implications for the fetal alcohol syndrome. *Alcoholism: Clinical and Experimental Research* 15(3):395–398, 1991. PMID: 1877725

Godin, E.A.; Dehart, D.B.; Parnell, S.E.; et al. Ventromedian forebrain dysgenesis follows early prenatal ethanol exposure in mice. *Neurotoxicology and Teratology* 33(2):231–239, 2011. PMID: 21074610

Godin, E.A.; O'Leary-Moore, S.K.; Khan, A.A.; et al. Magnetic resonance microscopy defines ethanol-induced brain abnormalities in prenatal mice: Effects of acute insult on gestational day 7. *Alcoholism: Clinical and Experimental Research* 34(1):98–111, 2010. PMID: 19860813

Golebiewska, A.; Atkinson, S.P.; Lako, M.; and Armstrong, L. Epigenetic landscaping during hESC differentiation to neural cells. *Stem Cells* 27(6):1298–1308, 2009. PMID: 19489095

Hashimoto-Torii, K.; Kawasawa, Y.I.; Kuhn, A.; and Rakic, P. Combined transcriptome analysis of fetal human and mouse cerebral cortex exposed to alcohol. *Proceedings of the National Academy of Sciences of the United States of America* 108(10):4212–4217, 2011. PMID: 21368140

Haycock, P.C., and Ramsay, M. Exposure of mouse embryos to ethanol during preimplantation development: Effect on DNA methylation in the h19 imprinting control region. *Biology of Reproduction* 81(4):618–627, 2009. PMID: 19279321

Hemberger, M.; Dean, W.; and Reik, W. Epigenetic dynamics of stem cells and cell lineage commitment: Digging Waddington's canal. *Nature Reviews. Molecular Cell* Biology 10(8):526–537, 2009. PMID: 19603040

Hicks, S.D.; Middleton, F.A.; and Miller, M.W. Ethanol-induced methylation of cell cycle genes in neural stem cells. *Journal of Neurochemistry* 114(6):1767–1780, 2010. PMID: 20626555

Hirabayashi, Y., and Gotoh, Y. Epigenetic control of neural precursor cell fate during development. *Nature Reviews. Neuroscience* 11(6):377–388, 2010. PMID: 20485363

Huang, H.S.; Matevossian, A.; Whittle, C.; et al. Prefrontal dysfunction in schizophrenia involves mixed-lineage leukemia 1-regulated histone methylation at GABAergic gene promoters. *Journal of Neuroscience* 27(42): 11254–11262, 2007. PMID: 17942719

Jiang, H.; Shukla, A.; Wang, X.; et al. Role for Dpy-30 in ES cell-fate specification by regulation of H3K4 methylation within bivalent domains. *Cell* 144(4):513–25, 2011. PMID: 21335234

Kaminen-Ahola, N.; Ahola, A.; Maga, M.; et al. Maternal ethanol consumption alters the epigenotype and the phenotype of offspring in a mouse model. *PLoS Genetics* 6(1):e1000811, 2010. PMID: 20084100

Kim, K.C.; Go, H.S.; Bak, H.R.; et al. Prenatal exposure of ethanol induces increased glutamatergic neuronal differentiation of neural progenitor cells. *Journal of Biomedical Science* 17:85, 2010. PMID: 21073715

Kim, J.S., and Shukla, S.D. Histone h3 modifications in rat hepatic stellate cells by ethanol. *Alcohol and Alcoholism* 40(5):367–372, 2005. PMID: 15939707

Ku, M.; Koche, R.P.; Rheinbay, E.; et al. Genomewide analysis of PRC1 and PRC2 occupancy identifies two classes of bivalent domains. *PLoS Genetics* 4(10):e1000242, 2008. PMID: 18974828

Lewis E.B. A gene complex controlling segmentation in Drosophila. *Nature* 276(5688):565–570, 1978. PMID: 103000

Lim, D.A.; Huang, Y.C.; Swigut, T.; et al. Chromatin remodelling factor Mll1 is essential for neurogenesis from postnatal neural stem cells. *Nature* 458(7237): 529–533, 2009. PMID: 19212323

Liu, Y.; Balaraman, Y.; Wang, G.; et al. Alcohol exposure alters DNA methylation profiles in mouse embryos at early neurulation. *Epigenetics* 4(7):500–511, 2009. PMID: 20009564

Lubitz, S.; Glaser, S.; Schaft, J.; et al. Increased apoptosis and skewed differentiation in mouse embryonic stem cells lacking the histone methyltransferase Mll2. *Molecular Biology of the Cell* 18(6):2356–2366, 2007. PMID: 17429066

Mo, Z.; Milivojevic, V.; and Zecevic, N. Enforced Pax6 expression rescues alcohol-induced defects of neuronal differentiation in cultures of human cortical progenitor cells. *Alcoholism: Clinical and Experimental Research,* 36(8):1374–1384, 2012. PMID: 22524987

Ouko, L.A.; Shantikumar, K.; Knezovich, J.; et al. Effect of alcohol consumption on CpG methylation in the differentially methylated regions of H19 and IG-DMR in male gametes: implications for fetal alcohol spectrum disorders. *Alcoholism: Clinical and Experimental Research* 33(9):1615–1627, 2009. PMID: 19519716

Pal-Bhadra, M.; Bhadra, U.; Jackson, D.E.; et al. Distinct methylation patterns in histone H3 at Lys-4 and Lys-9 correlate with up- & down-regulation of genes by ethanol in hepatocytes. *Life Sciences* 81(12):979–87, 2007. PMID: 17826801

Park, P.H.; Lim, R.W.; and Shukla, S.D. Involvement of histone acetyltransferase (HAT) in ethanol-induced acetylation of histone H3 in hepatocytes: Potential mechanism for gene expression. *American Journal of Physiology. Gastrointestinal and Liver Physiology* 289(6):G1124–G1136, 2005. PMID: 16081763

Parnell, S.E.; O'Leary-Moore, S.K.; Godin, E.A.; et al. Magnetic resonance microscopy defines ethanol-induced brain abnormalities in prenatal mice: Effects of acute insult on gestational day 8. *Alcoholism: Clinical and Experimental Research* 33(6):1001–1011, 2009. PMID: 19302087

Pasini, D.; Bracken, A.P.; Hansen, J.B.; et al. The polycomb group protein Suz12 is required for embryonic stem cell differentiation. *Molecular and Cellular Biology* 27(10): 3769–3779, 2007. PMID: 17339329

Pereira, J.D.; Sansom, S.N.; Smith, J.; et al. Ezh2, the histone methyltransferase of PRC2, regulates the balance between self-renewal and differentiation in the cerebral cortex. *Proceedings of the National Academy of Sciences of the United States of America* 107(36): 15957–15962, 2010. PMID: 20798045

Poux, S.; Horard, B.; Sigrist, C.J.; and Pirrotta, V. The Drosophila trithorax protein is a coactivator required to prevent re-establishment of polycomb silencing. *Development* 129(10):2483–2493, 2002. PMID: 11973279

Rifas, L.; Towler, D.A.; and Avioli, L.V. Gestational exposure to ethanol suppresses msx2 expression in developing mouse embryos. *Proceedings of the National Academy of Sciences of the United States of America* 94(14):7549–7554, 1997. PMID: 9207129

Riley, E.P.; Infante, M.A.; and Warren, K.R. Fetal alcohol spectrum disorders: An overview. *Neuropsychology Review* 21(2):73–80, 2011. PMID: 21499711

Roitbak, T.; Thomas, K.; Martin, A.; et al. Moderate fetal alcohol exposure impairs neurogenic capacity of murine neural stem cells isolated from the adult subventricular zone. *Experimental Neurology* 229(2):522–525, 2011. PMID: 21419122

Rugg-Gunn, P.J.; Cox, B.J.; Ralston, A.; and Rossant, J. Distinct histone modifications in stem cell lines and tissue lineages from the early mouse embryo. *Proceedings of the National Academy of Sciences of the United* States of America, 107(24):10783-10790, 2010. PMID: 20479220

Schuettengruber, B.; Chourrout, D.; Vervoort, M.; et al. Genome regulation by polycomb and trithorax proteins. *Cell* 128(4):735–745, 2007. PMID: 17320510

Steward, M.M.; Lee, J.S.; O'Donovan, A.; et al. Molecular regulation of H3K4 trimethylation by ASH2L, a shared subunit of MLL complexes. *Nature Structural & Molecular Biology* 13(9):852–854, 2006. PMID: 16892064

Swayze, V.W. 2nd; Johnson, V.P.; Hanson, J.W.; et al. Magnetic resonance imaging of brain anomalies in fetal alcohol syndrome. *Pediatrics* 99(2):232–240, 1997. PMID: 9024452

Taléns-Visconti, R.; Sanchez-Vera, I.; Kostic, J.; et al. Neural differentiation from human embryonic stem cells as a tool to study early brain development and the neuroteratogenic effects of ethanol. *Stem Cells and Development* 20(2):327–339, 2011. PMID: 20491543

Vangipuram, S.D., and Lyman, W.D. Ethanol affects differentiation-related pathways and suppresses Wnt signaling protein expression in human neural stem cells. *Alcoholism: Clinical and Experimental Research* 36(5):788–797, 2012. PMID: 22150777

Varambally, S.; Dhanasekaran, S.M.; Zhou, M.; et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature* 419(6907):624–629, 2002. PMID: 12374981

Veazey, K.J.; Carnahan, M.N.; Muller, D.; et al. Alcohol-induced epigenetic alterations to developmentally crucial genes regulating neural stemness and differentiation. *Alcoholism: Clinical and Experimental Research* doi: 10.111/acer.12080 [Epub ahead of print], March 12, 2013. PMID: 23488822

Viré, E.; Brenner, C.; Deplus, R.; et al. The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* 439(7078):871–874, 2006. PMID: 16357870

Wang, H.; Wang, L.; Erdjument-Bromage, H.; et al. Role of histone H2A ubiquitination in Polycomb silencing. *Nature* 431(7010):873–878, 2004. PMID: 15386022

Wysocka, J.; Swigut, T.; Milne, T.A.; et al. WDR5 associates with histone H3 methylated at K4 and is essential for H3 K4 methylation and vertebrate development. *Cell* 121(6):859–872, 2005. PMID: 15960974

Yu, B.D.; Hess, J.L.; Horning, S.E.; et al. Altered Hox expression and segmental identity in Mll-mutant mice. *Nature* 378(6556):505–508, 1995. PMID: 7477409

Zeisel, S.H. What choline metabolism can tell us about the underlying mechanisms of fetal alcohol spectrum disorders. *Molecular Neurobiology* 44(2):185–191, 2011. PMID: 21259123

Zhou, F.C.; Balaraman, Y.; Teng, M.; et al. Alcohol alters DNA methylation patterns and inhibits neural stem cell differentiation. *Alcoholism: Clinical and Experimental Research,* 35(4):735–746, 2011. PMID: 21223309

Zorn, A.M., and Wells, J.M. Vertebrate endoderm development and organ formation. *Annual Review of Cell and Developmental Biology* 25:221–251, 2009. PMID: 19575677

Epigenetic Control of Gene Expression in the Alcoholic Brain

Igor Ponomarev, Ph.D.

Igor Ponomarev, Ph.D., *is a research assistant professor at the Waggoner Center for Alcohol and Addiction Research and the College of Pharmacy, University of Texas at Austin, Austin, Texas.*

Chronic alcohol exposure causes widespread changes in brain gene expression in humans and animal models. Many of these contribute to cellular adaptations that ultimately lead to behavioral tolerance and alcohol dependence. There is an emerging appreciation for the role of epigenetic processes in alcohol-induced changes in brain gene expression and behavior. For example, chronic alcohol exposure produces changes in DNA and histone methylation, histone acetylation, and microRNA expression that affect expression of multiple genes in various types of brain cells (i.e., neurons and glia) and contribute to brain pathology and brain plasticity associated with alcohol abuse and dependence. Drugs targeting the epigenetic "master regulators" are emerging as potential therapeutics for neurodegenerative disorders and drug addiction. **KEY WORDS: Alcohol consumption; alcoholism; chronic alcohol exposure; alcohol use, abuse and dependence; epigenetics; epigenetic therapeutics; gene expression; brain; brain cells; brain pathology; behavior; DNA methylation; histone; microRNA; transcription; pharmacotherapy; animal models; human studies**

Whether a specific gene is transcribed or repressed is determined by the specific status (i.e., conformational state) of the complex of chromosomal DNA and proteins (i.e., the chromatin) and by the recruitment of specific proteins (i.e., transcription factors) to regulatory sites on the DNA (Copeland et al. 2010). Chromatin states can change as a result of enzyme-mediated covalent modifications of the DNA and structural chromatin proteins (i.e., histones) (Borrelli et al. 2008; Copeland et al. 2010). These changes in chromatin, which are often termed epigenetic marks, include such modifications as DNA methylation and histone methylation and acetylation. In addition, incorporation of histone variants, adenosine triphosphate (ATP)-dependent chromatin remodeling, and regulation of gene expression by noncoding RNAs also are considered epigenetic phenomena and play important roles in regulation of gene expression. It is becoming increasingly clear that epigenetic mechanisms play a key role in cellular differentiation and regulation of cell type–specific transcriptional programs, producing a remarkable heterogeneity of cellular transcriptomes[1] that reflect the physiological properties and functional state of individual cells.

The brain arguably is one of the most complex biological tissues and enables the organism to sense, remember, and respond to its environment. It constantly adapts to environmental stimuli through regulated changes in gene expression. Chronic alcohol exposure causes widespread changes in brain gene expression in humans and animal models (Mulligan et al. 2006; Ponomarev et al. 2012), and there is evidence that many of these changes mediate the processes of cellular adaptation leading to addiction (Mayfield et al. 2008). Until recently, the role of epigenetic processes in alcohol's effects on the central nervous system (CNS) has been largely understudied. However, in the past 5 years the number of studies that suggested a role for epigenetics in alcohol-related molecular and behavioral changes has grown considerably. This review summarizes evidence for the role of epigenetic modifications in alcohol's effects on brain gene expression and behavior.

[1] The first step of gene expression (i.e., transcription) involves the synthesis of intermediary molecules called messenger RNAs (mRNAs) that are copies of the gene(s) to be expressed and which serve as templates for the synthesis of the encoded protein(s) during the second step of gene expression (i.e., translation). A transcriptome is the entirety of all mRNAs found in a certain cell, organ, or organism.

DNA Methylation

DNA methylation generally is associated with transcriptional repression. It mainly occurs at sites where a cytosine and a guanosine nucleotide are located next to each other (i.e., CpG dinucleotides). If these CpG dinucleotides are located within regulatory sequences, such as promoter regions, their methylation can block the binding of transcription factors and/or establish a repressive chromatin state (Renthal

and Nestler 2009*b*). One of the first indications that DNA methylation may play a role in alcoholism can be traced back to 1940s and 1950s, to the work of Dr. Roger J. Williams, a biochemistry professor at the University of Texas at Austin. He showed for the first time that dietary changes could affect beverage alcohol (i.e., ethanol) consumption in rodents. Specifically, diets deficient in B vitamins (e.g., folic acid and choline) increased consumption of solutions containing 10 percent ethanol in some rats, whereas vitamin-enriched diets decreased it (Williams et al. 1949). It now is well established that folates and several other B vitamins are critical for one-carbon metabolism and the synthesis of a compound called *S*-adenosyl-methionine (SAM), which serves as the primary methyl group donor in most transmethylation reactions, including DNA methylation (Hamid et al. 2009). Therefore, it is possible that dietary changes in this early study affected alcohol consumption via changes in DNA methylation and methylation-regulated gene expression.

Chronic alcohol consumption causes well-documented vitamin B and folate deficiencies that negatively affect the biochemical reactions in which a chemical unit containing one carbon atom (e.g., a methyl group) is transferred through several steps from a donor to another compound, such as DNA (i.e., one-carbon metabolism). These effects on one-carbon metabolism can result in excess levels of the SAM precursor homocysteine in the blood (i.e., homocysteinemia) and decreased SAM production (Blasco et al. 2005; Hamid et al. 2009). In addition, alcohol can affect DNA methylation through several other mechanisms, including the following:

- The alcohol metabolite, acetaldehyde, may induce inhibition of an enzyme called DNA methyltransferase 1 (DNMT1) that mediates most DNA methylation reactions needed to maintain the cell's normal functioning (Garro et al. 1991).
- Alcohol-induced DNA damage and the resulting repair reactions can lead to demethylation of 5-methylcytosine nucleotides (Chen et al. 2011).

Both of these mechanisms can cause reduced levels of methylation throughout the DNA (i.e., global DNA hypomethylation), a chromatin state associated with many pathological conditions, including cancer (Pogribny and Rusyn 2012). Alcohol-induced global DNA hypomethylation has been reported in several peripheral tissues of alcohol-related models and may play a role in alcoholic liver disease, fetal alcohol syndrome, and colon cancer (Choi et al. 1999; Garro et al. 1991; Hamid et al. 2009; Lu et al. 2000; Shukla et al. 2008). However, the effect of chronic alcohol on global DNA methylation seems to be tissue specific because one study reported enhanced DNA methylation (i.e., global DNA hypermethylation) in a certain type of blood cells (i.e., peripheral mononuclear cells) in alcoholic patients undergoing early alcohol withdrawal (Bonsch et al. 2004).

Two recent studies (Manzardo et al. 2012; Ponomarev et al. 2012) have examined alcohol's effects on global DNA methylation in the brain. Both studies measured DNA methylation in the frontal cortex of chronic alcoholics and matched control cases, but using two different methods. Ponomarev and colleagues (2012) studied genomic regions that included DNA sequences called long terminal repeat (LTR)-containing retrotransposons, also known as endogenous retroviruses (ERVs), most of which are nonfunctional remnants of ancient retroviral infections (Antony et al. 2004). The investigators showed that these repeats, which usually are heavily methylated, were less methylated in alcoholic brains, which was associated with their increased expression. Because ERVs constitute a significant part of the human genome, the study concluded that alcohol abuse causes global DNA hypomethylation in the brain, which is consistent with the majority of previous studies on alcohol-induced changes in DNA methylation. Manzardo and colleagues (2012) used immunological methods (i.e., immunoprecipitation) to isolate methylated DNA from alcoholics and control subjects and then applied this DNA to microarrays containing genomic promoter regions to identify promoters for which the methylation patterns differed between the two groups. The analyses found no differences between the groups in total methylation at the whole-genome level; however, about 20 percent of all promoters were differentially methylated between the groups, with less than half of these promoters showing greater methylation in alcoholics.

These complementary findings suggest that chronic alcohol causes a general decrease in the overall number of methylated cytosines but also could lead to the de novo methylation of previously unmethylated nucleotides at the promoters of some genes. Such a combination of these processes already has been widely reported in studies of cancer, showing, for example, that methyl-deficient diets induce development of liver tumors (i.e., hepatocarcinogenesis) associated with global DNA hypomethylation and promoter hypermethylation at specific genes (Ehrlich 2005; Pogribny and Rusyn 2012). Hypomethylated states associated with cancer and other pathological conditions often are accompanied by a downregulation of the gene encoding DNMT1 (Hervouet et al. 2010), which also has been observed in the brains of chronic alcoholics (Ponomarev et al. 2012). These striking similarities point to some common mechanisms of methyl deficiency across tissues.

Studies assessing epigenetic regulation of individual genes in the brain have shown that alcohol's effects on DNA methylation depend on a variety of factors, including the specific gene targets, developmental stage of exposure, and type of neuronal tissue affected. Much of this work has focused on the central effects of prenatal alcohol exposure and on gene regulation in cell cultures. Prenatal exposure of rats

to alcohol resulted in DNA hypermethylation and a reduced expression of a protein called brain-derived neurotrophic factor (BDNF) in olfactory bulbs of rat pups, which was associated with loss of neurons in this brain region (Maier et al. 1999). Similar molecular results were obtained in a separate study where prenatal alcohol treatment of rats led to DNA hypermethylation and a decreased expression of a protein characteristically found in brain cells called astrocytes (i.e., glial fibrillary acidic protein [GFAP]) in the brains of the pups (Valles et al. 1997). In neural cell cultures, alcohol-induced downregulation of cell-cycle genes was paralleled by an increased DNMT activity and hypermethylation of the promoters of those genes (Hicks et al. 2010). Conversely, upregulation of the gene encoding a receptor subunit for the neurotransmitter glutamate (i.e., the NMDA NR2B receptor subunit) was associated with demethylation of CpG dinucleotides in the gene's promoter after chronic alcohol (Marutha Ravindran and Ticku 2004).

However, some reports suggest that the relationship between DNA methylation and the expression of neighboring genes may be even more complex than previously thought (Ehrlich 2005). For example, a recent study demonstrated an increased expression of a signaling molecule called prodynorphin (PDYN) that was associated with methylation of a CpG dinucleotide located in a DNA region behind the actual protein-coding region of the gene (i.e., in the 3′-untranslated region of the gene) in the brains of alcohol-dependent people (Taqi et al. 2011), although no causal link was established.

Specific DNA methylation patterns differ among tissues and cell types, and these differences contribute to establishing the cells' epigenetic landscape and transcriptional programs and defining cellular identity (Bernstein et al. 2007). Also, although alcohol's general effects on DNA methylation may be similar across various tissues, the specific genes affected by this regulation may differ depending on cell type. The epigenetic regulation of such proteins as GFAP, which is a marker of astrocytes, and the NR2B subunit, which generally is expressed in neurons, suggests that alcohol-induced epigenetic changes will affect molecular markers of individual cell types to a greater degree than other proteins. Many studies of alcohol's epigenetic modification of the chromatin have been conducted in blood cells obtained from alcoholics (Biermann et al. 2009; Bonsch et al. 2005; Hillemacher et al. 2009). Because of the concern regarding the tissue specificity of alcohol's epigenetic effects, however, the results of these important studies cannot be readily generalized to mechanisms in brain. Therefore, parallel measurements of the entirety of all alcohol-induced epigenetic changes (i.e., the epigenomic changes) in the blood and brain should be obtained and vigorously compared in animal models to detect common patterns, based on which generalization of results in humans can be made.

Histone Modifications

Histone proteins are the second major target of epigenetic changes. These proteins can be modified by a relatively large number of specific enzymes that mediate covalent attachment and removal of four classes of chemical groups: methyl, acetyl, phosphate, and ubiquitin (Bernstein et al. 2007; Borrelli et al. 2008). Studies of alcohol-induced modifications mainly have focused on two histone modifications: a trimethylation of histone 3 at the lysine 4 residue (H3K4me3), which is a promoter-enriched chromatin mark of actively transcribed genes, and acetylation of various residues of histones 3 and 4 (H3 and H4). Histone acetylation generally is associated with a more open, accessible structure of the chromatin and, consequently, increased transcription, whereas deacetylated histones can cause transcriptional repression (Bernstein et al. 2007).

Chronic alcohol abuse in humans can result in global and gene-specific increases in H3K4me3 in the brain cortex (Ponomarev et al. 2012) and in either increases or decreases of this modification in promoters of specific genes in the hippocampus (Zhou et al. 2011). The latter study used a combination of two techniques (i.e., chromatin immunoprecipitation followed by DNA sequencing [ChIP-Seq]) to detect individual genes with differences between alcoholics and control subjects in H3K4 promoter trimethylation and in parallel measured the levels of transcription of the same genes. Interestingly, differences in promoter methylation did not correlate with differences in gene expression, suggesting that H3K4me3 status alone is not a reliable predictor of genome-wide steady-state mRNA levels at a given time point. A possible explanation of these results is that the H3K4me3 mark in the promoter regions only indicates that the chromatin is in an open conformation that is accessible to regulatory or transcription factors but does not mean that transcription actually is initiated and the transcription machinery is present (Bernstein et al. 2007). A recent study (D'Addario et al. 2011) supports this hypothesis as well as previous findings showing mechanistically linked but temporally complex relationships between chromatin marks at gene promoters and mRNA abundance. The investigators explored the effects of ethanol and its metabolite acetaldehyde on various chromatin marks and the transcription of the *PDYN* gene in a human cell line derived from a tumor arising from nerve tissue cells (i.e., a neuroblastoma). The analyses suggested that the ethanol-induced increase in H3K4me3 that was observed after 72 hours of ethanol exposure did not result in initiation of *PDYN* transcription but kept the gene in a poised state for later reactivation. This is consistent with other findings regarding *PDYN* activation in human alcoholics (Taqi et al. 2011).

Most evidence to date on the role of central epigenetic processes in alcoholism has been collected from studies focusing on histone acetylation, often by

modifying the activities of the enzymes that add acetyl groups (i.e., histone acetyl transferases [HATs]) or remove acetyl groups (i.e., histone deacetylases [HDACs]). Particularly, small molecules that inhibit HDAC function (HDACis) and thus result in increased histone acetylation have been investigated intensely in recent years. These molecules are attractive because they can enter the brain via the blood (i.e., cross the blood–brain barrier) and exert a broad range of effects in the CNS, including enhanced memory formation as well as anti-inflammatory and neuroprotective effects (Kazantsev and Thompson 2008; Sweatt 2009). Several studies using HDACis demonstrated effects of altered histone acetylation on different alcohol-related behaviors, including withdrawal-related anxiety (Pandey et al. 2008), locomotor sensitization (Sanchis-Segura et al. 2009), alcohol consumption (Wostenholme et al. 2011), conditioned place aversion (Pascual et al. 2012), and rapid tolerance (Sakharkar et al. 2012). For example, Pandey and colleagues (2008) showed that acute ethanol increased H3K9 and H4K8 acetylation in rats, whereas anxiety-like behaviors during withdrawal after chronic alcohol exposure were associated with decreases in these acetylation marks, decreased expression of several proteins (e.g., CREB-binding protein [CBP] and neuropeptide Y [NPY]), and increased HDAC activity. However, treatment with the HDACi, trichostatin A (TSA), to block HDAC activation prevented the deficits in gene expression and the development of withdrawal-related anxiety. Sanchis-Segura and colleagues (2009) demonstrated that treatment of mice with another HDACi (i.e., sodium butyrate) altered some alcohol-related behaviors (e.g., enhanced ethanol-induced locomotor sensitization) but had no effect on others (e.g., ethanol tolerance or withdrawal). Finally, daily injections of TSA in mice that had continuous access to both water and an alcohol solution increased the animals' alcohol consumption (Wolstenholme et al. 2011).

Similar to DNA methylation, alcohol's effects on histone acetylation are tissue, brain region–, and cell type–specific. For example, a single dose of ethanol[2] into the stomach increased the levels of H3 acetylation in the liver, lungs, and testes but had no effects in other tissues, including whole brain, of rats (Kim and Shukla 2006). In the brain, ethanol-induced changes in H3/H4 acetylation were observed in the central and medial but not the basolateral nuclei of the amygdala (Pandey et al. 2008; Sakharkar et al. 2012); moreover, the increased histone acetylation appeared to be specific for neurons (Sakharkar et al. 2012).

Other factors that can affect alcohol-induced changes in histone acetylation include species, the organism's specific genetic makeup (i.e., genotype), age, the dose and route of ethanol administration, and duration of exposure. For example, ddY mice treated with chronic ethanol vapor showed increases of both global and gene-specific histone acetylation in the ventral midbrain during withdrawal that peaked around 10 hours post ethanol (Shibasaki et al. 2011). Also, intermittent alcohol exposure produced different effects on histone acetylation in adolescent and adult rats, with juvenile animals generally showing more changes (Pascual et al. 2009, 2012). Consistent with these studies was the finding that ethanol exposure during the early postnatal period in rats resulted in a marked reduction of CBP levels and histone acetylation in the developing cerebellum (Guo et al. 2011). In addition, possible interactions among various factors may result in different time courses for alcohol-induced changes, because histone acetylation measured 24 hours after the last of repeated alcohol injections was increased in some brain areas (e.g., frontal cortex and nucleus accumbens), decreased in others (e.g., striatum), and unchanged in still others (e.g., hippocampus) (Pascual et al. 2009).

Histone acetylation generally is associated with transcriptional activation, but similar to the H3K4me3 mark, the relationships between levels of histone acetylation and steady-state mRNA are complex, because activation of different genes is associated with acetylation of different residues of H3 and H4 at different time points (Renthal and Nestler 2009*a*). And although alcohol's effects on histone acetylation now are well established, the exact mechanisms underlying this influence on gene expression are not well understood. Alcohol-induced changes in histone acetylation are paralleled by regulation of several genes, including *CBP, NPY* (Pandey et al. 2008), *FosB* (Pascual et al. 2012), and *NR2B* (Qiang et al. 2011). One proposed mechanism involves the transcription factor CREB, to which CBP can bind (Moonat et al. 2010). CBP has intrinsic HAT activity and, when recruited by CREB, can promote transcriptional activation by acetylating histones. This mechanism has been shown to play a role in cocaine-induced regulation of *FosB* (Levine et al. 2005). A similar mechanism also was proposed to regulate H4 acetylation, transcription of the gene encoding the BK-type potassium channel, and tolerance to benzyl alcohol in the fruit fly, Drosophila (Wang et al. 2007).

Gene expression experiments have provided additional support for the role of histone acetylation in alcohol addiction. Several studies focusing on brain changes in human alcoholics have shown general downregulation of genes involved in histone acetylation and upregulation of genes promoting histone deacetylation. The latter group of genes includes those encoding proteins forming so-called transcription corepressor complexes (TCCs), which help suppress transcription by coupling HDAC activity with DNA methylation, thereby establishing a repressive chromatin state (McDonel et al. 2009). For example, transcripts of the genes encoding CREB and CBP were downregulated in alcoholics (Ponomarev et al. 2012). Conversely, transcripts of the gene *MBD3,* which encodes a key player in TCCs called methyl-CpG–binding protein, as well as many other TCC genes, such as *SIN3A, SIN3B,*

[2] The dose administered was 6 g/kg of a 32 percent ethanol solution.

player in TCCs called methyl-CpG–binding protein, as well as many other TCC genes, such as *SIN3A, SIN3B, MTA1, MTA2, RBBP4, GATAD2A, GATAD2B,* and *CHD4* were upregulated in alcoholics (Liu et al. 2006; Ponomarev et al. 2012; Zhou et al. 2011). Together, these observations validate previous findings that histone acetylation is decreased during alcohol withdrawal (Pandey et al. 2008) and suggest that TCCs are activated and play a role in the downregulation of some genes in the alcoholic brain.

MicroRNAs

MicroRNAs (miRNAs) comprise a specific class of noncoding RNAs that bind to complementary sequences on target mRNAs to repress translation and silence gene expression (Robison and Nestler 2011). Expression of miRNAs can alter the transcriptional potential of a gene in the absence of any change to the DNA sequence and therefore can be considered an epigenetic phenomenon. The most convincing evidence for the involvement of miRNAs in alcohol-related gene expression was presented by Pietrzykowski and colleagues (2008), who showed that alcohol upregulates expression of microRNA 9 (miR-9) in rat brain, which results in miR-9–dependent downregulation of BK channel variants with high sensitivity to alcohol. This mechanism is proposed to mediate the development of cellular tolerance and generally may contribute to neuronal adaptation to alcohol.

Additional evidence for the role of miRNAs in alcohol-induced regulation of gene expression and behavior comes from genomic studies measuring levels of multiple miRNAs after exposure to alcohol. Using neural cultures and a model of alcohol-induced teratogenesis, Sathyan and colleagues (2007) identified the first alcohol-sensitive miRNAs. Subsequent studies using miRNA microarrays detected multiple alcohol-regulated miRNAs in neural cultures (Yadav et al. 2011), fetal mouse brains (Wang et al. 2009), and brains of human alcoholics (Lewohl et al. 2011).

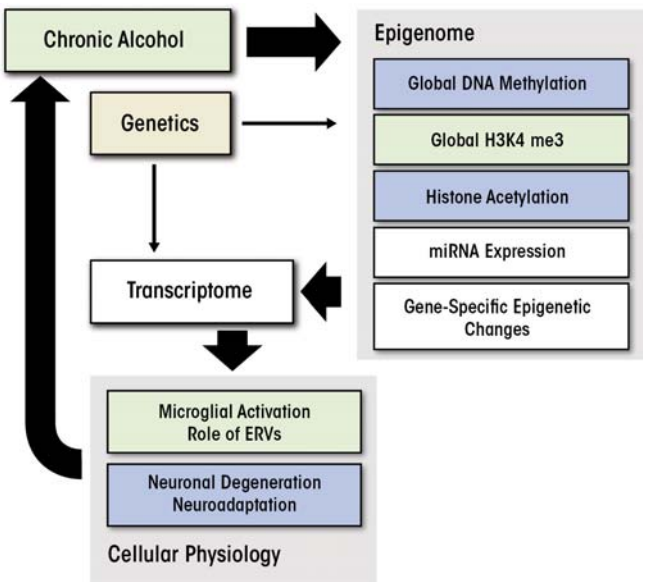


Figure A hypothetical diagram for the role of epigenetic modifications in alcohol addiction. Yellow color indicates general increase, up-regulation, or activation, whereas blue color indicates general decrease, down-regulation, or degeneration. White background implies bidirectional changes. Potential interactions between different components of the diagram are discussed in the text.

Summary and Future Directions

The findings reviewed in this article point to a central role of various epigenetic processes in controlling alcohol-induced changes in brain gene expression and behavior, which may play an important part in the development of alcohol addiction (see the figure). For example, chronic alcohol exposure can result in global DNA hypomethylation via several mechanisms, including vitamin B and folate deficiencies that can lead to an impairment of one-carbon metabolism and a decrease in SAM levels. However, these global effects of alcohol do not imply unidirectional changes across the whole genome, because many genes show the opposite epigenetic changes in their promoters.

Many of the observed chromatin modifications are mechanistically linked, resulting in a limited number of chromatin states (Jaenisch and Bird 2003). For example, trimethylation of H3K4 is mechanistically coupled with unmethylated DNA (Hashimoto et al. 2010), suggesting that the reduced DNA methylation observed in alcoholic brains can promote a general increase in the H3K4me3 levels. Histone actetylation patterns also are commonly altered by alcohol in a process that may be linked to DNA methylation. Thus, acute alcohol exposure promotes histone acetylation, whereas withdrawal from chronic alcohol often increases deacetylation of histones. Deacetylation via HDAC activity is coupled to DNA methylation through the actions of methyl-binding proteins and other TCC components. Chronic alcohol exposure leads to upregulation of TCC genes, which may serve to compensate for the reduced number of methylated CpGs. These cumulative changes in

ferent cell types and lead to activation of microglia, neuronal degeneration, and compensatory neuroadaptations in alcoholic brain. In summary, alcohol-induced epigenetically mediated changes in gene expression may underlie the brain pathology and adaptations in brain functioning (i.e., brain plasticity) associated with alcohol abuse and alcohol dependence and may contribute to alcohol relapse and craving.

To advance the current state of epigenetic research in alcoholism, future studies that look at both simplified models and entire regulatory systems (i.e., that use both reductionist and systems approaches) are needed. One focus of this research should be on understanding the exact mechanistic links between chronic alcohol exposure, epigenetic changes, and gene expression. Exploratory studies likely will first use discovery-driven approaches to investigate the mechanistic relationships between the epigenome and the transcriptome in animal models and formulate hypotheses at the single-gene, gene-network, and systems levels. Follow-up studies using both animal models and human postmortem material then can help test these hypotheses and validate functional predictions of the genome-wide experiments. Because of the complex temporal relationships between chromatin marks and transcriptional changes, time-course studies also will be required. The recently available epigenetic maps from the ENCODE (ENCyclopedia Of DNA Elements) Project (Dunham et al. 2012) should help accelerate these research efforts.

To address the causal relationships between epigenetic modifications and alcohol traits, it will be essential to use tools of both forward and reverse genetics. Forward-genetics approaches seek to determine the genetic basis of an observed trait (i.e., phenotype). Such approaches include mapping DNA regions that may contain disease-related genes (i.e., quantitative trait loci [QTLs]), using chromatin modifications as phenotypes. This can be achieved using genetic reference panels, such as recombinant inbred strains of mice and rats (Rosen et al. 2007). Many reference populations have been tested extensively for both expression of specific genes and alcohol-related behaviors and therefore can serve as powerful tools for integrating data across biological modalities and investigating mechanistic links between the genome and the entirety of all analyzed phenotypes (i.e., the phenome) through genetic mapping of the epigenome and the transcriptome. Conversely, reverse-genetics approaches study the phenotypes that arise as the result of alterations of particular genes. An example of a reverse-genetics approach is to assess alcohol-related behaviors in mice with genetic mutations of chromatin-binding proteins.

Another important research direction is to investigate the cellular specificity of alcohol-induced epigenetic changes. For example, future research should determine cell type–specific chromatin states that drive the unique molecular responses to alcohol in different neurons and glial cells and show how epigenetic modifications help establish functional states consistent with the pathophysiological changes observed in alcoholism. One example of this approach is the analysis of the role of epigenetically controlled ERVs in alcohol addiction (Ponomarev et al. 2012). Previous studies found that an ERV-encoded glycoprotein called syncytin can directly activate different types of glial cells (i.e., microglia and astrocytes) and induce neuroinflammation (Antony et al. 2004). Microglial activation, in turn, can result in neuronal degeneration (Crews et al. 2011), and syncytin-activated astrocytes can secrete compounds that are toxic to other glial cells (i.e., oligodendrocytes) and thus lead to myelin degeneration (Antony et al. 2004). Both of these effects are consistent with pathologies observed in alcoholics (Harper et al. 2003; Pfefferbaum et al. 2009; Zahr et al. 2011). Alcohol-induced neuroimmune responses have been suggested to be a critical factor in alcohol addiction (Crews et al. 2011), and Ponomarev and colleagues (2012) proposed a novel mechanism including the potential role for ERVs in neuroinflammation and brain pathophysiology of human alcoholism. Another approach to assessing the cell specificity of epigenetic processes is to compare alcohol-induced epigenetic changes across tissues and cell types. Human research often is limited to peripheral tissues (e.g., blood). To be able to draw parallels between peripheral and central mechanisms in humans, researchers first need to study the relationships between responses to alcohol in the brain and those in other tissues using animal models.

Other research efforts should focus on the potential exploitation of epigenetic mechanisms for alcoholism treatment. Epigenetic therapeutics, such as HDACis, offer unique advantages in treating diseases through chromatin-dependent changes in gene expression. These "master regulators" can affect expression of multiple genes. Therefore, in order to understand the effects of these agents on alcohol behaviors, it is important to study their mechanisms of action and identify the range of genes and molecular pathways affected. Large-scale genomic studies should focus on the global relationships between chromatin marks and gene expression in the context of chronic alcohol exposure and epigenetic therapeutics. Finally, multiple studies in humans and animal models have highlighted the importance of the genetic component in alcohol addiction (Crabbe 2008; Mayfield et al. 2008; Spanagel 2009). To better understand the interplay between genetic, epigenetic, and environmental factors in controlling gene expression in alcoholism, integrative approaches across studies are warranted. Many epigenetic therapeutics have been developed for other diseases, and understanding the functional relationships between epigenetic processes and the transcriptome in the alcoholic brain may lead to new molecular targets for medication development for human alcoholism. ■

Acknowledgments

This work was supported by a K-Award from the National Institute on Alcohol Abuse and Alcoholism (AA–017234). The author thanks Dr. Adron Harris for critical reading of the manuscript.

Financial Disclosure

The author declares that he has no competing financial interests.

References

Antony, J.M.; van Marle, G.; Opii, W.; et al. Human endogenous retrovirus glycoprotein-mediated induction of redox reactants causes oligodendrocyte death and demyelination. *Nature Neuroscience* 7(10):1088–1095, 2004. PMID: 15452578

Bernstein, B.E.; Meissner, A.; and Lander, E.S. The mammalian epigenome. *Cell* 128(4):669–681, 2007. PMID: 17320505

Biermann, T.; Reulbach, U.; Lenz, B.; et al. N-methyl-D-aspartate 2b receptor subtype (NR2B) promoter methylation in patients during alcohol withdrawal. *Journal of Neural Transmission* 116(5):615–622, 2009. PMID: 19350219

Blasco, C.; Caballeria, J.; Deulofeu, R.; et al. Prevalence and mechanisms of hyperhomocysteinemia in chronic alcoholics. *Alcoholism: Clinical and Experimental Research* 29(6):1044–1048, 2005. PMID: 15976531

Bonsch, D.; Lenz, B.; Kornhuber, J.; and Bleich, S. DNA hypermethylation of the alpha synuclein promoter in patients with alcoholism. *Neuroreport* 16(2):167–170, 2005. PMID: 15671870

Bonsch, D.; Lenz, B.; Reulbach, U.; et al. Homocysteine associated genomic DNA hypermethylation in patients with chronic alcoholism. *Journal of Neural Transmission* 111(12):1611–1616, 2004. PMID: 15565495

Borrelli, E.; Nestler, E.J.; Allis, C.D.; and Sassone-Corsi, P. Decoding the epigenetic language of neuronal plasticity. *Neuron* 60(6):961–974, 2008. PMID: 19109904

Chen, C.H.; Pan, C.H.; Chen, C.C.; and Huang, M.C. Increased oxidative DNA damage in patients with alcohol dependence and its correlation with alcohol withdrawal severity. *Alcoholism: Clinical and Experimental Research* 35(2):338–344, 2011. PMID: 21070251

Choi, S.W.; Stickel, F.; Baik, H.W.; et al. Chronic alcohol consumption induces genomic but not p53-specific DNA hypomethylation in rat colon. *Journal of Nutrition* 129(11):1945–1950, 1999. PMID: 10539767

Copeland, R.A.; Olhava, E.J.; and Scott, M.P. Targeting epigenetic enzymes for drug discovery. *Current Opinions in Chemical Biology* 14(4):505–510, 2010. PMID: 20621549

Crabbe, J.C. Review. Neurogenetic studies of alcohol addiction. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 363(1507):3201–3211, 2008. PMID: 18640917

Crews, F.T.; Zou, J.; and Qin, L. Induction of innate immune genes in brain create the neurobiology of addiction. *Brain, Behavior, and Immunity* 25(Suppl.) 54–512, 2011. PMID: 21402143

D'Addario, C.; Johansson, S.; Candeletti, S.; et al. Ethanol and acetaldehyde exposure induces specific epigenetic modifications in the prodynorphin gene promoter in a human neuroblastoma cell line. *FASEB Journal* 25(3):1069–1075, 2011. PMID: 21106935

Dunham, I.; Kundale, A.; Aldred, S.F.; et al. An integrated encyclopedia of DNA elements in the human genome. *Nature* 489(7414):57–74, 2012. PMID: 22955616

Ehrlich, M. The controversial denouement of vertebrate DNA methylation research. *Biochemistry (Moscow)* 70(5):568–575, 2005. PMID: 15948710

Garro, A.J.; McBeth, D.L.; Lima, V.; and Lieber, C.S. Ethanol consumption inhibits fetal DNA methylation in mice: Implications for the fetal alcohol syndrome. *Alcoholism: Clinical and Experimental Research* 15(3):395–398, 1991. PMID: 1877725

Guo, W.; Crossey, E.L.; Zhang, L.; et al. Alcohol exposure decreases CREB binding protein expression and histone acetylation in the developing cerebellum. *PLoS One* 6(5):e19351, 2011. PMID: 21655322

Hamid, A.; Wani, N.A.; and Kaur, J. New perspectives on folate transport in relation to alcoholism-induced folate malabsorption—association with epigenome stability and cancer development. *FEBS Journal* 276(8):2175–2191, 2009. PMID: 19292860

Harper, C.; Dixon, G.; Sheedy, D.; and Garrick, T. Neuropathological alterations in alcoholic brains. Studies arising from the New South Wales Tissue Resource Centre. *Progress in Neuro-psychopharmacology & Biological Psychiatry* 27(6):951–961, 2003. PMID: 14499312

Hashimoto, H.; Vertino, P.M.; and Cheng, X. Molecular coupling of DNA methylation and histone methylation. *Epigenomics* 2(5):657–669, 2010. PMID: 21339843

Hervouet, E.; Lalier, L.; Debien, E.; et al. Disruption of Dnmt1/PCNA/UHRF1 interactions promotes tumorigenesis from human and mice glial cells. *PLoS One* 5(6):e11333, 2010. PMID: 20613874

Hicks, S.D.; Middleton, F.A.; and Miller, M.W. Ethanol-induced methylation of cell cycle genes in neural stem cells. *Journal of Neurochemistry* 114(6):1767–1780, 2010. PMID: 20626555

Hillemacher, T.; Frieling, H.; Hartl, T.; et al. Promoter specific methylation of the dopamine transporter gene is altered in alcohol dependence and associated with craving. *Journal of Psychiatric Research* 43(4):388–392, 2009. PMID: 18504048

Jaenisch, R., and Bird, A. Epigenetic regulation of gene expression: How the genome integrates intrinsic and environmental signals. *Nature Genetics* 33(Suppl.): 245–254, 2003. PMID: 12610534

Kazantsev, A.G., and Thompson, L.M. Therapeutic application of histone deacetylase inhibitors for central nervous system disorders. *Nature Reviews. Drug Discovery* 7(10):854–868, 2008. PMID: 18827828

Kim, J.S., and Shukla, S.D. Acute in vivo effect of ethanol (binge drinking) on histone H3 modifications in rat tissues. *Alcohol and Alcoholism* 41(2):126–132, 2006. PMID: 16314425

Levine, A.A.; Guan, Z.; Barco, A.; et al. CREB-binding protein controls response to cocaine by acetylating histones at the fosB promoter in the mouse striatum. *Proceedings of the National Academy of Sciences of the United States of America* 102(52):19186–19191, 2005. PMID: 16380431

Lewohl, J.M.; Nunez, Y.O.; Dodd, P.R.; et al. Up-regulation of microRNAs in brain of human alcoholics. *Alcoholism: Clinical and Experimental Research* 35(11):1928–1937, 2011. PMID: 21651580

Liu, J.; Lewohl, J.M.; Harris, R.A.; et al. Patterns of gene expression in the frontal cortex discriminate alcoholic from nonalcoholic individuals. *Neuropsychopharmacology* 31(7):1574–1582, 2006. PMID: 16292326

Lu, S.C.; Huang, Z.Z.; Yang, H.; et al. Changes in methionine adenosyltransferase and S-adenosylmethionine homeostasis in alcoholic rat liver. *American Journal of Physiology: Gastrointestinal and Liver Physiology* 279(1):G178–G185, 2000. PMID: 10898761

Maier, S.E.; Cramer, J.A.; West, J.R.; and Sohrabji, F. Alcohol exposure during the first two trimesters equivalent alters granule cell number and neurotrophin expression in the developing rat olfactory bulb. *Journal of Neurobiology* 41(3):414–423, 1999. PMID: 10526319

Manzardo, A.M.; Henkhaus, R.S.; and Butler, M.G. Global DNA promoter methylation in frontal cortex of alcoholics and controls. *Gene* 498(1):5–12, 2012. PMID: 22353363

Marutha Ravindran, C.R., and Ticku, M.K. Changes in methylation pattern of NMDA receptor NR2B gene in cortical neurons after chronic ethanol treatment in mice. *Brain Research. Molecular Brain Research* 121(1-2):19–27, 2004. PMID: 14969733

Mayfield, R.D.; Harris, R.A.; and Schuckit, M.A. Genetic factors influencing alcohol dependence. *British Journal of Pharmacology* 154(2):275–287, 2008. PMID: 18362899

McDonel, P.; Costello, I.; and Hendrich, B. Keeping things quiet: Roles of NuRD and Sin3 co-repressor complexes during mammalian development. *International Journal of Biochemistry & Cell Biology* 41(1):108–116, 2009. PMID: 18775506

Moonat, S.; Starkman, B.G.; Sakharkar, A.; and Pandey, S.C. Neuroscience of alcoholism: Molecular and cellular mechanisms. *Cellular and Molecular Life Sciences* 67(1):73–88, 2010. PMID: 19756388

Mulligan, M.K.; Ponomarev, I.; Hitzemann, R.J.; et al. Toward understanding the genetics of alcohol drinking through transcriptome meta-analysis. *Proceedings of the National Academy of Sciences of the United States of America* 103(16):6368–6373, 2006. PMID: 16618939

Pandey, S.C.; Ugale, R.; Zhang, H.; et al. Brain chromatin remodeling: A novel mechanism of alcoholism. *Journal*

of Neuroscience 28(14):3729–3737, 2008. PMID: 18385331

Pascual, M.; Boix, J.; Felipo, V.; and Guerri, C. Repeated alcohol administration during adolescence causes changes in the mesolimbic dopaminergic and glutamatergic systems and promotes alcohol intake in the adult rat. *Journal of Neurochemistry* 108(4):920–931, 2009. PMID: 19077056

Pascual, M.; Do Couto, B.R.; Alfonso-Loeches, S.; et al. Changes in histone acetylation in the prefrontal cortex of ethanol-exposed adolescent rats are associated with ethanol-induced place conditioning. *Neuropharmacology* 62(7):2309–2319, 2012. PMID: 22349397

Pfefferbaum, A.; Rosenbloom, M.; Rohlfing, T.; and Sullivan, E.V. Degradation of association and projection white matter systems in alcoholism detected with quantitative fiber tracking. *Biological Psychiatry* 65(8):680–690, 2009. PMID: 19103436

Pietrzykowski, A.Z.; Friesen, R.M.; Martin, G.E.; et al. Posttranscriptional regulation of BK channel splice variant stability by miR-9 underlies neuroadaptation to alcohol. *Neuron* 59(2):274–287, 2008. PMID: 18667155

Pogribny, I.P., and Rusyn, I. Role of epigenetic aberrations in the development and progression of human hepatocellular carcinoma. *Cancer Letters* doi:10.1016/j.canlet.2012.01.038, 2012. [Epub ahead of print] PMID: 22306342

Ponomarev, I.; Wang, S.; Zhang, L.; et al. Gene coexpression networks in human brain identify epigenetic modifications in alcohol dependence,. *Journal of Neuroscience* 32(5):1884–1897, 2012. PMID: 22302827

Qiang, M.; Denny, A.; Lieu, M.; and Carreon, L.J. Histone H3K9 modifications are a local chromatin event involved in ethanol-induced neuroadaptation of the NR2B gene. *Epigenetics* 6(9):1095–1104, 2011. PMID: 21814037

Renthal, W., and Nestler, E.J. Histone acetylation in drug addiction. *Seminars in Cell & Developemental Biology* 20(4):387–394, 2009*a*. PMID: 19560043

Renthal, W., and Nestler, E.J. Chromatin regulation in drug addiction and depression. *Dialogues in Clinical Neuroscience* 11(3):257–268, 2009*b*. PMID: 19877494

Robison, A.J., and Nestler, E.J. Transcriptional and epigenetic mechanisms of addiction. *Nature Reviews. Neuroscience* 12(11):623–637, 2011. PMID: 21989194

Rosen, G.D.; Chesler, E.J.; Manly, K.F.; and Williams, R.W. An informatics approach to systems neurogenetics. *Methods in Molecular Biology* 401:287–303, 2007. PMID: 18368372

Sakharkar, A.J.; Zhang, H.; Tang, L.; et al. Histone deacetylases (HDAC)-induced histone modifications in the amygdala: A role in rapid tolerance to the anxiolytic effects of ethanol. *Alcoholism: Clinical and Experimental Research* 36(1):61–71, 2012. PMID: 21790673

Sanchis-Segura, C.; Lopez-Atalaya, J.P.; and Barco, A. Selective boosting of transcriptional and behavioral responses to drugs of abuse by histone deacetylase inhibition. *Neuropsychopharmacology* 34(13):2642–2654, 2009. PMID: 19727068

Sathyan, P.; Golden, H.B.; and Miranda, R.C. Competing interactions between micro-RNAs determine neural progenitor survival and proliferation after ethanol exposure: Evidence from an ex vivo model of the fetal cerebral cortical neuroepithelium. *Journal of Neuroscience* 27(32):8546–8557, 2007. PMID: 17687032

Shibasaki, M.; Mizuno, K.; Kurokawa, K.; and Ohkuma, S. Enhancement of histone acetylation in midbrain of mice with ethanol physical dependence and its withdrawal. *Synapse* 65(11):1244–1250, 2011. PMID: 21538550

Shukla, S.D.; Velazquez, J.; French, S.W.; et al. Emerging role of epigenetics in the actions of alcohol. *Alcoholism: Clinical and Experimental Research* 32(9):1525–1534, 2008. PMID: 18616668

Spanagel, R. Alcoholism: A systems approach from molecular physiology to addictive behavior. *Physiological Reviews* 89(2):649–705, 2009. PMID: 19342616

Sweatt, J.D. Experience-dependent epigenetic modifications in the central nervous system. *Biological Psychiatry* 65(3):191–197, 2009. PMID: 19006788

Taqi, M.M.; Bazov, I.; Watanabe, H.; et al. Prodynorphin CpG-SNPs associated with alcohol dependence: Elevated methylation in the brain of human alcoholics. *Addiction Biology* 16(3):499–509, 2011. PMID: 21521424

Valles, S.; Pitarch, J.; Renau-Piqueras, J.; and Guerri, C. Ethanol exposure affects glial fibrillary acidic protein gene expression and transcription during rat brain development. *Journal of Neurochemistry* 69(6):2484–2493, 1997. PMID: 9375681

Wang, L.L.; Zhang, Z.; Li, Q.; et al. Ethanol exposure induces differential microRNA and target gene expression and teratogenic effects which can be suppressed by folic acid supplementation. *Human Reproduction* 2493):562–579, 2009. PMID: 19091803

Wang, Y.; Krishnan, H.R.; Ghezzi, A.; et al. Drug-induced epigenetic changes produce drug tolerance. *PLoS Biology* 5(10):e265, 2007. PMID: 17941717

Williams, R.J.; Berry, L.J.; and Beerstecher, E. Individual metabolic patterns. Alcoholism. Genetotrophic Diseases. *Proceedings of the National Academy of Sciences of the United States of America* 35(6):265–271, 1949. PMID: 16588890

Wolstenholme, J.T.; Warner, J.A.; Capparuccini, M.I.; et al. Genomic analysis of individual differences in ethanol drinking: Evidence for non-genetic factors in C57BL/6 mice. *PLoS One* 6(6):e21100, 2011. PMID: 21598166

Yadav, S.; Pandey, A.; Shukla, A.; et al. miR-497 and miR-302b regulate ethanol-induced neuronal cell death through BCL2 protein and cyclin D2. *Journal of Biological Chemistry* 286(43):37347–37357, 2011. PMID: 21878650

Zahr, N.M.; Kaufman, K.L.; and Harper, C.G. Clinical and pathological features of alcohol-related brain damage. *Nature Reviews. Neurology* 7(5):284–294, 2011. PMID: 21487421

Zhou, Z.; Yuan, Q.; Mash, D.C.; and Goldman, D. Substance-specific and shared transcription and epigenetic changes in the human hippocampus chronically exposed to cocaine and alcohol. *Proceedings of the National Academy of Sciences of the United States of America* 108(16):6626–6631, 2011. PMID: 21464311

Epigenetic Events in Liver Cancer Resulting From Alcoholic Liver Disease

Samuel W. French, M.D.

Samuel W. French, M.D., *is a distinguished professor in the Department of Pathology at the Harbor UCLA Medical Center in Torrance, California.*

Epigenetic mechanisms play an extensive role in the development of liver cancer (i.e., hepatocellular carcinoma [HCC]) associated with alcoholic liver disease (ALD) as well as in liver disease associated with other conditions. For example, epigenetic mechanisms, such as changes in the methylation and/or acetylation pattern of certain DNA regions or of the histone proteins around which the DNA is wrapped, contribute to the reversion of normal liver cells into progenitor and stem cells that can develop into HCC. Chronic exposure to beverage alcohol (i.e., ethanol) can induce all of these epigenetic changes. Thus, ethanol metabolism results in the formation of compounds that can cause changes in DNA methylation and interfere with other components of the normal processes regulating DNA methylation. Alcohol exposure also can alter histone acetylation/deacetylation and methylation patterns through a variety of mechanisms and signaling pathways. Alcohol also acts indirectly on another molecule called toll-like receptor 4 (TLR4) that is a key component in a crucial regulatory pathway in the cells and whose dysregulation is involved in the development of HCC. Finally, alcohol use regulates an epigenetic mechanism involving small molecules called miRNAs that control transcriptional events and the expression of genes important to ALD. **KEY WORDS: Alcohol consumption; alcohol abuse; chronic alcohol use; alcoholic liver disease; ethanol metabolism; alcoholic liver disease; liver cancer; hepatocellular carcinoma; epigenetics; epigenetic mechanisms; DNA methylation; histone methylation; stem cells; micro RNAs**

The molecular pathogenesis of liver cancer (i.e., hepatocellular carcinoma [HCC]) is a multistep process that involves both genetic changes, such as chromosomal abnormalities and mutations of the DNA sequence (i.e., somatic mutations), and epigenetic mechanisms, such as chemical modifications of the DNA and the histone proteins around which the DNA is wrapped to form the chromosomes, microRNA post-transcriptional regulators, and changes in various signaling pathways (Wong et al. 2010). This review will focus on the epigenetic phenomena that contribute to the pathogenesis of HCC resulting from alcoholic liver disease (ALD).

Does ALD Lead to HCC Formation?

According to some studies, ALD is the most common cause of HCC, accounting for approximately one-third of all HCC cases (Morgan et al. 2004). Chronic alcohol use of greater than 80 g/day (or approximately three standard drinks or more per day) for more than 10 years increases the risk for HCC approximately fivefold. In patients with decompensated alcoholic cirrhosis, in whom the liver damage is so extensive that the functional portions of the organ can no longer compensate for the damaged ones, the risk of developing HCC approaches 1 percent per year, and this risk does not decrease with abstinence (Morgan et al. 2004). However, HCC also can occur in patients with noncirrhotic ALD. Finally, HCC is more likely to develop 1 to 10 years after the cessation of drinking by ALD patients. Therefore, HCC in these patients is not directly caused by alcohol consumption (Donato et al. 2002).

Alcohol abuse also has synergistic effects with other risk factors for the development of HCC, such as infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), diabetes, and obesity (Hassan et al. 2002; Loomb et al. 2010; Morgan et al. 2004). For example, studies in Italy (Tagger et al. 1999) and the United States (Hassan et al.

2002) found that in patients with HCV infection, alcohol consumption over 80 g/day increased the odds ratio of developing HCC by 7.3 and 4.5, respectively. Likewise, a study conducted in Africa (Mohamed et al. 1992) determined a synergism between HBV and alcohol consumption over 80 g/day in the development of HCC (odds ratio of 4.4).

What Do ALD, HCV, and HBV Have in Common?

The livers of patients who develop HCCs commonly are cirrhotic. Moreover, they often contain molecules (i.e., markers) indicating that the cells undergo changes in their structure and function to a less specialized (i.e., less differentiated) state. These progenitor/stem cell markers mainly are found in the cirrhotic portion of the liver and in the regions where the HCC develops. These changes and markers have been observed in the livers of patients developing HCC associated with ALD, HBV, and HCV (Oliva et al. 2010). The reversion of normal liver cells (i.e., hepatocytes) into progenitor and stem cells is caused by epigenetic mechanisms. For example, during the development of the progenitor and stem cells, changes occur in the expression of several genes that result from the addition of too many or fewer-than-normal methyl groups to the DNA (i.e., DNA hyper- and hypomethylation, respectively). This alteration of methylation patterns results in an epigenetic reprogramming of the cells (Alison et al. 2009; Collas 2009; Iacobuzio-Donohue 2009; Ohm and Baylin 2009; Richly et al. 2010; Sasaki 2006; Sawan et al. 2008). In addition, modification (i.e., methylation and the addition of acetyl groups [acetylation]) of the histone proteins play roles in the epigenetic modification of progenitor and stem cells that underlies the transformation into cancer cells (i.e., a carcinoma) (Iacobuzio-Donohue 2009). Alcohol excess can induce all of these epigenetic changes that contribute to the transformation of hepatocytes into progenitor or stem cells.

How Does Alcohol Generate Epigenetic Changes?

DNA Methylation

One step in the metabolism of beverage alcohol (i.e., ethanol) in the liver is the oxidation of ethanol by a molecule called cytochrome P450 2E1 (CYP2E1). During this reaction, highly reactive, oxygen-containing molecules (i.e., reactive oxygen species [ROS]) are generated (Bardag-Gorce et al. 2006). ROS are among the most potent agents and conditions that can alter methylation patterns in the liver, including DNA methylation. Thus, oxidative DNA damage caused by ROS, such as the formation of an abnormal variant of the DNA building block (i.e., nucleotide) deoxyguanine called 8-oxyguanine (8-OHdG), can result in a decrease in methylated DNA during DNA repair (Weitzman et al. 1994). 8-OHdG can be incorporated into DNA regions rich in the nucleotides cytosine and guanosine (i.e., CpG islands) in which the cytosine residues frequently are methylated. Incorporation of 8-OHdG into such CpG islands inhibits the methylation of adjacent cytosine residues by enzymes called methyl transferases, resulting in hypomethylation. Also, 8-OHdG formation can interfere with the normal function of DNA methyl transferases and prevent DNA re-methylation (Sagaki 2006). The relationship between alcohol, ROS formation, and DNA damage was demonstrated by studies in cultured liver cells (i.e., HepG2 cells) that were genetically modified to produce excessive levels of CYP2E1. When these cells were incubated with ethanol, ROS-induced DNA damage occurred as indicated by the formation of 8-OHdG (Bardag-Gorce 2006).

Ethanol also interferes with the metabolism of the amino acid methionine into a compound called S-adenosyl-methionine (SAMe) by several different methyl transferase reactions. SAMe, in turn, is needed as the methyl-group donor for many methylation reactions and is converted into S-adenosyl-homocysteine (SAH). Ethanol inhibits methionine adenosyl transferase, which converts methionine into SAMe, as well as enzymes that help regenerate methionine (i.e., betaine homocysteine methyltransferase and methionine synthase) (Seitz and Sticke 2007). This was shown in rodent models of ALD, where ethanol feeding decreased the SAMe/SAH ratio in the liver (Esfandari et al. 2010). The net effect of all these alcohol-induced reductions in methyl transferase activity is to reduce the synthesis of SAMe, which in turn leads to a decrease in DNA methylation.

The significance of reduced SAMe production in the development of HCC is supported by findings that SAMe feeding can inhibit tumor formation (Hitchler and Domann 2009). Furthermore, studies found that the SAMe content and the SAMe/SAH ratio were decreased in tissue regions that showed some damage but had not yet turned into cancer cells (i.e., in preneoplastic lesions). SAMe feeding blocked the transformation of these preneoplastic lesions into HCCs because it promoted global DNA methylation. Moreover, SAMe administration inhibited the expression of certain cancer-inducing genes (i.e., proto-oncogenes) called *c-myc, c-Ha-ras,* and *c-K-ras,* because the SAMe supplementation allowed for the methylation (and thus blockage) of the regulatory regions (i.e., promoters) for those genes. The potential role of SAMe in preventing tumor formation and survival also was supported by an in vitro study demonstrating that SAMe decreased the survival of a type of liver cell tumor Hepa 1-6 in a dose-dependent manner (Oliva et al. 2012). Finally, SAMe treatment prevented cultured liver tumor cells (i.e., H411e cells) from forming a tumor in a model of laboratory rats (Lu et al. 2009).

Histone Modifications

Histones, which exist in numerous variants, regulate gene expression, with the level of gene expression depending on the modifications that the histones undergo. These modifications may include methylation, acetylation, the addition of phosphate groups (i.e., phosphorylation), or the addition of a molecule called ubiquitin (i.e., ubiquitination). These modifications also can have an impact on tumor development. For example, the removal of acetyl groups (i.e., deacetylation) as well as hypermethylation is linked to the inactivation (i.e., silencing) of genes that can help repress tumor formation (i.e., tumor suppression genes) and as a result may promote tumor development (i.e., carcinogenesis). Thus, some cancers exhibit CpG island hypermethylation in combination with multiple histone modifications, such as deacetylation of histones H3 and H4, methylation of histone H3K9, trimethylation of histone H3K27, and a loss of trimethylation of histone H3K4 (Hamilton 2010).

Histone Acetylation. Alcohol exposure can alter histone acetylation and methylation patterns. Researchers have investigated these effects in rats that chronically were fed alcohol through a tube into the stomach (i.e., intragastric tube feeding). These studies identified several alterations in histone methylation and acetylation that correlated with the changes seen in HCCs. Thus, alcohol-treated animals showed increased acetylation of histone H3K18 (Bardag-Gorce et al. 2009) and histone H3K9 (Bardag-Gorce et al. 2007). Furthermore, the levels of several proteins (i.e., phospho c-Jun, phospho AKT threonine 308, p38, pERK, and phospho-SAPK/JNK) in the nucleus of HCC cells were reduced whereas the nuclear levels of a molecule called β-catenin were increased. (For a list of the genes and proteins and their main functions, see table 1.) An increase in β-catenin in the nucleus of hepatocytes indicates activation of a signaling pathway, known as the canonical WNT/β-catenin pathway,[1] that can be involved in tumor formation. This often is seen in HCCs related to ALD and HBV and HCV infection and leads to abnormal cell proliferation and survival (Hamilton 2010). The chronically alcohol-fed rats also had increased levels of an enzyme called histone acetyltransferase (HAT) p300, which is responsible for histone acetylation (Bardag-Gorce et al. 2007). This increase could explain the increased histone H3K4 and H3K9 acetylation, which, in turn, globally activates gene expression (Bardag-Gorce 2009). Simultaneously, the levels of a deacetylase (i.e., SIRT1) also were increased in the alcohol-fed animals (Bardag-Gorce et al. 2007; 2009). This change was accompanied by alterations in the levels of several other molecules, including increases in RARb and peroxisome proliferator–activated receptor (PPAR) C coactivator 1α (PGC1α) expression and a decrease in PPARγ expression.

The increase in HAT p300 levels observed in chronically alcohol-fed rats also could lead to an increase in a signaling molecule called p21WAF1/C, p1 (p21) through several direct and

[1] Among other functions, the WNT/β-catenin pathway is involved in the fate of stem cells and regulates whether stem cells proliferate or self-renew. Accordingly, there is a strong correlation between WNT/β-catenin signaling and the onset of cancer. Normally, β-catenin cannot travel to the nucleus and is degraded. Under certain conditions, however, the degradation of β-catenin is prevented and it enters the nucleus. This can lead to excessive stem cell renewal and proliferation, predisposing the cells to the formation of tumors.

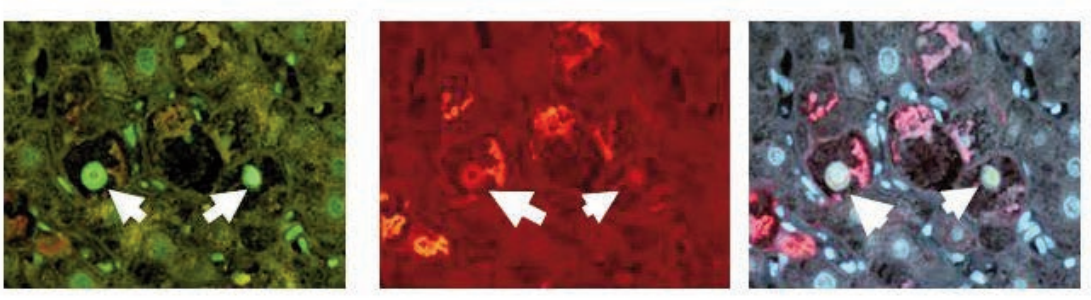


Figure 1 Histone deacetylase 1 (HDAC1) is upregulated in the nuclei of liver cells (i.e., hepatocytes) that form Mallory-Denk bodies (MDBs), which are indicative of liver damage. The image shown is from a liver biopsy from a patient with alcoholic hepatitis. The liver section was IHC double stained for HDAC1 (green nuclei arrows) **(A)**, ubiquitin to identify cells with MDBs (red, arrows) **(B),** and tricolor **(C)**. Magnification: ×350.

indirect mechanisms[2] (Fang et al. 2007). p21 and a related protein called p27 are enzyme complexes that can mediate the phosphorylation of certain other proteins (i.e, protein kinase complexes) and which cause delays in the cycle progression at various stages of the cell cycle, thereby preventing the cells from dividing and multiplying normally. This leads to cell-cycle arrest, genetic instability, programmed cell death (i.e., apoptosis), and oncogenic effects (Abbas and Dutta 2011; Serres et al. 2011, 2012). p21 expression is regulated by histone acetylation, with greater acetylation promoting p21 expression. This process is regulated by a protein complex that is associated with the p21 promoter and which includes an enzyme called histone deacetylase-1 (HDAC1) that reduces acetylation (Dokmanovic et al. 2007) and, thus, p21 expression. Agents that promote acetylation by inhibiting deacetylation (i.e., HDAC inhibitors) accordingly also induce p21 expression, causing cell-cycle arrest (Dokmanovic et al. 2007; Gui et al. 2004). For this reason, HDAC inhibitors are used to treat cancers (Drummond et al. 2005). Liver cells that show signs of ALD—that is, which form Mallory-Denk bodies (MDBs)[3]—show increased HDAC1 levels in their nuclei compared with adjacent normal hepatocytes (see figure 1) (French et al. 2010). The HDAC inhibitor trichstatin A inhibited formation of MDBs in cell cultures from the livers of drug-primed mice (Oliva et al. 2008), indicating that these agents also may be able to prevent the development of liver disease.

The induction of p21 by alcohol abuse may explain why HCC more often only occurs after the patient has stopped drinking. As mentioned above, alcohol consumption induces p21 expression, causing the cell-cycle arrest. After prolonged abstinence, this induction no longer persists in the liver, eliminating the cell cycle arrest and promoting cell multiplication and, thus, tumor formation.

The role of p21 and p27 in HCC also is supported by studies showing that both proteins are overexpressed in alcoholic hepatitis and in rats chronically fed ethanol (Crary and Albrecht 1998; French et al. 2012; Koteish et al. 2007) For example, immunohistochemical studies of liver samples from patients with alcoholic hepatitis found that many of the cells were positive for p27 (see figure 2), and additional analyses indicated that cell-cycle progression was blocked in these cells as indicated by low numbers of nuclei showing expression of (i.e., positive for) a protein called ki-67 (French et al. 2012). Another study demonstrated that both p21 and p27 overexpression inhibit the regeneration of the liver in rats whose liver had been partially removed (Koteish et al. 2007).

Histone Methylation. The levels of methylated histone H3K4 (H3K4me2), as well as histone H3K27 (H3K27me3), are increased in the nuclei of liver cells from rats fed ethanol intragastrically for 1 month (Bardag-Gorce et al. 2009), as demonstrated by intense nuclear staining in immunohistochemical analyses of liver samples. H3K4me2 is associated with active transcription, which seems to have beneficial effects. In particular, the

[2] Direct mechanisms would include HAT p300-induced histone acetylation as seen in human HCC cells, whereas indirect mechanisms could include the induction of a molecule called integrin β17 through regulatory elements called Sp1 sites (Fang et al. 2007).

[3] MDBs are inclusions found in the cytoplasm of liver cells and are indicative of liver damage; they are most commonly found in patients with ALD.

Table 1 Abbreviations of the Protein Names Mentioned in This Article, Their Full Names, and Their Main Functions

Acronym	Full Name	Function
DNACD133	Prominin 1	Cancer stem cell marker
CD49f	Integrin α 6	Cell adhesion Cell signaling
ERK	Extracellular signal-regulated kinase	Signaling pathway for growth of cells
EZH2	Enhancer of Zeste homology 2	Methylates DNA
MyD88	Myeloid differentiation response gene	Activates NFκB
Nanog	Named after Tir NanOg legend	Stem cell renewal
Oct 4	Octamer-binding transcription factor 4	Self-renewal of embryonal cells
p21 Waf1/C.p1	Type of p21 Cip/kip family	Regulates the cell cycle
p27	Type of p27 member Cip/kip	Regulates the cell cycle
pERK	Phosphorylated ERK	Activated ERK
Phospho AKT Threonine 308	AKT-mouse forming thymomas	Regulates cell survival
Phospho cJun	Early response transcription factor	Activates cJun Stimulates cell growth
Phospho-SAPK/JNK	Stress-activated protein kinase Jun-amino kinase	Activates fetal liver formation
PPARPGC1α	PPAR γ coactivator 1 α	Regulates energy metabolism
PPARγ	Peroxisome proliferator-activated receptor γ	Regulates fatty acid storage
RARβ	Retinoic acid receptor β	Regulates cellular growth
SOX 2	SRY (Sex determination region Y) box 2	Induces pluripotential cells
TLR4	Toll-like receptor 4	Innate immunity
β catenin	Cadherin associated protein	Wnt signaling pathway

combination of H3K4me2 with acetylated histone H3K18, which is seen in the alcohol-fed rats, would correlate with an improved prognosis in cancers. A loss of H3K4me2 impairs the body's ability to control DNA damage in cancer because it increases the risk of mutations and, consequently, cancer development (Lennartsson and Ekwall 2009).

Whereas histone acetylation is a highly dynamic process, modification of histones by methylation of one or more lysine amino acids (i.e., mono-, di-, and trimethylation) is thought to be a more lasting change that forms a "cellular memory." Methylation is performed by enzymes known as methyl transferases, and the activity of these enzymes may be specific to certain histones. The enzymes that generate persistent methylation patterns and other histone modifications are known as histone code writers.[4] One such enzyme called EZH2 has intrinsic histone H3K27 methyl transferase activity; it assembles into a multiprotein complex called polycomb repressive complex 2 (PRC2) that, together with another protein complex (i.e., PRC1), maintains a state of transcriptional repression and plays an important role in gene silencing (Muntean and Hess 2009). One role of EZH2/H3K27me3 is to target PRCs to sites of transcriptional regulation and DNA replication. The latter provides the means of perpetuating the characteristics (i.e., phenotypes) of the dividing cell to the daughter cells.

The gene-silencing pathway mediated by H3K27 methylation is linked to the second major silencing pathway (i.e., DNA methylation) via a deacetylase called SIRT 1 that is recruited by the PRC2 complex and contributes to gene silencing (Muntean and Hess 2009). SIRT 1 levels are increased in the alcohol intragastric tube-feeding rat model cited above. In contrast, when SIRT 1 activity is decreased, EZH2 levels increase, which enhances the EZH2-mediated repression of target genes (Lu et al. 2011). Upregulation of EZH2 expression in tumors appears to correlate with disease progression by maintaining a stem cell-like phenotype. Overexpression of EZH2 can lead to cancer progression mediated by deregulation of epigenetic mechanisms (Muntean and Hess 2009). However, EZH2 levels do not seem to be affected by alcohol and other factors that can induce liver damage. For example, EZH2 levels were not changed in mice that exhibited a precursor stage to HCC (as characterized by the presence of balloon cells and MDBs) after drug treatment, in liver biopsies of patients with alcoholic hepatitis, or in MDB-forming HCCs (French et al. 2012) (see figure 3). However, in all three cases there were increases in a modified form of EZH2 (i.e., phosphorylated EZH2 [pEZH2]), which is degraded more rapidly in the cells than unmodified EZH2 and is located in the MDBs as demonstrated by immunohistochemistry. This degradation of pEZH2 occurs at cell components called proteasomes. However, proteasomes are inhibited by ethanol excess; as a result,

[4] The term histone code refers to the hypothesis that modifications of different histones have different effects on gene expression—that is, whereas methylation of some histones activates gene expression, methylation of other histones may inhibit it. It is thought that the histone modifications serve to recruit other regulatory proteins that specifically recognize the modified histones. These recruited proteins then act to alter chromatin structure actively or to promote transcription. Enzymes that generate the histone modifications that make up this histone code are referred to as code writers.

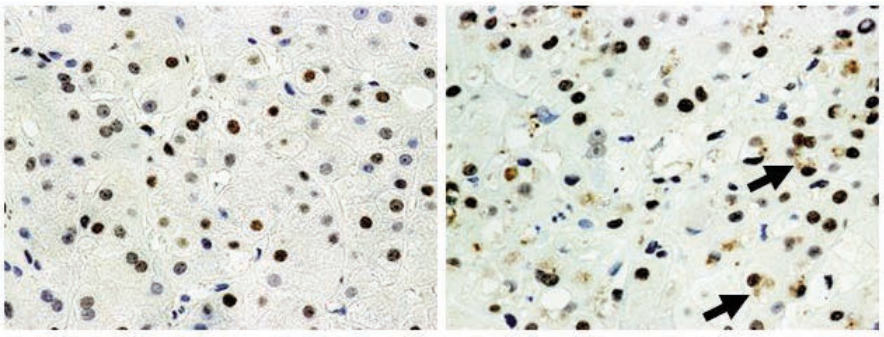


Figure 2 The signaling molecule p27 is upregulated in the nuclei of liver cells (i.e., hepatocytes) in a liver biopsy from two patients with alcoholic hepatitis. The livers were stained with an immunoperoxidase-labeled antibody that recognizes p27. The hepatocyte nuclei positive for p27 appear brown; those that are negative for p27 appear blue. **(A** and **B)** Most of the nuclei stained positive. **(B)** The Mallory-Denk bodies (MDBs) also stained brown (arrows), indicating that p27 also is sequestered in the MDBs. Magnification ×520.

pEZH2 levels are increased in MDBs. Moreover, in all three cases, the levels of H3K27me3 were reduced in the nuclei of the damaged liver cells (i.e., cells that were ballooned or formed MDBs) compared with neighboring normal liver cells as shown by different experimental approaches (Bardag-Gorce et al. 2010; French et al. 2012). Paradoxically, when tumors form, they overexpress EZH2. High expression of EZH2 in tumors is associated with poor survival (Gieni and Hendzel 2009). Thus, EZH2 overexpression represses expression of the product of a tumor suppressor gene called E cadherin that causes cells to stick to each other. Accordingly, loss of E cadherin expression by tumor cells may cause loss of cell cohesion, which would promote metastasis and thus a more unfavorable prognosis.

How Are Stem Cells Converted to Cancer Stem Cells in ALD?

In individuals with HCC associated with ALD, focal progenitor cell/stem cell formation occurs both in portions of the liver that show cirrhosis and in the HCC cells as indicated by the expression of certain proteins (i.e., Nanog, Yapi-1, Igf2bp, and Sox2) (see figure 4 A, B, C,). This raises the question whether the liver cells that are transformed into progenitor cells/stem cells in the cirrhotic liver subsequently are transformed in a second step into cancer stem cells in HCC. One of the regulatory molecules involved in this process is called Nanog. It is a transcription factor that is thought to play a crucial role in the self-renewal of embryonic stem cells and helps them maintain their ability to subsequently differentiate into numerous other cell types. Cancer stem cells can express both EZH2/H3k27me3 and Nanog, and the epigenetic balance between these factors determines the further fate of the cells. When the levels of Nanog are high and those of EZH2/H3K27me3 are low, the cells exhibit self-renewal activity—that is, they multiply and a tumor can develop. Paradoxically, when the reverse is true, the cancer stem cells differentiate into cells that no longer proliferate (Villasante et al. 2011). Also, EZH2-mediated epigenetic silencing of tumor suppressor genes leads to the activation of the WNT/β-catenin signaling pathway mentioned earlier, which culminates in the proliferation of HCC cells (Cheng et al. 2011). EZH2 overexpression occurs in many different cancers, where it acts as a classical oncogene that can promote tumor formation by silencing several tumor suppressor genes, such as E cadherin. These suppressor genes play a role for both tumor cells and cancer stem cells (Crea 2011).

What Role Do Epigenetic Changes in TLR4 Play in Stem Cell Transformation?

Another molecule that is involved in the epigenetic mechanisms contributing to ALD-related HCC and which helps regulate the activity of Nanog is called toll-like receptor 4 (TLR4). Studies in a certain line of genetically modified mice (i.e., HCV core transgenic mice) that were chronically fed alcohol found that TLR4 activation leads to up regulation of Nanog in stem cells (Machida et al. 2009). This TLR4–Nanog pathway promotes the development of liver tumors induced by a variety of factors, including alcohol, diabetes, and HCV (Machida et al. 2012). The activation of TLR4 is regulated both at the transcriptional level (i.e., via molecules called lipopolysaccharides [LPS]) and at the epigenetic level (i.e., via acetylation of histones and methylation of DNA). For instance,

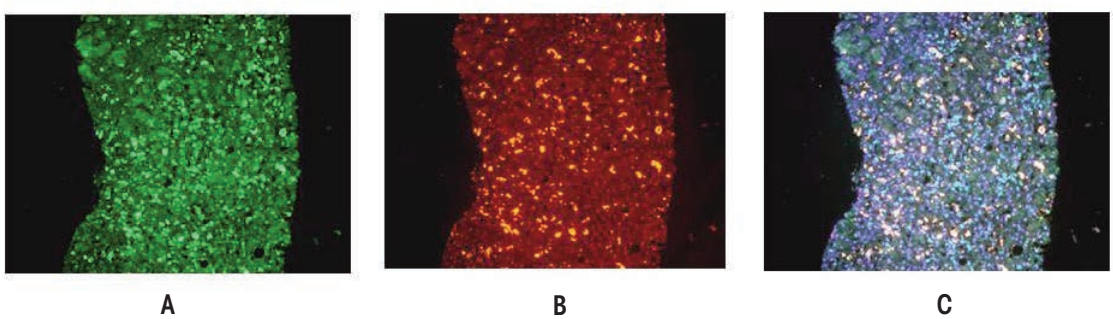


Figure 3 These images show a double-immunostained liver biopsy from a patient with alcoholic hepatitis where most of the hepatocytes had formed Mallory-Denk bodies (MDBs). The MDBs stained positive for **(A)** pEZHZ (green), **(B)** ubiquitin (red), and **(C)** merged (yellow), indicating that the pEZH2 colocalized in the MDBs. Magnification ×350.

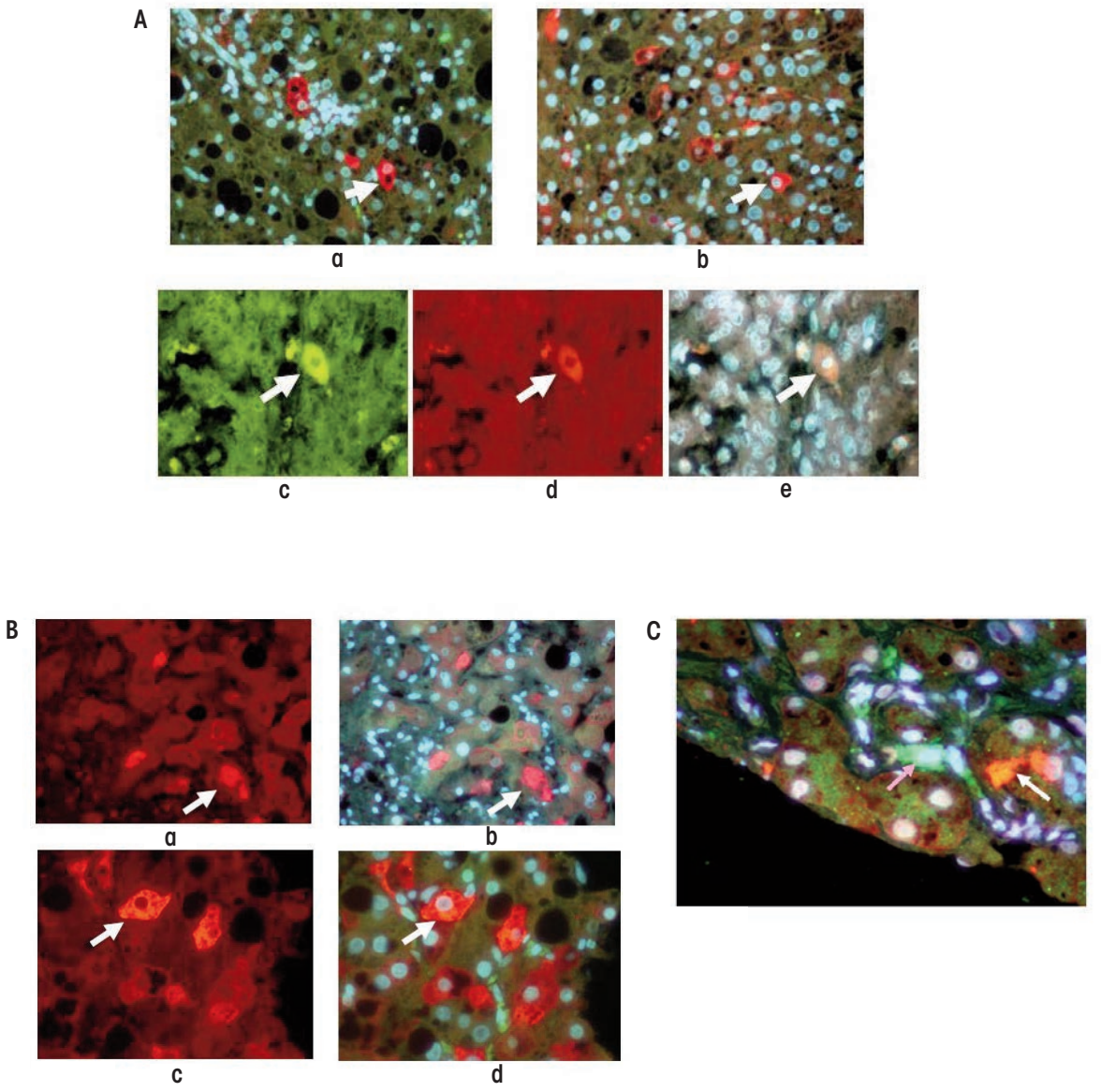


Figure 4 Analysis of different marker proteins in stem cell/progenitor cells located in the livers of patients with alcoholic liver disease with cirrhosis and associated hepatocellular carcinoma (HCC). **(A)** Liver cirrhosis and HCC samples stained for both YAP-1 (green) and IGF2bp3 (red). a) Cirrhosis; b) HCC (magnification ×350); c) HCC; d) HCC; e) Tricolor image merged from c and d (magnification ×525). **(B)**, a and b) Liver cirrhosis sample double stained for Nanog (green) and SOX2 (red). Note the Mallory-Denk bodies (MDBs) (arrow) stain positive for SOX 2. c and d) Liver cells stained for Yap 1 (green) and SOX 2 (red). The liver cells/progenitor cells stain positive for SOX2 (arrows). Magnification ×780. **(C)** Liver sample from a patient with alcoholic hepatitis double stained for the Nanog protein (green) and ubiquitin (red). The stem cell stains positive for Nanog (pink arrow) and an MDB stained positive for ubiquitin (white arrow). Magnification ×780.

increased methylation of regulatory DNA regions in front of the gene encoding TLR4 was found in embryonic stem cells. Moreover, increased methylation suppressed TLR promoter activity in reporter gene assays (Zampetaki et al. 2006). In addition, other assays (i.e., CHiP assays) in embryonic stem cells demonstrated that histones H3 and H4 had lower-than-normal acetylation levels (i.e., were hypoacetylated) in the TLR promoter region. Treatment with inhibitors of DNA methylation or deacetylase partially relieved repression of the TLR4 gene and increased its responsiveness to LPS (Zampetaki et al. 2006). The combined inhibition of DNA methylation and histone deacetylase activity leads to a robust induction of TLR4 with return of LPS responsiveness.

Rats fed ethanol intragastrically for 1 month had increased levels of TLR4 and another molecule called MyD88 in their livers. This effect could be prevented by feeding the animals SAMe together with the alcohol, which, as mentioned earlier, is required for methylation. These findings indicate that methylation can prevent the alcohol-induced changes in TLR4 and MyD88 levels (Oliva et al. 2012). Similar changes in TLR4 expression and protein levels were found in mice that developed liver tumors after being fed a compound called diethyl 1,4-dihydro-2,4,6-trimethyl-3,5pyridinedicarboxylate (DDC). Again, the changes could be prevented by also feeding the animals SAMe (Bardag-Gorce et al. 2010). More detailed analyses determined reductions in the levels of H3k27me3 that also could be prevented by SAMe feeding. These findings indicate that DDC feeding causes histone demethylation, which in turn results in increased TLR4 expression (Bardag-Gorce et al. 2010). In fact, the mice exhibited numerous epigenetic changes of histone methylation and acetylation (Bardag-Gorce et al. 2008), as well as DNA methylation of the gene encoding interleukin 12A (Oliva and French 2012).

Researchers also have studied the TLR4-Nanog pathway in another line of genetically modified mice (i.e., HCV Ns5a transgenic mice) that were fed alcohol; under these conditions, liver tumors form in the animals that contain cancer stem cells (Machida et al. 2012). During this process, cells that normally differentiate into hepatocytes (i.e., hepatic stem cells) are transformed into tumor-initiating stem-like cells (TISCs), which then may develop further into cancer cells and cause tumor formation in other tissues. For example, TISC cells isolated from alcoholic patients induced tumor formation in cultured tissues (i.e., in vitro) and after transplantation into laboratory animals (i.e., in a xenograft model). The role of TLR4 and Nanog in this process was demonstrated by findings that when TLR4 or Nanog were silenced, the tumor-initiating properties of the TISCs were attenuated. Further studies found that Nanog upregulated the expression of two genes encoding molecules called Yap 1 and activator Igf2bp3, which in turn inhibited transforming growth factor-β signaling in the TISCs. Transforming growth factor-β signaling inhibits the growth of liver cells; accordingly, its inhibition would favor the proliferation of TISCs to form liver tumors. These observations suggest that TLR4 may be a universal proto-oncogene that is responsible for the development of TLR/Nanog-dependent TISCs. By staining tissue samples with specific markers researchers demonstrated that TISCs can be found in patients with cirrhosis and HCCs caused by alcoholism as well as by nonalcoholic hepatitis and HBV or HCV infection (Bardag-Gorce et al. 2008; French et al. 2011; Oliva et al. 2010) (figures 4A–C).

Machida and colleagues (2012) also found that TISCs isolated from the livers of alcohol-fed HCV Ns5a transgenic mice and from alcoholic patients carried molecules called CD133 and CD49f (i.e., were $CD133^{+}/CD49f^{+}$ cells). CD49f enhances the cell's ability to differentiate into different cell types (i.e., multipotency) and maintains the cells' stem-cell–like characteristics by directly controlling the regulatory molecules OCT4 and SOX2 (Yu et al. 2012). In addition, CD49f activates a signaling pathway called the phosphatidylinositol 3-kinase (P13K) AKT pathway and suppresses the levels of a protein called p53, which regulates the cell cycle and acts to prevent tumor formation (i.e., is a tumor suppressor gene). Immunohistochemical analyses of liver biopsies from patients with alcoholic hepatitis that contained numerous MDBs found that these cells expressed high levels of CD49f in the cytoplasm and the nuclei (see figure 5). This finding supports the concept that MDB-forming hepatocytes have progenitor and pluripotential properties and eventually may transform into TISC cells. Furthermore, in mice that were fed DCC and subsequently developed MDBs and, ultimately, HCC, CD49f, in combination with other molecules, induced MDB formation. This process could be blocked by inhibiting the phosphorylation of ERK and thus the activation of this protein as well as MDB formation (Wu et al. 2005).

What About MicroRNAs?

MicroRNAs (miRNAs) are a class of small noncoding RNAs that, in general, negatively regulate gene expression at the posttranscriptional level. Each miRNA controls a specific set of target genes. miRNAs have been identified in various tumor types, including HCCs. miRNAs also are encoded by specific genes in the DNA. miRNA genes that harbor CpG islands can undergo methylation-mediated silencing, similar to many tumor suppressor genes. As a result, the miRNAs are not produced and therefore cannot inhibit the expression of their target genes. In one study examining the expression of 11 miRNA genes in HCCs, three of those genes were silenced (i.e., those encoding miRNAs miR-124, miR-203, and miR-375) (Furuta et al. 2010). For miR-124 and miR-203, the methylation frequently was tumor specific and was not found in nontumor tissue.

Thus, these miRNAs were suppressive miRNAs for HCC that could be silenced epigenetically. This silencing resulted in the activation of multiple target genes (i.e., those encoding CDK6, vimentin, SET, and MYNO domain) (Furuta et al. 2010). Conversely, for other miRNAs the levels were increased in HCC, including miR-21, miR-34a, miR-221/222, miR-224, miR-106a, miR-92, miR-17-5 p, miR-20, and miR-18 (Bracconi and Patel 2008; Murakami et al. 2006). Finally, one miRNA (i.e., miR-126) was specific to HCC and alcohol use (Ladeiro et al. 2008).

Studies have shown that alcohol use regulates miRNAs that control transcriptional events and the expression of genes important to ALD (Mandrekar 2011). Mice fed alcohol as part of a liquid diet showed decreases in the levels of 1 percent of the total known miRNAs and increases in the levels of 3 percent of the miRNAs (Dolaniuc et al. 2009). For example, the levels of miR-182, miR-183, and miR-199a-3P were decreased, whereas those of miR-705 and miR-122 were increased. So far the miRNAs associated with HCC do not overlap with those associated with experimental ALD. However, there is overlap of changes in miRNA expression observed in mice fed a methyl-deficient diet for 12 weeks and those identified in HCCs (i.e., in both models the levels of miR-34a and miR-122 are changed) (Pogribny et al. 2009). In the methyl-deficient mice these miRNAs were associated with more extensive liver damage. These data mechanistically link alterations in microRNA expression to the pathogenesis of HCC and strongly suggest that differences in the susceptibility to liver carcinogenesis may be determined by differences in the miRNA expression response to factors such as methyl deficiency.

Summary

ALD is a major cause of HCC, which usually develops long after alcohol abuse has ceased and when cirrhosis has developed. This clinical pattern suggests that changes in epigenetic liver cellular memory occur that affect differentiation and cellular renewal, as well as the transformation to HCC. Progenitor hepatocytes develop during the cirrhotic process from normal cells through epigenetic mechanisms, such as changes in DNA hyper- or hypomethylation, histone acetylation and methylation, and epigenetic reprogramming. For example, oxidative DNA damage from ethanol-induced ROSs leads to loss of methylated DNA. Chronic ethanol feeding leads to altered methionine metabolism and reduced DNA methylation because the levels of the major methyl donor SAMe are lowered. This process can be prevented by feeding SAMe or another compound called betaine together with ethanol.

Likewise, chronic ethanol feeding alters the methylation and acetylation of histones in the liver. Histone acetylation leads to upregulation of p21, causing cell cycle arrest and DNA damage. This, in turn, results in loss of DNA methylation, as demonstrated in experimental rat models as well as in human alcoholic hepatitis and HCCs. Histone H3K27me3, together with EZH2, regulates stem cell renewal and differentiation of progenitor stem cells in the liver. The balloon cells, which form MDBs in alcoholic hepatitis and HCCs, show a decrease in nuclear H3K27me3 and an increase in pEZH2 in the MDBs. This supports the role of MDB-forming cells as progenitor cells that give rise to HCC transformation. This concept is supported by findings that the MDB-forming cells also express proteins that are markers of embryonal stem cells (i.e., SOX2 and

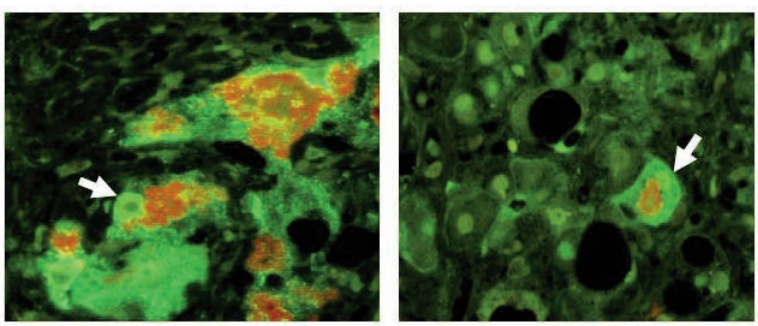


Figure 5 Immunohistochemical analysis of a liver biopsy obtained from a patient with alcoholic hepatitis with Mallory-Denk body (MDB) formation. The samples were stained for the presence of CD49f (integrin subunit α6) (green) and ubiquitin (red). Note that the MDBs stain both red for ubiquitin and green for CD49f. The arrows point to the nuclei that stain green except for the nucleolus. The yellow fringe on the MDB indicates colocalization of both proteins at the interface of the MDBs. The round black holes are macrovesicular fat globules in the hepatocytes. **A)** (magnification ×700) shows a cluster of MDB-forming cells. **B)** (magnification ×1,050) shows a single cell forming an MDB.

CD49f). The transformation of these progenitor cells to HCC is driven by the TLR4 signaling pathway, which is upregulated by increases in LPS levels in the liver that result from alcohol abuse. This upregulation of the TLR4 pathway, which has been demonstrated in rats chronically fed ethanol, can be prevented by SAMe supplementation. These and other findings support the concept that TLR4 may be a proto-oncogene responsible for the transformation of progenitor cells into HCC in ALD as well as HCV infection. ■

Acknowledgements

The author acknowledges that the manuscript was typed by Adriana Flores and the immunohistochemistry was done by Barbara French.

Financial Disclosure

The author declares that he has no competing financial interests.

References

Abbas, T., and Dutta, A. CRL4^{Cdt2}: Master coordinator of cell cycle progression and genome stability. *Cell Cycle* 10(2):241–249, 2011. PMID: 21212733

Alison, M.R.; Islam, S.; and Lim, S. Stem cells in liver regeneration, fibrosis and cancer: The good, the bad and the ugly. *Journal of Pathology* 217(2):282–298, 2009. PMID: 18991329

Bardag-Gorce, F.; French, B.A.; Joyce, M.; et al. Histone acetyltransferase p300 gene expression in an epigenetic manner at high blood alcohol levels. *Experimental and Molecular Pathology* 82(2):197–202, 2007. PMID: 17208223

Bardag-Gorce, F.; French, B.A.; Nan, L.; et al. CYP2E1 induced by ethanol causes oxidative stress, proteasome inhibition and cytokeratin aggresome (Mallory body-like) formation. *Experimental and Molecular Pathology* 81(3):191–201, 2006. PMID: 17034788

Bardag-Gorce, F.; Oliva, J.; Dedes, J.; et al. Chronic ethanol feeding alters hepatocyte memory which is not altered by acute feeding. *Alcoholism: Clinical and Experimental Research* 33(4):684–692, 2009. PMID: 19170665

Bardag-Gorce, F.; Oliva, J.; Lin, A.; et al. SAMe prevents the up regulation of toll-like receptor signaling in Mallory-Denk body forming hepatocytes. *Experimental and Molecular Pathology* 88(3):376–379, 2010. PMID: 20206621

Bardag-Gorce, F.; Oliva, L.; Villegas, J.; et al. Epigenetic mechanisms regulate Mallory Denk body formation in the livers of drug-primed mice. *Experimental and Molecular Pathology* 84(2):113–121, 2008. PMID: 18281034

Braconi, C., and Patel, T. MicroRNA expression profiling: A molecular tool for defining the phenotype of hepatocellular tumors. *Hepatology* 47(6):1807–1809, 2008. PMID: 18506877

Cheng, A.S.; Lan, S.S.; Chen, Y.; et al. EZH2-mediated concordant repression of Wnt antagonists promotes β-catenin-dependent hepatocarcinogenesis. *Cancer Research* 71(11):4028–4039, 2011. PMID: 21512140

Collas, P. Epigenetic states in stem cells. *Biochemica et Biophysica Acta* 1790(9):900–905, 2009. PMID: 19013220

Crary, G.S., and Albrecht, J.H. Expression of cyclin-dependent kinase inhibitor p21 in human liver. *Hepatology* 28(3):738–743, 1998. PMID: 97311566

Crea, F. EZH2 and cancer stem cells: Fact or fiction? *Epigenomics* 3(2):127–128, 2011. PMID: 22122274

Dokmanovic, M.; Clarke, C.; and Marks, P.A. Histone deacetylase inhibitors: Overview and perspectives. *Molecular Cancer Research* 5(10):981–989, 2007. PMID: 17951399

Dolganiuc, A.; Petrasek, J.; Kodys, K.; et al. Micro RNA expression profile in Lieber-DeCarli diet-induced alcoholic and methionine choline deficient diet-induced nonalcoholic steatohepatitis models in mice. *Alcoholism: Clinical and Experimental Research* 33(10):1704–1710, 2009. PMID: 19572984

Donato, F.; Tagger, A.; Gellati, U.; et al. Alcohol and hepatocellular carcinoma. The effect of lifetime intake and hepatitis virus infections in men and women. *American Journal of Epidemiology* 155(4):323–331, 2002. PMID: 11836196

Drummond, D.C.; Noble, C.O.; Kirpotin, D.B.; et al. Clinical development of histone deacetylase inhibitors as anticancer agents. *Annual Review of Pharmacology and Toxicology* 45:495–528, 2005. PMID: 15822187

Esfandiari, F.; Medici, V.; Wong, D.H.; et al. Epigenetic regulation of hepatic endoplasmic reticulum stress pathways in the ethanol-fed cystathionine beta synthase-deficient mouse. *Hepatology* 51(3):932–941, 2010. PMID: 19957376

Fang, Z.; Fu, Y.; Liang, Y.; et al. Increased expression of integrin in β1 subunit enhances p21$^{WAF1/Cip1}$ transcription through the Sp1 sites and p300-mediated histone acetylation in human hepatocellular carcinoma cells. *Journal of Cellular Biochemistry* 101(3):654–664, 2007. PMID: 17211849

French, B.A.; Oliva, J.; Bardag-Gorce, F.; et al. Mallory-Denk bodies form when EZH2/H3K27me3 fails to methylate DNA in the nuclei of human and mice liver cells. *Experimental and Molecular Pathology* 92(3):318–326, 2012. PMID: 22465358

French, S.W.; Bardag-Gorce, F.; French, B.A.; et al. The role of innate immunity in the pathogenesis of preneoplasia in drug-induced chronic hepatitis based on a mouse model. *Experimental and Molecular Pathology* 91(3):653–659, 2011. PMID: 21820428

French, S.W.; Bardag-Gorce, F.; Li, J.; et al. Mallory-Denk body pathogenesis revisited. *World Journal of Hepatology* 2(8):295–301, 2010. PMID: 21161012

Furuta, M.; Kozaki, K.I.; Tanaka, S.; et al. miR-124 and miR-203 are epigenetically silenced tumor-suppressive microRNAs in hepatocellular carcinoma. *Carcinogenesis* 31(5):766–776, 2009. PMID: 19843643

Gieni, R.S., and Hendzel, M.J. Polycomb group protein gene silencing, non-coding RNA, stem cells, and cancer. *Biochemistry and Cell Biology* 87(5):711–746, 2009. PMID: 19898523

Gui, C.Y.; Ngo, L.; Xu, W.S.; et al. Histone deacetylase (HDAC) inhibitor activation of p21^{WAF1} involves changes in promoter-associated proteins, including HDAC1. *Proceedings of the National Academy of Sciences of the United States of America* 101(5):1241–1246, 2004. PMID: 14734806

Hamilton, J.P. Epigenetic mechanisms involved in the pathogenesis of hepatobiliary malignancies. *Epigenomics* 2(2):233–243, 2010. PMID: 20556199

Hassan, M.M.; Hwang, L.Y.; Hatten, C.J.; et al. Risk factors for hepatocellular carcinoma: Synergism of alcohol with viral hepatitis and diabetes mellitus. *Hepatology* 36(5): 1206–1213, 2002. PMID: 12395331

Hitchler, M.J., and Domann, F.E. Metabolic defects provide a spark for the epigenetic switch in cancer. *Free Radical Biology & Medicine* 47(2):115–127, 2009. PMID: 19362589

Iacobuzio-Donohue, C.A. Epigenetic changes in cancer. *Annual Review of Pathology* 4:229–249, 2009. PMID: 18840073

Koteish, A.; Yang, S.; Lin, H.; et al. Ethanol induces redox-sensitive cell-cycle inhibitors and inhibits liver regeneration after partial hepatectomy. *Alcoholism: Clinical and Experimental Research* 26(11):1710–1718, 2002. PMID: 12436061

Ladeiro, Y.; Couchy, G.; Balabaud, C.; et al. MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor mutations. *Hepatology* 47(6):1955–1963, 2008. PMID: 18433021

Lennartsson, A., and Ekwall, K. Histone modification patterns and epigenetic codes. *Biochemica et Biophysica Acta* 1790(9):863–868, 2009. PMID : 19168116

Loomba, R.; Yang, H.I.; Su, J.; et al. Obesity and alcohol synergize to increase the risk of incident hepatocellular carcinoma in men. *Clinical Gastroenterology and Hepatology* 8(10):891–898, 2010. PMID: 20621202

Lu, L.; Li, L.; Lu, X.; et al. Inhibition of SIRT1 increases EZH2 protein level and enhances the repression of EZH2 on target gene expression. *Chinese Medical Sciences Journal* 26(2):77-84, 2011. PMID: 21703114

Lu, S.C.; Ramani, K.; Ou, X.; et al. S-adenosylmethionine in the chemoprevention and treatment of hepatocellular carcinoma in a rat model. *Hepatology* 50(2):462–471, 2009. PMID: 19444874

Machida, K.; Tsukamoto, H.; Mkrtchyan, H.; et al. Toll-like receptor 4 mediates synergism between alcohol and HCV in hepatic oncogenesis involving stem cell marker Nanog. *Proceedings of the National Academy of Sciences of the United States of America* 106(5):1548–1553, 2009. PMID: 19171902

Machida, K.; Chen, C.L.; Liu, J.C.; et al. Cancer stem cells generated by alcohol, diabetes and hepatitis C virus. *Journal of Gastroenterology and Hepatology* 27(Suppl. 2):19–22, 2012. PMID: 22320911

Mandrekar, P. Epigenetic regulation in alcoholic liver disease. *World Journal of Gastroenterology* 17(20):2456–2464, 2011. PMID: 21633650

Mohamed, A.E.; Kew, M.C.; and Groeneveld, H.T. Alcohol consumption as a risk factor for hepatocellular carcinoma in urban Southern Africa Blacks. *International Journal of Cancer* 51(4):537–541, 1992. PMID: 1318267

Morgan, T.R.; Mandayam, S.; and Jamal, M.M. Alcohol and hepatocellular carcinoma. *Gastroenterology* 127(5 Suppl 1):S87–S96, 2004. PMID: 15508108

Muntean, A.G., and Hess, J.L. Epigenetic dysregulation in cancer. *American Journal of Pathology* 175(4):1353–1361, 2009. PMID: 19717641

Murakami, Y.; Yasuda, T.; Saigo, K.; et al. Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. *Oncogene* 25(17):2537–2545, 2006. PMID: 16331254

Ohm, J.E., and Baylin, S.B. Stem cell chromatin patterns and DNA hypermethylation in cancer drug discovery and development. In: Bagley, R.G., and Teicher, B.A., eds. *Stem Cells and Cancer.* New York: Humana Press, 2009, pp. 85–97.

Oliva, J., and French, S.W. Changes in IL12A methylation pattern in livers from mice fed DDC. *Experimental and Molecular Pathology* 92(2):191–193, 2012. PMID: 22273483

Oliva, J.; Bardag-Gorce, F.; French, B.A.; et al. FAT10 is an epigenetic marker for liver preneoplasia in a drug-primed mouse model of tumorigenesis. *Experimental and Molecular Pathology* 84(2):102–112, 2008. PMID: 18280469

Oliva, J.; French, B.A.; Qing, X.; and French, S.W. The identification of stem cells in human liver diseases and hepatocellular carcinoma. *Experimental and Molecular Pathology* 88(3):331–340, 2010. PMID: 20080086

Oliva, J.; Zhong, J.; Buslon, V.S.; and French, S.W. The effect of SAMe and betaine on Hepa 1-6, C34 and E47 liver cell survival *in vitro*. *Experimental and Molecular Pathology* 92(1):126–130, 2012. PMID: 22032937

Pogribny, I.P. MicroRNA dysregulation during chemical carcinogenesis. *Epigenomics* 1(2):281–290, 2009. PMID: 22122703

Richly, H.; Lange, M.; Simboeck, E.; and Croce, L. Setting and resetting of epigenetic marks in malignant transformation and development. *Bioassays* 32(8):669–679, 2010. PMID: 20658705

Sasaki, Y. Does oxidative stress participate in the development of hepatocellular carcinoma? *Journal of Gastroenterology* 41(12):1135–1148, 2006. PMID: 17287893

Sawan, C.; Vaissiere, T.; Murr, R.; and Herceg, Z. Epigenetic drivers and genetic passengers on the road to cancer. *Mutation Research* 642(1-1):1–13, 2008. PMID: 18471836

Seitz, H.K., and Stickel, F. Molecular mechanisms of alcohol-mediated carcinogenesis. *Nature Reviews. Cancer* 7(8):599–612, 2007. PMID: 17646865

Serres, M.P.; Kossatz, U.; Chi, Y.; et al. p27 (Kip1) controls cytokinesis via the regulation of citron kinase activation. *Journal of Clinical Investigation* 122(3):844–858, 2012. PMID: 22293177

Serres, M.P.; Zlotek-Zlotkiewicz, E.; Concha, C.; et al. Cytoplasmic p27 is oncogenic and cooperates with Ras both *in vivo* and *in vitro*. *Oncogene* 30(25):2846–2858, 2011. PMID: 21317921

Starland-Davenport, A.; Tryndyak, V.; Kosyk, O.; et al. Dietary methyl deficiency, microRNA expression and susceptibility to liver carcinogenesis. *Journal of Nutrigenetics and Nutrigenomics* 3(4-6):259–266, 2010. PMID: 21474957

Tagger, A.; Donato, F.; Ribero, M.L.; et al. Case-control study on hepatitis C virus (HCV) as a risk factor for hepatocellular carcinoma: The role of HCV genotypes and the synergism with hepatitis B virus and alcohol. Bresica HCC Study. *International Journal of Cancer* 81(5):695–699, 1999. PMID: 10328218

Villasante, A.; Piazzolla, D.; Li, H.; et al. Epigenetic regulation of Nanog expression by EZH2 in pluripotent stem cells. *Cell Cycle* 10(9):1488–1498, 2011. PMID: 21490431

Weitzman, S.A.; Turk, P.W.; Milowski, D.H.; and Kozlowski, K. Free radical adducts induce alterations in DNA cytosine methylation. *Proceedings of the National Academy of Sciences of the United States of America* 91(4):1261–1264, 1994. PMID: 8108398

Wong, C.M.; Yam, J.W.; and Ng, I.O. Molecular Pathogenesis of hepatocellular carcinoma. In: Wang, X.W.; et al., (eds). *Molecular Genetics in Liver Neoplasms, Cancer Genetics.* New York: Springer Science and Business Media, LLC, pp. 373–396, 2010.

Wu, Y.; Nan, L.; Bardag-Gorce, F.; et al. The role of laminin- integrin signaling in triggering MB formation. An *in vivo* and *in vitro* study. *Experimental and Molecular Pathology* 79(1):1–8. 2005. PMID: 15896771

Yu, K.R.; Yang, S.R.; Jung, J.W.; et al. CD49f enhances multipotency and maintains stemness through the direct regulation of OCT4 and SOX2. *Stem Cells* 30(5):876–887, 2012. PMID: 2231137

Zampetaki, A.; Xiao, Q.; Zeng, L.; et al. TLR4 expression in mouse embryonic stem cells and in stem cell-derived vascular cells is regulated by epigenetic modifications. *Biochemical and Biophysical Research Communications* 347(1):87–99, 2006. PMID: 16814255

Epigenetic Effects of Ethanol on the Liver and Gastrointestinal System

Shivendra D. Shukla, Ph.D., and Robert W. Lim, Ph.D.

Shivendra D. Shukla, Ph.D., *is Margaret Proctor Mulligan Professor, and* **Robert W. Lim, Ph.D.,** *is an associate professor in the Department of Medical Pharmacology & Physiology, School of Medicine, University of Missouri, Columbia, Missouri.*

The widening web of epigenetic regulatory mechanisms also encompasses ethanol-induced changes in the gastrointestinal (GI)–hepatic system. In the past few years, increasing evidence has firmly established that alcohol modifies several epigenetic parameters in the GI tract and liver. The major pathways affected include DNA methylation, different site-specific modifications in histone proteins, and microRNAs. Ethanol metabolism, cell-signaling cascades, and oxidative stress have been implicated in these responses. Furthermore, ethanol-induced fatty liver (i.e., steatohepatitis) and progression of liver cancer (i.e., hepatic carcinoma) may be consequences of the altered epigenetics. Modification of gene and/or protein expression via epigenetic changes also may contribute to the cross-talk among the GI tract and the liver as well as to systemic changes involving other organs. Thus, epigenetic effects of ethanol may have a central role in the various pathophysiological responses induced by ethanol in multiple organs and mediated via the liver–GI axis.

KEY WORDS: Ethanol; ethanol metabolism; alcohol consumption; epigenetics; epigenetic effects; epigenetic mechanisms; alcohol-induced epigenetic alterations; liver; gastrointestinal system; immune system; DNA methylation; histone acetylation; microRNAs (miRNAs); cell-signaling; oxidative stress; alcoholic liver disease; steatohepatitis; liver cancer; hepatic carcinoma

Epigenetic modifications are emerging as important dynamic mechanisms contributing to both transient and sustained changes in gene expression. In some cases, epigenetic changes even can be inherited, although the mechanism for this remains elusive. Several types of epigenetic modifications have been studied in recent years. For example, several laboratories have actively examined modifications, of one end (i.e., the N-terminus) of the histone proteins around which the DNA is wrapped in the cell nucleus to form the chromatin. After their initial synthesis (i.e., after translation), histones can undergo a variety of modifications, such as acetylation, methylation, or phosphorylation, at different sites and under different conditions with diverse consequences. Another frequently studied type of epigenetic modification is the methylations of DNA at regions rich in cytosine and guanosine nucleotides (i.e., CpG islands), which has been found to affect, for example, cancer genes. Small RNA molecules called micro-RNAs (miRNAs) that cause inhibition of the first step of gene expression (i.e., transcription) or degradation of RNA also are considered to be master regulators involved in the modification of gene expression in abnormal conditions or disease states. Furthermore, all of these epigenetic mechanisms are influenced by foreign substances to which the body is exposed (i.e., xenobiotics) and environmental conditions.

The accumulation of all these findings has led to a dramatic shift from a genetic to an epigenetic basis in the conceptual thinking about the causes of disease. This also applies to the causes underlying ethanol-induced conditions, and new developments particularly have highlighted the importance of epigenetic mechanisms in mediating ethanol's actions in the liver and gastrointestinal (GI) tract (see figure 1). These developments are the focus of this review.

Alcohol-Induced Epigenetic Alterations in the Liver and GI Tract

Histone Acetylation, Methylation, and Phosphorylation

Evidence for the ethanol-induced epigenetic modifications of histone H3 first was obtained by Park and colleagues (2003) who demonstrated H3 acetylation in primary cultures of rat liver cells (i.e., hepatocytes). Other researchers subsequently determined that ethanol altered methylation of histone H3 at two lysine residues (i.e., lys-4 and lys-9) (Pal-Bhadra et al. 2007) and that phosphorylation of histone H3 at two serine residues (i.e.,

ser-10 and ser-28) was increased in ethanol-exposed hepatocytes (Lee and Shukla 2007). Additional studies have established that these changes occur not only in cultured hepatocytes but also in vivo in the liver and other organs (see Kim and Shukla 2006; Shukla and Aroor 2006) as well as in other liver cell types (e.g., hepatic stellate cells) (Kim and Shukla 2005). Alcohols other than ethanol that can be found as contaminants in adulterated alcoholic drinks also can modify histones (Choudhury et al. 2008). Finally, by interfering with single-carbon metabolism, ethanol may potentiate the epigenetic effects of toxins released by certain bacteria in the GI tract (i.e., lipopolysaccharide or endotoxin). These toxins promote methylation of histone H3 at lys-4 (Ara et al. 2008), which could in turn contribute to the progression of alcoholic liver disease (ALD).

The histone proteins form larger complexes called nucleosomes around which the DNA is wound in the cell nucleus. Modifications at different sites in histone H3 (e.g., lys-4, lys-9, ser-10, ser-28, etc.) may occur on nucleosomes located in the same or different domains of the chromatin (James et al. 2012). These site-specific modifications, in turn, will be associated with changes in the expression of different genes with diverse effects. Thus, ethanol can influence an intricate network of epigenetic modifications.

It should be noted that although the observed global ethanol-induced changes in histone modifications suggest that they would result in large-scale, perhaps genome-wide, alterations in gene expression, epigenetic changes also can be limited to selected subsets of genes, depending to some degree on the method and mode of ethanol administration. Indeed, a gene-specific increase in H3K9 acetylation has been observed in rat liver in response to chronic ethanol feeding even in the absence of obvious global changes in histone acetylation (Park et al. 2012). Ethanol-induced histone modification is associated with altered expression of several genes, including those encoding the ethanol-metabolizing enzyme alcohol dehydrogenase (ADH), the cancer-promoting gene (i.e., oncogene) *c-jun,* and the gene encoding a protein called plasminogen activator inhibitor 1 (PAI-1), which is involved in the dissolution of blood clots and in various diseases (e.g., fibrosis and certain types of cancer) (see table 1).

Changes in miRNAs

miRNAs are RNA molecules that do not serve as templates for protein production but have regulatory functions (for more information on miRNAs, see the article by Balamaran et al., pp. 18–24). To date, hundreds of miRNAs have been identified (Miranda et al. 2010) whose expression may be altered by various stimuli and as a result of changes in internal or environmental conditions. For example, chronic

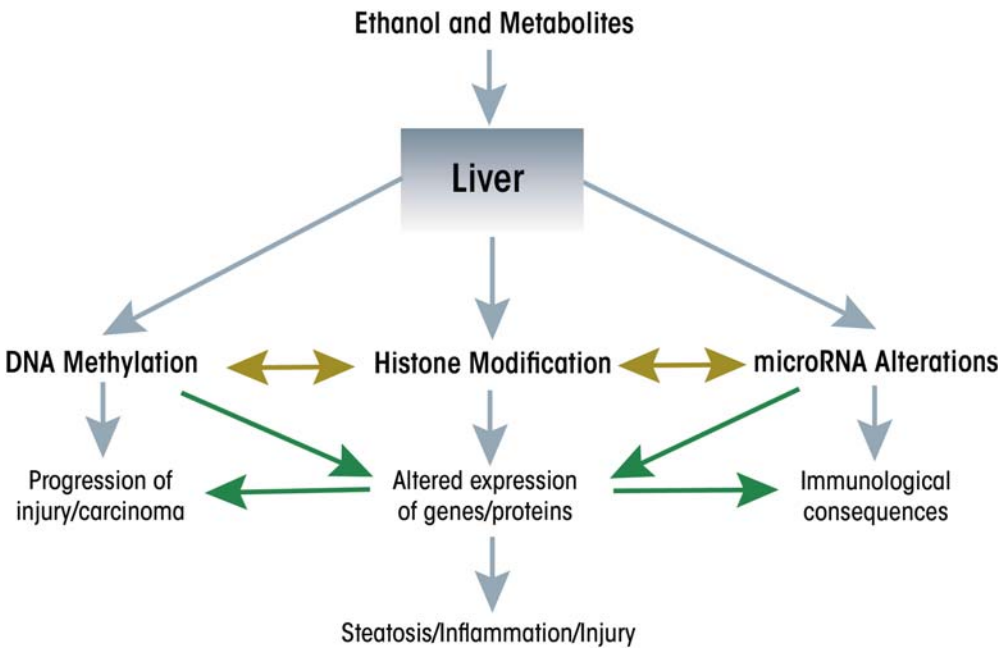


Figure 1 Ethanol and its metabolites modify epigenetic pathways in the liver.

ethanol feeding results in up- or downregulation of 1 percent or more of known miRNAs in the liver of mice (Dolganiuc et al. 2009) and rats (Dippold et al. 2013). Among those that were upregulated in rat liver by ethanol exposure were miR-34a, miR-103, miR-107, and miR-122 (Dippold et al. 2013), which have been implicated in the regulation of lipid metabolism (Esau et al. 2006; Lee et al. 2010), iron (Castoldi et al. 2011), and maintenance of glucose levels (i.e., glucose homeostasis) (Trajkovski et al. 2011). Conversely, the levels of miR-200b and miR-19b were downregulated under the same experimental conditions (Dippold et al. 2013). Similar results were observed in mice, where chronic ethanol feeding with a liquid Lieber-DeCarli diet led to upregulation of miR-705 and miR-1224 and downregulation of miR-182, miR-183, and miR-199a-3p in the liver. However, the biological targets of these miRNAs in the context of alcohol consumption still need to be determined (Dolganiuc et al. 2009; see table 1).

Ethanol exposure also influences miRNA expression in response to other changes in the organism. For example, the levels of a miRNA called miR-21 normally increase after a part of the animal's liver is removed (i.e., after partial hepatectomy), which had been thought to contribute to the regeneration of the liver. Ethanol enhances this increase in miR-21 but paradoxically interferes with the regenerative process (Dippold et al. 2012). The significance of the miR-21 increase therefore remains to be elucidated.

Chronic ethanol feeding of mice and exposure of mouse hepatocytes to ethanol in vitro also induces miR-217 (Yin et al. 2012), which has been proposed to be linked to excess fat accumulation in the liver. Interestingly, this effect on fat metabolism seems to be correlated with reduced expression of an enzyme involved in histone modification (i.e., the class IV histone deacetylase [HDAC], SIRT-1). SIRT-1 is a molecular target not only of miR-217 but also of miR-34a (Lee et al. 2010) which, as indicated above, also is upregulated by ethanol (Dippold et al. 2013). Likewise, expression of another miRNA, miR-101, can downregulate the level of another enzyme involved in histone modification called histone methyltransferase Ezh2 (Cao et al. 2010). Although it is not known if miR-101 expression is affected by ethanol, these studies point to the intriguing possibility that change, in miRNA levels also could indirectly affect other epigenetic changes such as histone acetylation and methylation.

Changes in miRNA levels in response to ethanol are not limited to the hepatocytes but also affect other types of cells found in the liver and GI tract. For example, ethanol feeding leads to up-regulation of miR-20 and miR-203 as well as down-regulation of miR-135 and miR-199 in liver sinusoidal endothelial cells (Yeligar et al. 2009), and increases the levels of miR-132 and miR-155 in Kupffer cells (Bala et al. 2011). In addition, elevated levels of miR-212 have been detected in intestinal epithelial cells of patients with ALD (Tang et al. 2008). These changes in miRNA levels are correlated with altered expression of certain proteins in these cells, including increased expression of endothelin-1 (ET-1) and ET-1 receptor (ET-BR) in endothelial cells (Yeligar et al. 2009), increased expression of the proinflammatory cytokine tumor necrosis factor-α (TNF α) in Kupffer cells (Bala et al 2011), and reduced expression of a protein called zonula occludens 1 (ZO1), which helps ensure the tight connection between intestinal epithelial cells (Tang et al. 2008). As will be discussed later in this article, these

Table 1 Epigenetic Parameters Altered by Ethanol in the Liver and Gastrointestinal System

Component	Molecular Alterations/ Entity	Possible Effect On
DNA	DNA methylation via DNA methyl transferase (DNMT) enzymes DNMT1, DNMT3a, and DNMT3b	Alcohol dehydrogenase (ADH), genes for folate metabolism
Histone	**Type of modification**	
	Acetylation	ADH, LSD
	Methylation	LSD
	Phosphorylation	C-jun, plasminogen activatory inhibitor 1 (PAI-1)
	Modifying enzymes	
	Histone acetyl transferases (HATs)	
	GCN5	
	p300	
	MOZ	
	Histone deacetylases (HDACs)	
	HDAC 1,3,5,6,7,9,10,11	
	SIRT-1	
micro-RNA	**Upregulation**	
	miR 03,20,21,29A,34a,101,103 miR107, 122, 132,148, 152, 155 miR 212, 217, 349, 705, 1224 miR 1256	Lipogenesis
	Downregulation	Immune response
	miR 19b, 135, 182, 183, 200b miR 199a-3P	

changes in turn may contribute to the cross-talk between the liver and the GI and immune systems that ultimately may be responsible for the development of ALD.

Although changes in miRNA levels can affect expression of enzymes involved in other epigenetic modifications, it is equally clear that expression of miRNAs themselves can be subject to regulation by histone modifications and/or DNA methylation at the DNA regions that regulate miRNA expression (i.e., at their promoters). For example, the ethanol-induced expression of miR-155 seems to be regulated by the recruitment of a regulatory protein called nuclear factor κB (NFκB) to the miR-155 promoter (Bala et al. 2011), presumably accompanied by epigenetic changes associated with gene activation. In other studies, removal of methyl groups from (i.e., demethylation of) cytosine nucleotides at the promoters of miR-29a and miR-1256 correlated with upregulation of these miRNAs in prostate cancer cells (Li et al. 2012). Although it is not yet known whether miRNAs regulated by ethanol also may be regulated by DNA methylation, these studies clearly point to the intriguing possibility of cross-talk among molecular components involved in different types of epigenetic modifications (see figure 1).

Changes in DNA Methylation Patterns

Ethanol also can alter the methylation patterns of DNA in liver, thereby influencing gene expression. For example, genes encoding enzymes involved in ethanol metabolism (e.g., ADH) are regulated by DNA methylation (Dannenberg et al. 2006). It therefore is likely that reduced levels of DNA methylation (i.e., hypomethylation) in response to ethanol will modulate the transcription of these genes. This effect is particularly relevant in patients with late-stage ALD, where ethanol is involved in the promotion of hepatic carcinoma. Like changes in miRNA expression, alcohol-induced changes in DNA methylation also have been observed in organs other than the liver. For example, chronic ethanol feeding in rats affects methylation of genes regulating absorption of the vitamin folate in the intestine (Wani et al. 2012). Folate is an important cofactor in single-carbon metabolism; therefore, its deficiency in turn could affect methylation reactions in various other organs, including the liver.

Kutay and colleagues (2012) found that ethanol affects methylation patterns by reducing the levels and activity of key DNA methylation enzymes, DNA methyl transferase (DNMT) 1 and 3b, without altering their mRNA levels. However, chronic ethanol feeding did not reveal any detectable methylation at the CpG islands in the promoters of several genes examined in liver (e.g., genes called *Agpat 9, Lepr,* and *Pparα*), suggesting that promoter methylation may not be involved in regulating the expression of these genes. Instead, transcriptional activation or chromatin modification may be the predominant mechanism involved in ethanol-induced gene expression. This possibility has yet to be confirmed in additional studies, including studies in human liver.

Several observations suggest that changes in DNA methylation induced by diet, folate deficiency, or alcohol exposure may represent important epigenetic mechanisms. For example, chronic exposure to ethanol has been shown to produce DNA hypomethylation throughout the genome in the colonic mucosa in rats, and this hypomethylation may constitute a pathway by which carcinogenesis is enhanced (Choi et al. 1999). Other studies have focused on the role of a compound known as S-adenosylmethionine (SAMe), which acts as a methyl donor, in liver injury. Ethanol-induced alterations in SAMe levels can affect the methylation of histones or DNA, which in turn can modify gene expression, thereby contributing to liver injury (Lu and Mato 2012).

Role of Ethanol Metabolism and Oxidative Stress in Ethanol-Related Epigenetic Mechanisms

The actions of ethanol in the liver are complex because it is metabolized via both oxidative and nonoxidative pathways that result in the generation of several metabolites, such as acetaldehyde and acetate. Interestingly, both of these metabolites, as well as ethanol itself, increase histone H3 acetylation. This observation is supported by studies investigating the effects of inhibitors of ADH (i.e., 4-methyl pyrazole) and of another alcohol-metabolizing enzyme called aldehydyde dehydrogenase (i.e., methyl cynamide). These inhibitors prevented acetaldehyde and acetate formation and also reduced ethanol-induced increases in histone acetylation (Park et al. 2003), suggesting that ethanol metabolism has a role in this effect. Other findings suggest that ethanol-derived acetate may increase histone acetylation by increasing the available levels of acetyl groups for these reactions. Thus, studies in a cultured macrophage cell line found that downregulation of an enzyme that converts acetate into acetyl CoA, which then is used for histone acetylation, ameliorates the acetate effect on histone modification (Kendrick et al. 2010). However, the significance of this observation in vivo is unclear because the changes in acetyl-CoA levels following alcohol consumption are rather modest and transient.

Another important consequence of ethanol metabolism in the liver is the production of reactive oxygen species (ROS), leading to oxidative stress. ROS have been shown to play a role in ethanol-induced histone acetylation. Antioxidants that selectively interfere with different steps of ROS production affect this response. For example, general antioxidants (e.g., resveratrol or quercetin) inhibit histone acetylation. Conversely, inhibitors of certain enzyme complexes that are involved in ROS productions, such as rotenone (which inhibits mitochondrial complex 1) and antimycin (which inhibits

mitochondrial complex 3) increase histone acetylation (Choudhury et al. 2010). These observations are consistent with the view that ROS contribute to the epigenetic effects of alcohol consumption.

Role of Cell-Signaling Pathways in Ethanol-Related Epigenetic Mechanisms

The cellular actions of ethanol, including its epigenetic effects, are mediated via several signaling pathways (Mandarekar and Szabo 2009). One of these involves several enzymes called mitogen-activated protein (MAP) kinases (MAPKs) and therefore is known as the MAP kinase cascade. There are several different MAP kinase pathways that involve different MAPKs and which differentially affect ethanol-induced epigenetic modifications. For example, histone H3 phosphorylation is dependent on p38 MAPK (Lee and Shukla 2007), whereas histone H3 acetylation is regulated by a MAP kinase cascade involving MAPKs called ERK1/2 and JNK (Park et al. 2005). Even more intriguing is the finding that acetate-induced acetylation of histone H3 is MAPK independent (Park et al. 2005; Aroor et al. 2010). Thus, the involvement of different signaling pathways likely adds another level of regulatory control on histone modifications by ethanol and its metabolites (Shukla et al. 2013). These remarkable differences in signaling pathways utilized by ethanol and acetate may underlie the different modes of histone modifications and consequences of ethanol and its metabolites. This issue remains to be addressed in future studies.

Role of Epigenetic Mechanisms in Ethanol-Induced Steatosis, Steatohepatitis, and Carcinoma

Excessive alcohol consumption can lead to a range of liver disorders, including fatty liver (i.e., steatosis), steatosis accompanied by inflammation of the liver (i.e., steatohepatitis), and progressing in some cases to liver cancer (i.e., carcinoma). Histone modifications, DNA methylation, and miRNA expression may all play roles in ethanol-related steatosis and inflammatory responses. For example, ethanol affects the activity of enzymes called histone acetyl transferases (HATs) that mediate histone acetylation. One of these ethanol-regulated HATs is called GCN5 (Choudhury et al. 2011); it modulates the expression of a protein called PGC1β, which is involved in fat metabolism in the liver (Kelly et al. 2009). Furthermore, chronic intragastric ethanol feeding of rats leads to an increase in the levels of another HAT called p300 in the cell nuclei at peak blood alcohol level, which is correlated with increased acetylation of H3-lys-9 (Bardag-Gorce et al. 2007).

Histone modifications, DNA methylation, and miRNA expression may all play roles in ethanol-related steatosis and inflammatory responses.

Another type of histone-modifying enzyme are the HDACs. Chronic feeding of mice with an ethanol liquid diet downregulates the activity of the HDAC SIRT-1 and increases the expression of lipin-1, an important regulator of lipid synthesis in the liver (Yin et al. 2012) In contrast, other studies indicated that the transcription levels of SIRT-1 and PGC1β—another regulatory protein involved in lipid metabolism—are increased by chronic intragastric ethanol feeding in rats (Oliva et al. 2008). Recent studies also have shown that liver-specific knockout of the gene encoding HDAC3 in mice leads to severe hepatic steatosis and increased expression of lipogenic genes, although whether HDAC3 expression or function is altered by ethanol has yet to be elucidated (Sun et al. 2011). Increasing evidence thus suggest that both HATs and HDACs are likely to play a role in ethanol-induced liver injury (see Kirpich et al. 2012; Park et al. 2005; Pochareddy et al. 2012; Shepard et al. 2008; Yin et al. 2012). In addition to changes in lipid metabolism two molecules involved in inflammatory reactions (i.e., interleukin [IL] 8 and PAI-1) also are influenced by ethanol-induced histone modifications. Finally, ethanol-induced DNA hypomethylation has been implicated in the development of steatosis (Kutay et al. 2012) as well as hepatic carcinoma, an end consequence of ALD (Lambert et al. 2011).

miRNAs also mediate some of ethanol's effects in causing liver disorders. For example, the down regulation of SIRT-1 in mice in response to ethanol feeding described above appears to be mediated by miR217 (Yin et al. 2012). A high-content screening of 327 human miRNAs identified 11 that when overexpressed in human hepatocytes lead to either increased or decreased intracellular lipid droplets, with miR-181d being the most efficacious inhibitor of lipid droplet formation (Whittaker et al. 2010). As discussed above, the immunological responses of liver macrophages are thought to involve miR-155 (Bala et al. 2012). Moreover, several miRNAs have been postulated to play a role in ethanol-induced intestinal defects (Tang et al. 2008) which could also indirectly exacerbate liver injury (see further discussion below).

Time Dependence and Persistence of Alcohol-Induced Epigenetic Changes

Interestingly, the various epigenetic modifications observed in cultured hepatocytes in response to ethanol follow different time courses. For example, phosphorylation of H3 starts before acetylation and methylation of this histone (see figure 2). Furthermore, although the global changes

more, although the global changes in histone modifications appear to be transient, with the effect peaking at about 24 hours following initial ethanol exposure, it is likely that these changes may trigger secondary changes in gene expression (including those of miRNA) or DNA modification that are much longer lasting. To date, little is known about the time course and sustainability of these other epigenetic modifications. It also is possible that even when the overall global changes in histone modification have subsided, some of the secondary changes may persist in nucleosomes associated with specific genes and may continue to influence expression of these genes.

The responses to ethanol consumption in vivo also have not yet been fully elucidated. Chronic ethanol treatment definitely results in abundant epigenetic changes months after the ethanol feeding began. How long these changes remain after withdrawal of alcohol has not been carefully evaluated with respect to the GI tract and liver. Studies in other organ systems, however, suggest that some of these changes could indeed persist for a long time. For example, prenatal exposure of rat fetuses to ethanol resulted in the development of hepatic insulin resistance in the offspring 3 months after birth, which was correlated with an increase in HDAC activity and decrease in HAT activity in the liver (Yao and Nyomba 2008). Furthermore, exposure of males to ethanol was correlated with hypomethylation of normally hypermethylated regions in the DNA of the sperm corresponding to various paternally imprinted genes (Ouko et al. 2009). Epigenetic changes in these imprinted genes could be transmitted to the progeny following fertilization and thus affect the development and perhaps physiological functions of different organs, including the liver. Epigenetic effects of alcohol thus might even be able to exert long-lasting transgenerational effects in the offspring.

Relationship to the Immune System

Evidence gathered in the past decade has clearly shown that ethanol alters several immunological parameters. One important participant in ethanol's actions is a group of regulatory molecules called macrophage toll-like receptors (TLRs), particularly TLR 4. Ethanol's effects on TLRs likely are mediated via miRNAs because, as mentioned earlier, ethanol increases the levels of several of these noncoding RNAs. Other studies have shown that ethanol influences the activities of different classes of TLR-regulated genes through distinct epigenetic histone modifications (Foster et al. 2007). Specifically, several pro-inflammatory genes are selectively deacetylated during the development of immune tolerance and are no longer inducible in the tolerant macrophages. It is tempting to speculate that by affecting histone modifications, ethanol could interfere with the development of tolerance and thus promote a chronic inflammatory state. Consistent with this idea, exposure of cultured macrophages to ethanol or ethanol metabolites resulted in increased production of TNF- (Shen et al. 2009), although whether this involves increased histone modification at the TNF- promoter remains to be established. In addition to the involvement of Kupffer cells, it is likely that interactions between activated hepatic stellate cells and hepatocytes also contribute to a pro-inflammatory environment by increasing the production of cytokines. This cross-talk between stellate cells and hepatocytes appears to be inhibited by deacetylase inhibitors, such as trichostatin (Coulouarn et al. 2012).

It should be pointed out that ethanol's effects on the immune system likely are rather complex. In contrast to the enhanced inflammatory response seen during steatohepatitis following chronic ethanol administration, acute exposure to ethanol in vivo suppresses various inflammatory responses (e.g., leukocyte recruitment and endothelial cell activation) (Saeed et al. 2004). It is not completely clear if this anti-inflammatory effect is related to epigenetic changes; however, other studies have shown that

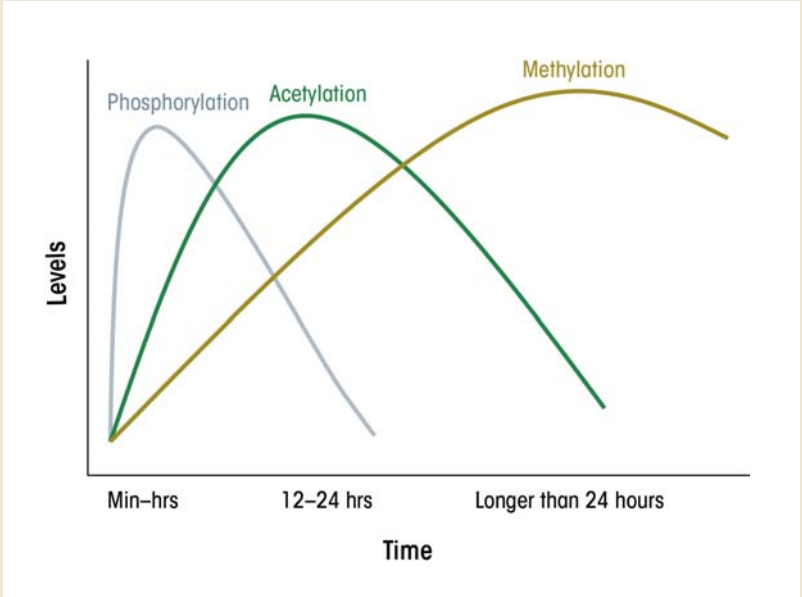


Figure 2 A schematic presentation of the kinetics of histone modifications by ethanol in liver.

treatment with HDAC inhibitors likewise inhibits the migration of macrophages in response to an inflammation-inducing stimulus (i.e., exposure to lipopolysaccharide) (Maa et al. 2010). Thus, it appears that ethanol may exert potent effects on the immune system, which likely are related to its epigenetic action, and that chronic and acute ethanol treatment could elicit different outcomes (Shukla et al. 2013).

Cross-Organ Talk Between the Liver and GI Tract

The nutrients and xenobiotics taken up orally pass through the intestinal system and then to the liver, the major metabolic organ in the body. Ethanol can alter the permeability of the intestine, a condition known as leaky gut. This alcohol-induced gut leakiness is an important factor in ALD because it allows endotoxin to enter the circulation and initiate liver damage (Keshavarzian et al. 2009). The alcohol-induced gut leakiness may in part be caused by epigenetic changes to genes coding for proteins involved in joining epithelial cells to each other (i.e., epithelial cell junction proteins) (Tang et al. 2008). For example, alcohol induced overexpression of miR-212 and downregulated expression of the ZO1 protein. A decrease in ZO1 disrupts intestinal permeability and integrity, resulting in gut leakiness (Tang et al. 2008).

The response of the liver to ethanol and endotoxin is a complex process involving macrophage-like Kupffer cells, hepatocytes, and stellate cells. Alcohol's effects on the activities of these cells may lead to liver injury and ultimately carcinoma. Ethanol causes epigenetic alterations in these cells that could result in changes in expression of genes associated with modified histones, including genes coding for various cytokines. Increases in the expression of these cytokines may occur in the liver, resulting in increased cytokine levels that then are circulated through the blood to other organs (e.g., heart or kidney) and in turn affect the functions of these organs. Thus, alcohol-induced epigenetic effects in the liver eventually may influence the cross-talk among these organs (see figure 3). This will be a fruitful topic for future studies to fully comprehend the role of ethanol-induced epigenetic alterations in the GI–hepatic system and its link to the responses of other organs.

Conclusions and Future Strategy

The consequences of ethanol-induced epigenetic alterations can be positive or negative, depending on the type and duration of the epigenetic changes. Furthermore, the epigenetic responses to ethanol and its metabolites (e.g., acetate) also can differ with a variety of consequences. This diversity remains to be examined thoroughly. Additionally, modifications in DNA and histones located in specific nucleosomes or chromatin domains may differ in their transcriptional effects on various genes, consequently exhibiting varying effects. Alterations in the expression levels of a plethora of miRNAs will add another level of regulatory control over these responses. Finally, it is fair to assume that the diverse epigenetic pathways cross-influence each other, leading to a highly complex regulatory network. The consequences of these epigenetic alterations in the GI

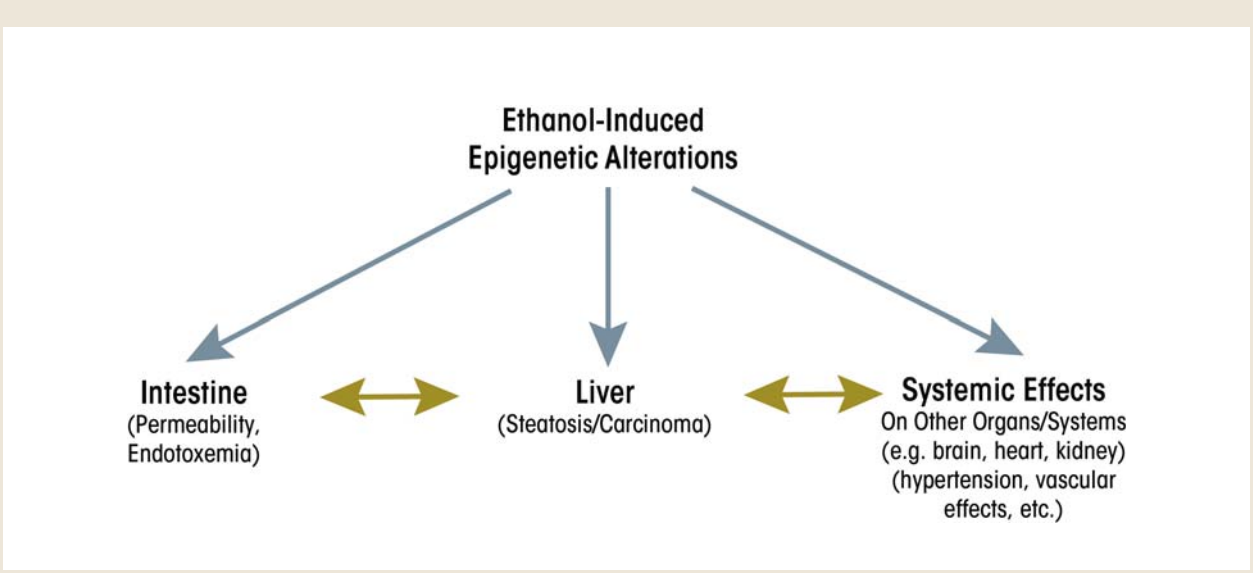


Figure 3 Ethanol-induced epigenetic alterations and cross-organ talk through the gastrointestinal–liver axis.

tract and liver likely have a systemic impact, influencing other organs and their functions as well, although these interactions are as yet relatively unexplored. Thus, many questions remain that need to be addressed by future research into this area. ■

Financial Disclosure

The authors declare that they have no competing financial interest.

References

ARA, A.I.; XIA, M.; RAMANI, K.; ET AL. S-adenosylmethionine inhibits lipopolysaccharide-induced gene expression via modulation of histone methylation. *Hepatology* 47(5):1655–1666, 2008. PMID: 18393372

AROOR, A.R.; JAMES, T.T.; JACKSON, D.E.; AND SHUKLA, S.D. Differential changes in MAP kinases, histone modifications, and liver injury in rats acutely treated with ethanol. *Alcoholism: Clinical and Experimental Research* 34(9):1543–1551, 2010. PMID: 20586759

BALA, S.; MARCOS, M.; KODYS, K.; ET AL. Up-regulation of microRNA-155 in macrophages contributes to increased tumor necrosis factor (TNFα) production via increased mRNA half-life in alcoholic liver disease. *Journal of Biological Chemistry* 286(2):1436–1444, 2011. PMID: 21062749

BARDAG-GORCE, F.; FRENCH, B.A.; JOYCE, M.; ET AL. Histone acetyltransferase p300 modulates gene expression in an epigenetic manner at high blood alcohol levels. *Experimental and Molecular Pathology* 82(2):197–202, 2007. PMID: 17208223

CAO, P.; DENG, Z.; WAN, M.; ET AL. MicroRNA-101 negatively regulates EZh2 and its expression is modulated by androgen receptor and HIF-1 alpha/HIF-1 beta. *Molecular Cancer* 9:108, 2010. PMID: 20478051

CASTOLDI, M.; VUJIC SPASIC, M.; ALTAMURA, S.; ET AL. The liver-specific microRNA miR-122 controls systemic iron homeostasis in mice. *Journal of Clinical Investigation* 121(4):1386–1396, 2011. PMID: 21364282

CHOI, S.W.; STICKEL, F.; BAIK, H.W.; ET AL. Chronic alcohol consumption induces genomic but not p53-specific DNA hypomethylation in rat colon. *Journal of Nutrition* 129(11):1945–1950, 1999. PMID: 10539767

CHOUDHURY, M., AND SHUKLA, S.D. Surrogate alcohols and their metabolites modify histone H3 acetylation: Involvement of histone acetyl transferase and histone deacetylase. *Alcoholism: Clinical and Experimental Research* 32(5):829–839, 2008. PMID: 18336638

CHOUDHURY, M.; PANDEY, R.S.; CLEMENS, D.L.; ET AL. Knock down of GCN5 histone acetyltransferase by siRNA decreases ethanol-induced histone acetylation and affects differential expression of genes in human hepatoma cells. *Alcohol* 45(4):311–324, 2011. PMID: 21367571

CHOUDHURY, M.; PARK, P.H.; JACKSON, D.; AND SHUKLA, S.D. Evidence for the role of oxidative stress in the acetylation of histone H3 by ethanol in rat hepatocytes. *Alcohol* 44(6):531–540, 2010. PMID: 20705415

COULOUARN, C.; CORLU, A.; GLAISE, D.; ET AL. Hepatocyte–stellate cell cross-talk in the liver engenders a permissive inflammatory microenvironment that drives progression in hepatocellular carcinoma. *Cancer Research* 72(10):2533–2542, 2012. PMID: 22419664

DANNENBERG, L.O.; CHEN, H.J.; TIAN, H.; AND EDENBERG, H.J. Differential regulation of the alcohol dehydrogenase 1B (ADH1B) and ADH1C genes by DNA methylation and histone deacetylation. *Alcoholism: Clinical and Experimental Research* 30(6):928–937, 2006. PMID: 16737450

DIPPOLD, R.P.; VADIGEPALLI, R.; GONYE, G.E.; AND HOEK, J.B. Chronic ethanol feeding enhances mir-21 induction during liver regeneration while inhibiting proliferation in rats. *American Journal of Physiology. Gastroinstestinal and Liver Physiology* 303(6):G733–G743, 2012. PMID: 22790595

DIPPOLD, R.P.; VADIGEPALL, R.; GONYE, G.E.; ET AL. Chronic ethanol feeding alters miRNA expression dymanics during liver regeneration. *Alcoholism: Clinical and Experimental Research,* 37(Suppl 1):E59-E69, 2013. PMID: 22823254

DOLGNIUC, A.; PETRASEK, J.; KODYS, K.; ET AL. MicroRNA expression profile in Lieber-DeCarli diet-induced alcoholic and methionine choline deficient diet-induced nonalcoholic steatohepatitis models in mice. *Alcoholism: Clinical and Experimental Research* 33(10):1704–1710, 2009. PMID: 19572984

ESAU, C.; DAVIS, S.; MURRAY, S.F.; ET AL. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metabolism* 3(2):87–98, 2006. PMID: 16459310

FOSTER, S.L.; HARGREAVES, D.C.; AND MEDZHITOV, R. Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature* 447(7147):972–978, 2007. PMID: 17538624

JAMES, T.T.; AROOR, A.R.; LIM, R.W.; AND SHUKLA, S.D. Histone H3 phosphorylation (Ser10, Ser28) and phosphoacetylation (K9S10) are differentially associated with gene expression in liver of rats treated in vivo with acute ethanol. *Journal of Pharmacology and Experimental Therapeutics* 340(2):237–247, 2012. PMID: 22025646

KELLY, T.J.; LERIN, C.; HAAS, W.; ET AL. GCN5-mediated transcriptional control of the metabolic coactivator PGC-1β through lysine acetylation. *Journal of Biological Chemistry* 284(30):19945–19952, 2009. PMID: 19491097

KENDRICK, S.F.; O'BOYLE, G.; MANN, J.; ET AL. Acetate, the key modulator of inflammatory responses in acute alcoholic hepatitis. *Hepatology* 51(6):1988–1997, 2010. PMID: 20232292

KESHAVARZIAN, A.; FARHADI, A.; FORSYTHY, C.B.; ET AL. Evidence that chronic alcohol exposure promotes intestinal oxidative stress, intestinal hyperpermeability and endotoxemia prior to development of alcoholic steatohepatitis in rats. *Journal of Hepatology* 50(3):538–547, 2009. PMID: 19155080

KIM, J., AND SHUKLA, S.D. Acute in vivo effect of ethanol (binge drinking) on histone H3 modifications in rat tissues. *Alcohol and Alcoholism* 41(2):126–132, 2006. PMID: 16314425

KIM, J., AND SHUKLA, S.D. Histone H3 modifications in rat hepatic stellate cells by ethanol. *Alcohol and Alcoholism* 40(5):367–372, 2005. PMID: 15939707

KIRPICH, I.; GHARE, S.; ZHANG, J.; ET AL. Binge alcohol-induced microvesicular liver steatosis and injury are associated with down-regulation of hepatic Hdac 1, 7, 9, 10, 11 and up-regulation of Hdac 3. *Alcoholism: Clinical and Experimental Research* 36(9):1578–1586, 2012. PMID: 22375794

KUTAY, H.; KLEPPER, C.; WANG, B.; ET AL. Reduced susceptibility of DNA methyltransferase 1 hypomorphic (Dnmt1(N/+)) mice to hepatic steatosis upon feeding liquid alcohol diet. *PLoS One* 7(8):e41949, 2012. PMID: 22905112

LAMBERT, M.P.; PALIWAL, A, VAISSIERE, T. ; ET AL. Aberrant DNA methylation distinguishes hepatocellular carcinoma associated with HBV and HCV infection and alcohol intake. *Journal of Hepatology* 54(4) :705-715, 2011. PMID :21146512

LEE, J.; PADHYE, A.; SHARMA, A.; ET AL. A pathway involving farnesoid X receptor and small heterodimer partner positively regulates hepatic sirtuin 1 levels via microRNA-34a inhibition. *Journal of Biological Chemistry* 285(17): 12604–12611, 2010. PMID: 20185821

LEE, Y., AND SHUKLA, S.D. Histone H3 phosphorylation at serine 10 and serine 28 is mediated by p38 MAPK in rat hepatocytes exposed to ethanol and acetaldehyde. *European Journal of Pharmacology* 573(1-3):29–38, 2007. PMID: 17643407

LI, Y.; KONG, D.; AHMAD, A.; ET AL. Epigenetic deregulation of miR-29a and miR-1256 by isoflavone contributes to the inhibition of prostate cancer cell growth and invasion. *Epigenetics* 7(8):940–949, 2012. PMID: 22805767

LU, S.C., AND MATO, J.M. S-Adenosylmethionine in liver health, injury, and cancer. *Physiological Reviews* 92(4):1515–1542, 2012. PMID: 23073625

MAA, M.C.; CHANG, M.Y.; HSIEH, M.Y.; ET AL. Butyrate reduced lipolpolysaccharide-mediated macrophage migration by suppression of Src enhancement and focal adhesion kinase activity. *Journal of Nutritional Biochemistry* 21(12):1186–1192, 2010. PMID: 20149623

MANDREKAR, P., AND SZABO, G. Signalling pathways in alcohol-induced liver inflammation. *Journal of Hepatology* 50(6):1258–1266, 2009. PMID: 19398236

MIRANDA, R.C.; PIETRZYKOWSKI, A.Z.; TANG, Y.; ET AL. MicroRNAs: Master regulators of ethanol abuse and toxicity? *Alcoholism: Clinical and Experimental Research* 34(4):575–587, 2010. PMID: 20102566

OLIVA, J.; FRENCH, B.A.; LI, J.; ET AL. Sirt1 is involved in energy metabolism: The role of chronic ethanol feeding and resveratrol. *Experimental and Molecular Pathology* 85(3):155–159, 2008. PMID: 18793633

OUKO, L.A.; SHANTIKUMAR, K.; KNEZOVICH, J.; ET AL. Effect of alcohol consumption on CpG methylation in the differentially methylated regions of H19 and IG-DMR in male gametes: Implications for fetal alcohol spectrum disor-

ders. *Alcoholism: Clinical and Experimental Research* 33(9):1615–1627, 2009. PMID: 19519716

Pal-Bhadra, M.; Bhadra, U.; Jackson, D.E.; et al. Distinct methylation patterns in histone H3 at Lys-4 and Lys-9 correlate with up- & down-regulation of genes by ethanol in hepatocytes. *Life Sciences* 81(12):979–987, 2007. PMID: 17826801

Park, P.H.; Lim, R.W.; and Shukla, S.D. Gene selective histone H3 acetylation in the absence of increase in global histone acetylation in liver of rats chronically fed alcohol. *Alcohol and Alcoholism* 47(3):233–239, 2012. PMID: 22301686

Park, P.H.; Lim, R.W.; and Shukla, S.D. Involvement of histone acetyltransferase (HAT) in ethanol-induced acetylation of histone H3 in hepatocytes: Potential mechanism for gene expression. *American Journal of Physiology. Gastrointestinal and Liver Physiology* 289(6):G1124–G1136, 2005. PMID: 16081763

Park, P.H.; Miller, R.; and Shukla, S.D. Acetylation of histone H3 at lysine 9 by ethanol in rat hepatocytes. *Biochemical and Biophysical Research Communications* 306(2):501–504, 2003. PMID: 12804592

Pochareddy, S., and Edenberg, H.J. Chronic alcohol exposure alters gene expression in HepG2 cells. *Alcoholism: Clinical and Experimental Research* 36(6):1021–1033, 2012. PMID: 22150570

Saeed, R.W.; Varma, S.; Peng, T.; et al. Ethanol blocks leukocyte recruitment and endothelial cell activation in vivo and in vitro. *Journal of Immunology* 173(10): 6376–6383, 2004. PMID: 15528377

Shen, Z.; Ajmo, J.M.; Rogers, C.Q.; et al. Role of SIRT1 in regulation of LPS- or two ethanol metabolites-induced TNF-α production in cultured macrophage cell lines. *American Journal of Physiology. Gastrointestinal and Liver Physiology* 296(5):G1047–G1053, 2009. PMID: 19299582

Shepard, B.D.; Joseph, R.A.; Kannarkat, G.T.; et al. Alcohol-induced alterations in hepatic microtubule dynamics can be explained by impaired histone deacetylase 6 function. *Hepatology* 48(5):1671–1679, 2008. PMID: 18697214

Shukla, S.D., and Aroor, A.R. Epigenetic effects of ethanol on liver and gastrointestinal injury. *World Journal of Gastroenterology* 12(33):5265–5271, 2006. PMID: 16981253

Shukla, S.D.; Pruett, S.B.; Szabo, G.; and Arteel, G.E. Binge ethanol and liver: New molecular developments. *Alcoholism: Clinical and Experimental Research*, Epub ahead of print, 2013. PMID: 23347137

Shukla, S.D.; Velazquez, J.; French, S.W.; et al. Emerging role of epigenetics in the actions of alcohol. *Alcoholism: Clinical and Experimental Research* 32(9):1525–1534, 2008. PMID: 18616668

Sun, Z.; Feng, D.; Everett, L.J.; et al. Circadian epigenomic remodeling and hepatic lipogenesis: Lessons from HDAC3. *Cold Spring Harbor Symposia on Quantitative Biology* 76:49–55, 2011. PMID: 21900149

Tang, Y.; Banan, A.; Forsyth, C.B.; et al. Effect of alcohol on miR-212 expression in intestinal epithelial cells and its potential role in alcoholic liver disease. *Alcoholism: Clinical and Experimental Research* 32(2):355–364, 2008. PMID: 18162065

Trajkovski, M.; Hausser, J.; Soutschek, J.; et al. MicroRNAs 103 and 107 regulate insulin sensitivity. *Nature* 474(7353):649–653, 2011. PMID: 21654750

Wani, N.A.; Hamid, A.; and Kaur, J. Alcohol-associated folate disturbances result in altered methylation of folate-regulating genes. *Molecular and Cellular Biochemistry* 363(1-2):157–166, 2012. PMID: 22147198

Whittaker, R.; Loy, P.A.; Sisman, E.; et al. Identification of microRNAs that control lipid droplet formation and growth in hepatocytes via high-content screening. *Journal of Biomolecular Screening* 15(7):798–805, 2010. PMID: 20639500

Yao, X.H., and Nyomba, B.L. Hepatic insulin resistance induced by prenatal alcohol exposure is associated with reduced PTEN and TRB3 acetylation in adult rat offspring. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* 294(6): R1797–R1806, 2008. PMID: 18385463

Yeligar, S.; Tsukamoto, H.; and Kalra, V.K. Ethanol-induced expression of ET-1 and ET-BR in liver sinusoidal endothelial cells and human endothelial cells involves hypoxia-inducible factor-1alpha and microRNA-199. *Journal of Immunology* 183:5232–5243, 2009. PMID: 19783678

Yin, H.; Hu, M.; Zhang, R.; et al. MicroRNA-217 promotes ethanol-induced fat accumulation in hepatocytes by down-regulating SIRT1. *Journal of Biological Chemistry* 287(13):9817–9826, 2012. PMID: 223080

In Utero Alcohol Exposure, Epigenetic Changes, and Their Consequences

Michelle Ungerer; Jaysen Knezovich, M.Sc.; and Michele Ramsay, Ph.D.

Michelle Ungerer *is a Masters student and* **Jaysen Knezovich, M.Sc.,** *is a Ph.D. student and lecturer in the Division of Human Genetics, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand and the National Health Laboratory Service in Johannesburg, South Africa.*

Michele Ramsay, Ph.D., *is a professor in the Division of Human Genetics, National Health Laboratory Service, School of Pathology, Faculty of Health Sciences, and Interim Director of the Syndey Brenner Institute for Molecular Bioscience at the University of the Witwatersrand, Johannesburg, South Africa.*

Exposure to alcohol has serious consequences for the developing fetus, leading to a range of conditions collectively known as fetal alcohol spectrum disorders (FASD). Most importantly, alcohol exposure affects the development of the brain during critical periods of differentiation and growth, leading to cognitive and behavioral deficits. The molecular mechanisms and processes underlying the teratogenic effects of alcohol exposure remain poorly understood and are complex, because the specific effects depend on the timing, amount, and duration of exposure as well as genetic susceptibility. Accumulating evidence from studies on DNA methylation and histone modification that affect chromatin structure, as well as on the role of microRNAs in regulating mRNA levels supports the contribution of epigenetic mechanisms to the development of FASD. These epigenetic effects are difficult to study, however, because they often are cell-type specific and transient in nature. Rodent models play an important role in FASD research. Although recent studies using these models have yielded some insight into epigenetic mechanisms affecting brain development, they have generated more questions than they have provided definitive answers. Researchers are just beginning to explore the intertwined roles of different epigenetic mechanisms in neurogenesis and how this process is affected by exposure to alcohol, causing FASD. **KEY WORDS: Prenatal alcohol exposure; fetal alcohol spectrum disorders; epigenetics; epigenetic mechanisms; epigenetic modifications; brain development; cognitive deficits; behavioral deficits; DNA methylation; histone modification; chromatin; microRNAs; rodent models**

Alcohol exposure of the developing embryo and fetus in utero can have a wide range of detrimental effects collectively referred to as fetal alcohol spectrum disorders (FASD). Researchers are intensively investigating the mechanisms that may contribute to alcohol's effects on the developing organism and to the resulting consequences, particularly with respect to the cognitive and behavioral deficits associated with FASD. These studies have yielded increasing evidence that epigenetic mechanisms play an important role in these processes. This article reviews the current knowledge regarding the contributions of epigenetic modifications to the manifestations of FASD, much of which has been obtained using rodent models in which the timing, frequency, duration, and amount of alcohol exposure can be tightly controlled. This discussion also touches on the concepts of developmental reprogramming, the role of preconception alcohol exposure, and transgenerational transmission of the effects of alcohol exposure.

FASD

FASD can be associated with a variety of symptoms that differ widely in severity depending on the specific conditions of alcohol exposure. The most severe outcome is fetal alcohol syndrome (FAS), which can manifest variably with diverse combinations of craniofacial, growth, central nervous system (CNS), and neurobehavioral abnormalities (Jones et al. 1973; Sampson et al. 1997). Associated psychosocial problems include learning difficulties,

attention deficit–hyperactivity disorder (ADHD), and mental retardation (Burd et al. 2003; O'Leary 2004). Given that alcohol consumption is voluntary, FASD is said to be the most preventable cause of birth defects and mental retardation. FASD is a global health concern, and worldwide approximately 1 to 3 per 1,000 births is thought to be suffering from FAS. In the United States, FAS prevalence ranges between 0.5 and 2.0 per 1,000 live births (Abel 1995; May and Gossage 2001). The highest rates of FAS have been reported in mixed-ancestry communities in the Western Cape of South Africa, where between 68.0 and 89.2 per 1,000 school-age children display FAS symptoms (May et al. 2007).

Between 5 and 10 percent of offspring who have been exposed to alcohol prenatally display alcohol-related developmental anomalies (Abel 1995), with the severity of the outcome determined by the dose, timing, and duration of exposure (Padmanabhan and Hameed 1988; Pierce and West 1986; Sulik 1984). However, the proportion of affected offspring may be considerably higher in unfavorable circumstances, including instances of malnutrition of the mother and thus, the fetus. The genetic makeup of both mother and fetus, in conjunction with other factors (e.g., gender, diet, and social environment), also plays an important role in the manifestation of FASD (Chernoff 1980; Ogawa et al. 2005).

The effects of prenatal alcohol exposure are more similar in identical (i.e., monozygotic) twins than in fraternal (i.e., dizygotic) twins, suggesting a heritable component (Abel 1988; Chasnoff 1985; Streissguth and Dehaene 1993). Genetic studies have shown that different variants of the genes encoding various alcohol-metabolizing enzymes—such as alcohol dehydrogenases (ADHs), aldehyde dehydrogenases (ALDHs), and cytochrome P450 2E1 (CYP2E1)—in the mother and their offspring can affect alcohol metabolism and contribute to subsequent alcohol-related damage (Gemma et al. 2007; Warren and Li 2005). For example, variants at the ADH1B locus that result in an altered amino acid sequence and function of the encoded enzyme can influence the severity of the adverse effects on the developing fetus (i.e., teratogenesis) in different ethnic populations (for a review see Ramsay 2010). However, to date few studies have supported a role for genetics in the development of FASD.

Rodent models have provided a valuable tool for investigating genetic influences on the observable outcomes (i.e., phenotypes) associated with FASD. For example, the effects of in utero alcohol exposure differ between inbred and selectively bred mice. These findings highlight the contribution of a genetic predisposition to the susceptibility to the detrimental effects of prenatal ethanol exposure and provide additional support for the importance of genetic factors in the development of FASD (Boehm et al. 1997; Gilliam et al. 1989; Ogawa et al. 2005).

Although studies have investigated the genetic susceptibility to FASD, the underlying cause(s) of these disorders still remains unclear. The wide range of clinical features observed in people affected by in utero alcohol exposure underlines the importance of investigating the mechanisms of alcohol-related teratogenesis at a molecular level. Because FASD is a developmental abnormality, disruptions in normal cellular differentiation driven by changes in gene expression that in turn are regulated by epigenetic mechanisms are most likely involved in FASD pathogenesis.

Epigenetic Modifications

The term epigenetics, first defined by Waddington in 1942 (as reprinted in Waddington 2012), refers to the changes in gene expression that occur without changes in the DNA sequence itself. Epigenetics plays a vital role in regulating key developmental events, allowing for tissue-specific gene expression, genomic imprinting,[1] and stem-cell maintenance. Tissue-specific gene expression patterns are established and maintained through two mechanisms; structural chromatin modifications (i.e., DNA methylation and histone modifications) and RNA interactions (i.e., the actions of non-coding RNAs [ncRNAs]). In eukaryotes, the genome is present in the cell nucleus in the form of chromatin—a DNA–protein complex that packages DNA into a highly condensed form. The structural building blocks of chromatin are the nucleosomes, each of which consists of 147 base pairs of DNA wrapped around a core of 8 histone proteins (Ooi and Henikoff 2007). The octamer core comprises two copies each of histone proteins H2A, H2B, H3, and H4. Moreover, the nucleosomes are connected with each other by a linker histone H1 that offers stability to the packaged structure. Modifications of the chromatin structure affect the first step of gene expression (i.e., transcription). ncRNAs, on the other hand, act at the posttranscriptional level.

Chromatin Remodeling

DNA Modifications. Both DNA and protein components of the nucleosome are subject to a variety of modifications that can influence chromatin conformation and accessibility. The best-characterized epigenetic mark, DNA methylation, involves the covalent addition of a methyl (CH_3) group to one of the four DNA nucleotides (i.e., cytosine [C]) to form 5-methylcytosine (5mC). In eukaryotes, methylation usually affects C that are followed by the nucleotide guanine (G) (i.e., that are part of a CpG dinucleotide) (Rodenhiser and Mann, 2006). At these sites, enzymes called DNA methyltransferases (DNMTs) mediate the methylation

[1] Genomic imprinting is a genetic phenomenon in which only a gene copy inherited from one parent is expressed in the offspring. Each person inherits two copies of each gene, one inherited from the mother and one inherited from the father. In general, both of these gene copies can be active in the offspring. For some genes, however, one gene copy is "shut off" by genomic imprinting, so that only the non-imprinted copy remains active. For certain genes, the nonimprinted, active copy is always the one inherited from the mother, whereas for other genes the nonimprinted, active copy is always the one inherited from the father.

of C residues, thereby acting as critical modulators of fetal development (Li et al. 1992). For these DNA methylation reactions, DNMTs use methyl groups produced by a sequence of reactions known as the folate pathway (Friso et al. 2002). Generally, DNA methylation is associated with a condensed chromatin conformation, which effectively silences gene expression because the enzymes needed for transcription cannot access the DNA.

More recent studies have found that 5mC can be further modified by enzymes called ten-eleven translocation (Tet) proteins, in a process referred to as iterative oxidation. This results in the formation of several reaction products (i.e., derivatives), including 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) (Ito et al. 2011; Tahiliani et al. 2009). Although the role of these methylation derivatives still remains unclear (Branco et al. 2012) they seem to serve different functions than 5mC. Thus, the conversion of 5mC to 5hmC has been implicated in active DNA demethylation (Wu and Zhang, 2010). Furthermore, whereas 5mC typically is found in regions regulating the expression of specific genes (i.e., in promoters), 5hmC is associated with the bodies of the affected genes or with promoters of developmental regulatory genes (Wu et al. 2011). Finally, 5hmC appears to play an important role in reprogramming the paternal genome following fertilisation (Hackett and Surani 2013). (Reprogramming will be discussed in the following section.)

Histone Modifications. The histones making up the core of the nucleosome have unstructured N-terminal tails that protrude from the nucleosome and which are subject to modifications. Histone modifications are varied and include acetylation, methylation, phosphorylation, ubiquitinylation, ADP-ribosylation, and sumoylation at specified residues (for a review, see Kouzarides 2007). Importantly, these modifications are dynamic—that is, they can be removed again by specific enzymes.

These histone modifications, together with DNA methylation, influence chromatin structure and have a profound influence on gene regulation. Both of these types of epigenetic modifications work together to remodel the chromatin and partition the genome into two different functional domains—transcriptionally active regions collectively known as euchromatin and transcriptionally inactive regions collectively called heterochromatin. Euchromatic regions are modified to allow an open conformation, rendering the regions accessible to cellular proteins favoring transcription. In contrast, heterochromatic regions, such as the ends of chromosomes (i.e., telomeres) and regions around the center of the chromosome (i.e., pericentric regions), generally exhibit a closed conformation that limits interactions between the DNA and cellular proteins, thereby silencing gene activity (for a review, see Schneider and Grosschedl 2007). Additionally, chromatin structure, and thus gene expression, is influenced by the specific combination of histone variants in a nucleosome, the spacing between nucleosomes (i.e., nucleosome occupancy), and the position of each nucleosome within the nucleus (i.e., nuclear architecture) (Cairns 2009).

Developmental Reprogramming

Epigenetic reprogramming is a process that involves the erasure and then re-establishment of chromatin modifications during mammalian development. It serves to erase random changes in epigenetic marks (i.e., epimutations) that have occurred in the germ cells (i.e., gametes) and to restore the ability of the fertilized egg cell (i.e., zygote) to develop into all the different cell types and tissues (Reik et al. 2001). Epigenetic modifications are modulated in a temporal and spatial manner and act as reversible switches of gene expression that can lock genes into active or repressed states. In addition, these modifications allow the zygote to give rise to the cellular lineages that will form the embryo. Reprogramming occurs in two phases during in utero development, one shortly after fertilization and the other in the developing gametes of the fetus. The first phase takes place after fertilization in the preimplantation embryo (i.e., the blastocyst). During this phase, embryonic epigenetic patterns are re-established in a lineage-specific manner in the inner cell mass of the blastocyst (figure 1). The second phase occurs in the gametes, where rapid genome-wide demethylation is initiated to erase existing parental methylation patterns, followed by re-establishment of epigenetic marks in a sex-specific manner (Reik et al. 2001).

Researchers recently have begun to investigate epigenetic mechanisms as key contributors to the development of FASD. This research was prompted by the observation that periods of increased vulnerability to in utero alcohol exposure coincide with those of reprogramming events. In addition, evidence suggests that environmental factors, and specifically alcohol, are able to alter epigenetic modifications. This provides a link between the genotype, environment, and disease.

Alcohol and Biological Pathways

As mentioned previously, DNA methylation reactions rely on the folate pathway to supply the necessary methyl groups. Excessive alcohol exposure is known to interfere with normal folate metabolism and reduce its bioavailability (Halsted and Medici 2012). Folate is required as a coenzyme to supply methyl groups needed for the formation of a compound called S-adenosylmethionine (SAMe), which in turn participates in reactions in which the methyl group is transferred to another molecule (i.e., transmethylation reactions). In the folate-dependent pathway, the enzyme methionine synthase (MS), which requires vitamin

B12 to function properly, is responsible for transferring the methyl group contained within the 5-methyl-tetrahyrofolate compound to homocysteine, which ultimately generates methionine (Friso et al. 2002). The methionine is converted to SAMe by methionine adenosyltransferase (MAT), and the SAMe then is used for the methylation of DNA. As early as 1974, research on alcohol-fed rats described reduced MS activity and subsequent reduction of the levels of both methionine and SAMe (Barak et al. 1987; Finkelstein 1974; Trimble et al. 1993). Additionally, ethanol appears to enhance the loss of methyl groups, which in turn disrupts subsequent SAMe-dependent transmethylation reactions (Schalinske and Nieman 2005).

Rodent Models of Prenatal Ethanol Exposure

The teratogenic effects of prenatal alcohol exposure have been examined in rodent models for several decades. Studies have shown that in utero exposure to alcohol in these animals results in a wide range of anomalies, including growth retardation, CNS malformations, mental disability, and distinct craniofacial dysmorphology (Anthony et al. 2010; Boehm et al. 1997; Boggan et al. 1979; Bond and Di Giusto 1977; Klein de Licona et al. 2009; McGivern 1989; Parnell et al. 2009).

The FASD-like phenotypes observed in these rodent models have been associated with alterations in global gene expression, particularly in the developing brain (Hard et al. 2005; Hashimoto-

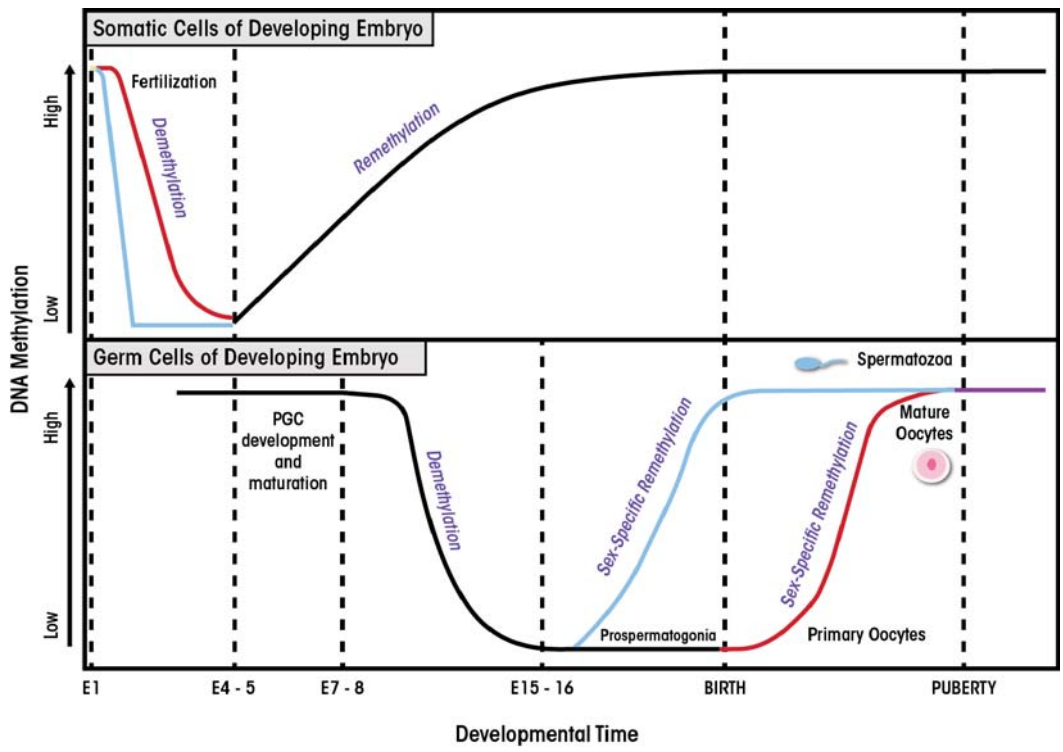


Figure 1 Reprogramming in mammalian development. Two waves of epigenetic reprogramming occur during embryo development. The first phase of reprogramming occurs in the normal body cells (i.e., somatic cells) of the developing embryo. In mice, following fertilization, the embryo undergoes genome-wide demethylation that is completed by embryonic day 5 (E5). The paternal genome (blue line) undergoes rapid, active demethylation, whereas in the maternal genome (pink line), demethylation occurs via a passive process. Remethylation of the embryonic genome begins at day E5 and is completed prior to birth. The second wave of epigenetic reprogramming occurs in the germ cells of the developing embryo, which will ultimately give rise to gametes that contain sex-specific epigenetic signatures. The primordial germ cells (PGCs) of the developing embryo contain the methylation signatures of the parental genomes. At approximately E7–8, the PGCs undergo rapid demethylation that is complete by E15–16. Following this, sex-specific methylation is re-established. In the male germline, reprogramming is complete at birth (blue line), whereas in females, reprogramming continues until puberty (pink line).

SOURCE: Adapted from Reik et al. 2001; Smallwood and Kelsey 2012.

Torii et al. 2011, 2011; Kleiber et al. 2012). This association, in conjunction with the vital role that epigenetic mechanisms play in controlling gene expression, suggests that normal epigenetic regulation by DNA methylation, histone modifications, and ncRNAs is disrupted as a result of ethanol insult.

Prenatal Ethanol Exposure and DNA Methylation

A direct link exists between ethanol exposure and aberrations in DNA methylation. For example, in a mouse model evaluating the effects of in utero ethanol exposure from days 9 to 11 of gestation, this acute ethanol administration resulted in lower-than-normal methylation throughout the genome (i.e., in global hypomethylation) of fetal DNA (Garro 1991). Furthermore, the ethanol-exposed fetuses displayed significantly reduced levels of DNA methylase activity. Ethanol-induced reductions in DNA methylation affect not only the fetus but also the placenta in pregnant mice exposed to alcohol (Haycock and Ramsay 2009). More recently, researchers evaluated the effect of prenatal alcohol exposure on DNA methylation of five imprinted genes in male offspring; these analyses detected a decrease in DNA methylation at a single locus in the *H19* imprinting control region in the sperm of these males (Stouder et al. 2011). Finally, in utero ethanol exposure in mice hinders the acquisition of DNA methylation in a brain region called the dentate gyrus, which is associated with developmental retardation (Chen et al. 2013).

Other analyses have looked at methylation patterns of specific genes rather than global DNA methylation. For example, a gene called *Agouti* has been used extensively as a model to study the effects of environmental (i.e., dietary) exposures on DNA methylation. The murine *Agouti* (*A*) locus regulates the animals' coat color; animals that carry two copies of the common variant, referred to as the wild-type allele, (i.e., *a/a* mice) display a pseudoagouti (i.e., brown) coat. A gene variant called A^{vy} is a dominant mutation that is caused by the insertion of a DNA sequence known as an intracisternal A-particle (IAP) retrotransposon in front of (i.e., upstream of) the *Agouti* gene. Animals that carry one mutant and one wild-type gene copy (i.e., heterozygous A^{vy}*/a mice*) display a variety of coat colors, ranging from yellow to mottled and brown, even though they are genetically identical. A^{vy} expression is strongly correlated to the DNA methylation profile of the inserted IAP. If the IAP shows hypomethylation, the *Agouti* gene is constantly expressed (i.e., shows constitutive ectopic *Agouti* expression) and the animals have a yellow coat. Conversely, hypermethylation correlates with promoter silencing and a pseudoagouti coat (Dolinoy et al. 2010). Kaminen-Ahola and colleagues (2010) investigated the effect of gestational ethanol exposure in A^{vy} heterozygous mice, demonstrating that ethanol exposure increased the proportion of pseudoagouti-colored offspring. This change in the proportion of coat colors was linked to transcriptional silencing of the mutant allele, which in turn correlated with hypermethylation of the A^{vy} locus. This study highlights the ability of prenatal alcohol exposure to alter the fetal epigenotype (albeit only at a specific locus) and, consequently, the adult phenotype.

In addition to the aberrant expression at the *Agouti* locus in the A^{vy} heterozygous mice, Kaminen-Ahola and colleagues (2010) noted altered gene expression profiles in the livers of their ethanol-exposed wild-type (*a/a*) siblings, as well as growth restriction and certain craniofacial dysmorphologies that are reminiscent of human FAS symptoms. Together, the findings that ethanol exposure can alter DNA methylation at the *Agouti* locus and elicit an associated phenotype (i.e., altered coat color), suggests that other epigenetic targets and associated gene expression also may be disrupted and may be responsible for the occurrence of a phenotype that corresponds to FAS in humans.

Similar studies have demonstrated the association of ethanol exposure with changes in DNA methylation and concurrent alterations in the expression of other genes. Downing and colleagues (2010) found that in utero ethanol exposure resulted in reduced methylation in the embryo at the *Igf2* locus, which encodes insulin-like growth factor 2, with a concomitant change in *Igf2* gene expression. These changes in gene expression were accompanied by skeletal malformations similar to those observed in FAS patients. In other studies, alcohol exposure resulted in neural tube defects in conjunction with genome-wide bidirectional methylation changes (i.e., occurrence of both hypo- and hypermethylation) (Liu et al. 2009). These altered methylation profiles were associated with significant changes in the expression of several genes associated with multiple functions, including chromatin remodeling, neuronal morphogenesis, synaptic plasticity, and neuronal development.

Together, these findings provide compelling evidence for alcohol-induced alterations of DNA methylation patterns in exposed fetuses that elicit a phenotype that is at least in part similar to that observed in FASD.

Prenatal Ethanol Exposure and Histone Modifications

Rodent models of alcoholism and in utero exposure to ethanol, as well as studies using cultured cells (i.e., in vitro experiments) have provided significant insights into the effects of alcohol on protein modifications, particularly to histones. Excess alcohol intake can exert its effect on protein modifications either directly or indirectly by disrupting the epigenetic machinery.

As with DNA methylation, some of these mechanisms involve folate, which as mentioned earlier serves as methyl group donor for histone methylation. Folate deficiency is a common clinical sign of chronic alcohol abuse and has been implicated in the development of alcoholism-related complications, such as alcoholic liver disease (Eichner et al. 1971). These deficiencies have been associated with significant alterations

in histone modifications, particularly at lysine residues (Esteller 2008; Kim and Shukla 2005; Park et al. 2003; Shukla et al. 2008). Altered histone modification, in turn, is associated with altered gene expression (Pal-Bhadra et al. 2007).

In in vitro studies using cultured rat liver cells (i.e., hepatocytes), ethanol exposure has been associated with bidirectional changes in histone methylation, including increased methylation at lysine 4 of histone H3 (i.e., increased H3K4me2) and decreased methylation at lysine 9 of histone H3 (i.e., decreased H3K9me2) (Pal-Bhadra et al. 2007). In addition, ethanol exposure led to selective acetylation of H3K9 (Park et al. 2003). These findings have been supported by in vivo models that have demonstrated increased H3K9 acetylation in the liver, lung, and spleen of adult rats acutely exposed to alcohol (Kim and Shukla 2006). Chronic alcohol exposure in adult rats also has been associated with increases in histone H3 and H4 acetylation in the amygdala of the brain that subsequently led to changes in the expression of the gene encoding a signaling molecule known as neuropeptide Y (Pandey et al. 2008). This increase in acetylation may result either from a decrease in the activity of the enzyme that removes acetyl groups (i.e., histone deacetylase) or an increase in the activity of the enzyme that adds acetyl groups (i.e., histone acetylase). Finally, in utero models have revealed that embryos exposed to acute levels of alcohol at mid-gestation showed elevated H3K9/18 acetylation as well as increased programmed cell death, referred to as apoptosis, of the fetal lung (Wang et al. 2010).

Zhong and colleagues (2010) investigated the effects of high and low levels of alcohol exposure on H3 acetylation and subsequent expression of genes related to heart development (i.e., *GATA4, Mef2c*, and *Tbx5*) in cardiac progenitor cells. Results indicated that low levels of alcohol increased H3 acetylation but did not significantly change the expression of the heart-development–related genes. In contrast, high levels of alcohol induced both H3 acetylation and significant gene-expression changes. These findings suggest that alterations to histone modifications are a potential mechanism for alcohol-induced cardiac disease (Zhong et al. 2010). An additional study by Guo and colleagues (2011) assessed the effects of alcohol on histone modifications in the cerebellum. The investigators found that perinatal alcohol exposure decreased the expression and function of one type of histone acetyl transferase called CREB binding protein (CBP). Altered CBP function resulted in decreased lysine acetylation on histones H3 and H4 within the cerebellum, which may contribute to the motor-activity deficits observed in FAS/FASD patients.

Another epigenetic mechanism by which alcohol could exert an effect on the epigenome is though the action of microRNAs (miRNAs).

More recently, researchers investigated the effects of alcohol exposure on certain fetal neuronal stem cells (i.e., fetal cerebral cortical neuroepithelial stem cells) and associated gene expression. These analyses found that ethanol exposure led to significant reductions in the levels of H3K4me3 (which activates gene expression) and H3K27me3 (which represses gene expression) (Veazey et al. 2013). Despite the reduction in expression-activating H3K4me3 levels, both increased and decreased transcription was observed in the genes investigated. Furthermore, loss of the repressive methylation mark, H3K27me3, did not result in altered transcription levels.

Altered protein modifications in response to alcohol exposure also may involve proteins other than histones that contribute to other manifestations of FAS, including proteins involved in insulin signaling. People with FAS often exhibit an underdeveloped cerebellum (i.e., cerebellar hypoplasia) that is associated with impaired insulin-stimulated survival signaling. This impaired signaling is mediated by the body's inability to properly respond to insulin (i.e., insulin resistance) (Soscia et al. 2006). It has been posited that chronic in utero ethanol exposure produces both insulin resistance in the CNS and oxidative stress, which is thought to play a major role in alcohol-related neurobehavioral teratogenesis (de la Monte and Wands 2010). In an in vivo model, adult rats prenatally exposed to alcohol exhibited reduced insulin signaling and increased expression of genes that regulate insulin (i.e., genes encoding proteins called TRB3 and PTEN) in the liver (Yao and Nyomba 2008). The analyses further suggested that the observed hepatic insulin resistance induced by alcohol exposure was associated with reduced acetylation of the TRB3 and PTEN proteins.

Taken together these findings suggest that alcohol-induced protein, and particularly histone, alterations continue to provide alternative or additional layers of complexity to an epigenetic etiology for FASD.

Prenatal Ethanol Exposure and ncRNA Dysregulation

Another epigenetic mechanism by which alcohol could exert an effect on the epigenome is though the action of microRNAs (miRNAs). These small ncRNAs play a critical role in several key biological processes, especially during in utero development, including cell-cycle regulation, differentiation, and organ formation (i.e., organogenesis). Individual miRNAs can affect many target genes, silencing their expression either by preventing translation of the intermediate molecules (i.e., messenger RNAs [mRNAs]) that are generated during transcription or by causing mRNA cleavage. Experimental evidence indicates that the expression of miRNAs is altered following exposure

to alcohol during development, and this may be one of the mechanisms underlying alcohol-related teratogenesis (Sathyan et al. 2007; Wang et al. 2009).

miRNAs have been implicated in the development of brain damage in response to prenatal alcohol exposure. Miranda and colleagues (2010) have hypothesized that ethanol causes brain damage during development by promoting the cell cycle of neural stem cells. This would accelerate the maturation of these progenitor cells and result in their premature depletion. This hypothesis is compatible with the observation that when clusters of neural stem cells (i.e., neurospheres) are grown in culture, differentiating neuroblasts from these clusters show increased migration and depletion of stem cells when they are exposed to ethanol compared with their unexposed counterparts. This observed behavior suggests the involvement of a large network of genes controlling complex biological outcomes. In order to examine the trigger for this behavior, researchers examined miRNA expression levels in alcohol-exposed and nonexposed neural stem cells. A preliminary screen of miRNAs in neural stem cells identified four miRNAs (i.e., miR9, miR21, miR135 and miR355) that were suppressed in the presence of ethanol exposure (Sathyan et al. 2007). These miRNAs were found to act both antagonistically and synergistically, both reducing and promoting apoptosis. Normally, these miRNAs favor normal development by balancing cell survival and cell proliferation. Following alcohol exposure, however, the reduction in their levels leads to an imbalance with detrimental effects.

In another study (Wang et al. 2009), pregnant mice were exposed to ethanol from day 6 to day 15 of gestation, and fetal brain tissue was examined for differential miRNA expression. Under these conditions, seven miRNAs were upregulated and eight were downregulated in response to ethanol exposure, with miR10a and miR10b showing the highest level of overexpression. It is biologically plausible that overexpression of these two miRNAs can disrupt developmental processes because they are thought to regulate expression of a group of genes called the *Hoxb* gene family (Wang et al. 2009). This group of genes is involved in the regulation and establishment of body patterning during embryonic development. Interestingly, there was no overlap in the miRNAs between this study and those identified in the study by Sathyan and colleagues (2007), suggesting that different models for alcohol exposure as well as the investigation of different tissues and different developmental time periods of exposure may have varying impacts on diverse miRNA targets.

Taken together, the preliminary studies suggest that miRNA plays a crucial role in normal development and that this process can be disrupted by alcohol exposure during critical periods, especially during neurogenesis.

Role of Preconception Alcohol Exposure in FASD

Although studies of FASD etiology predominantly have focused on maternal exposure during pregnancy, evidence also exists in support of contributions of paternal exposure. For example, FAS-like effects have been observed in children of alcoholic fathers even in the absence of gestational alcohol exposure, suggesting the possibility that preconception alcohol exposure may affect offspring development (Abel and Tan 1988; Lemoine et al. 1968). Studies conducted in rodents 100 years ago have supported these findings (Stockard 1913; Stockard and Papanicolaou 1916), and more recent analyses also reported that paternal preconception alcohol exposure was associated with neurobehavioral abnormalities, low birth weights, congenital malformations, and growth retardation in offspring (Friedler 1996; Jamerson et al. 2004). Additional studies have implicated a role for altered sperm DNA methylation in paternally-mediated effects of preconception ethanol exposure on offspring development (Knezovich and Ramsay 2012).

Transgenerational Transmission of the Effects of Alcohol Exposure

Altered epigenetic modifications (i.e., epimutations) may also be passed on from one generation to the next. There are two modes in which such a transmission of epimutations can occur (Skinner 2008):

- Multigenerational inheritance, in which several generations are affected because they all are exposed to the same factor (e.g., alcohol) and thus are prone to the same modifications; and

- Transgenerational inheritance, which involves a reprogramming event in the germline in response to a specific factor (e.g., alcohol exposure), resulting in an altered epigenome that would be inherited by future generations even if they are not themselves exposed to the same factor.

It was previously believed that transgenerational epigenetic inheritance would be unlikely because, as mentioned previously, epigenetic reprogramming occurs in the germline. However, increasing evidence indicates that transgenerational epigenetic inheritance does indeed happen (Anway et al. 2005; Crepin et al. 2012; Stouder and Paoloni-Giacobino 2010). Most of the work conducted thus far in this area has focused on the effects of agents that can interfere with the body's normal hormone systems (e.g., vinclozolin, which affects sex hormone levels and has been shown to have transgenerational effects). The potential transgenerational effects of alcohol and their role in the etiology and perpetuation of FAS/FASD symptoms in affected individuals and their progeny, however, still need to be determined.

Conclusions

Evidence is rapidly accumulating in support of an epigenetic etiology in the development of FASD (figure 2). All three types of epigenetic modulators—DNA methylation, histone modifications and regulation by ncRNAs—are perturbed by ethanol exposure. These ethanol-related changes can affect gene expression of critical developmental genes and pathways, impacting cell proliferation and differentiation.

The phenotypic consequences of in utero ethanol exposure are significantly correlated with the molecular consequences of ethanol's effects on epigenetic regulatory mechanisms. A complex picture of locus-specific and cell-type–restricted effects is emerging. In particular, many studies have focused on ethanol's effects on mechanisms that regulate neurogenesis, leading to the most devastating consequences of alcohol exposure during development. The range of effects appears to be significantly influenced by the timing and level of exposure, leading to a wide range of outcomes and combinations of phenotypic indicators.

In mouse models, ethanol exposure can be carefully controlled and other environmental parameters, such as diet and stress, can be kept constant. This allows for careful investigation of the effects of alcohol exposure on epigenetic regulatory mechanism and their association with FAS-like symptoms. Drinking patterns in pregnant women, in contrast, are seldom accurately documented and often occur throughout gestation, which, not surprisingly, leads to a vast array of phenotypes now recognized under the banner of FASD. Thus, discerning the role of epigenetic mechanisms in these processes will be much more challenging. ■

Acknowledgments

Michelle Ungerer and Jaysen Knezovich contributed equally to writing this review.

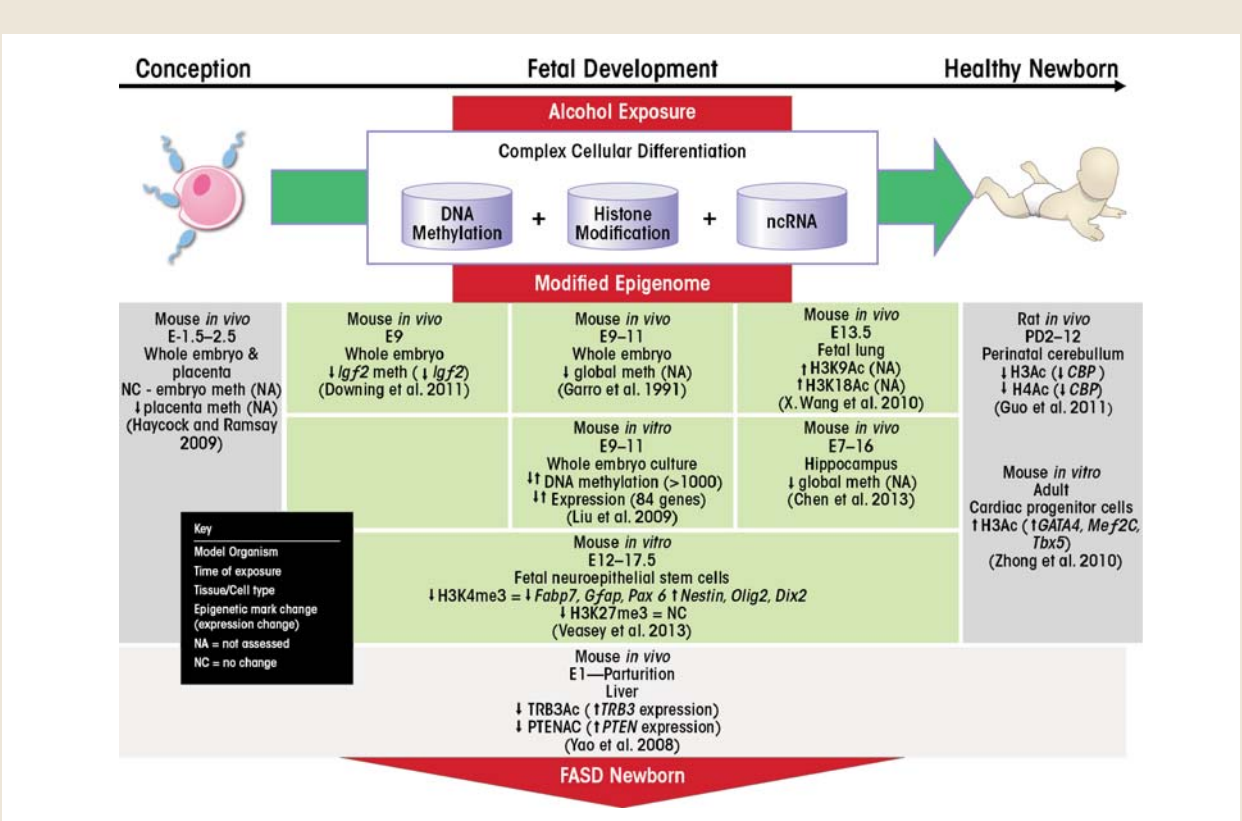


Figure 2 Epigenetic contributions to FASD. Following conception, a complex orchestration of epigenetic mechanisms ensures normal cellular differentiation and embryonic development (green horizontal arrow). These mechanisms include DNA methylation, histone modifications, and non-coding RNAs (ncRNAs) to modulate gene expression in a specified temporal and spatial manner. Alcohol exposure in utero (red downward arrow) has been shown to alter these epigenetic modulators, which may consequently dysregulate gene expression patterns as indicated by the study findings listed and affect normal embryonic development and phenotype outcome. By these mechanisms, alcohol-induced epigenetic aberrations may contribute to the etiology of fetal alcohol spectrum disorders (FASD).

Financial Disclosure

The authors declare that they have no competing financial interests.

References

Abel, E.L. Fetal alcohol syndrome in families. *Neurotoxicology and Teratology* 10(1):1–2, 1988. PMID: 3352564

Abel, E.L. An update on incidence of FAS: FAS is not an equal opportunity birth defect. *Neurotoxicology and Teratology* 17(4):437–443, 1995. PMID: 7565490

Abel, E.L., and Tan, S.E. Effects of paternal alcohol consumption on pregnancy outcome in rats. *Neurotoxicology and Teratology* 10(3):187–192, 1988. PMID: 3211095

Anthony, B.; Vinci-Booher, S.; Wetherill, L.; et al. Alcohol-induced facial dysmorphology in C57BL/6 mouse models of fetal alcohol spectrum disorder. *Alcohol* 44(7–8): 659–671, 2010. PMID: 20570474

Anway, M.D.; Cupp, A.S.; Uzumcu, M.; and Skinner, M.K. Epigentic transgenerational actions of endocrine disruptors and male fertility. *Science* 308(5727):1466–1469, 2005. PMID: 15933200

Barak, A.J.; Beckenhauer, H.C.; Tuma, D.J.; and Badakhsh, S. Effects of prolonged ethanol feeding on methionine metabolism in rat liver. *Biochemistry and Cell Biology* 65(3):230–233, 1987. PMID: 3580171

Boehm, S.L., 2nd; Lundahl, K.R.; Caldwell, J.; and Gilliam, D.M. Ethanol teratogenesis in the C57BL/6J, DBA/2J, and A/J inbred mouse strains. *Alcohol* 14(4):389–395, 1997. PMID: 9209555

Boggan, W.O.; Randall, C.L.; and Dodds, H.M. Delayed sexual maturation in female C57BL/6J mice prenatally exposed to alcohol. *Research Communications in Chemical Pathology and Pharmacology* 23(1):117–125, 1979. PMID: 441506

Bond, N.W., and Di Giusto, E.L. Prenatal alcohol consumption and open-field behaviour in rats: Effects of age at time of testing. *Psychopharmacology* 52(3):311–312, 1977. PMID: 406636

Branco, M.R.; Ficz, G.; and Reik, W. Uncovering the role of 5-hydroxymethylcytosine in the epigenome. *Nature Reviews. Genetics* 13(1):7–13, 2011. PMID: 22083101

Burd, L.; Martsolf, J.T.; Klug, M.G.; and Kerbeshian, J. Diagnosis of FAS: A comparison of the Fetal Alcohol Syndrome Diagnostic Checklist and the Institute of Medicine Criteria for Fetal Alcohol Syndrome. *Neurotoxicology and Teratology* 25(6):719–724, 2003. PMID: 14624971

Cairns, B.R. The logic of chromatin architecture and remodelling at promoters. *Nature* 461(7261):193–198, 2009. PMID: 19741699

Chasnoff, I.J. Fetal alcohol syndrome in twin pregnancy. *Acta Geneticae Medicae et Gemellologiae* (Roma) 34(3-4):229–232, 1985. PMID: 3832736

Chen, Y.; Ozturk, N.C.; and Zhou, F.C. DNA methylation program in developing hippocampus and its alteration by alcohol. *PLoS One* 8(3):e60503, 2013. PMID: 23544149

Chernoff, G.F. The fetal alcohol syndrome in mice: Maternal variables. *Teratology* 22(1):71–75, 1980. PMID: 7003793

Crepin, M.; Dieu, M.C.; Lejeune, S.; et al. Evidence of constitutional MLH1 epimutation associated to transgenerational inheritance of cancer susceptibility. *Human Mutation* 33(1):180–188, 2012. PMID: 21953887

de la Monte, S.M., and Wands, J.R. Role of central nervous system insulin resistance in fetal alcohol spectrum disorders. *Journal of Population Therapeutics and Clinical Pharmacology* 17(3):e390–404, 2010. PMID: 21063035

Dolinoy, D.C.; Weinhouse, C.; Jones, T.R.; et al. Variable histone modifications at the A^{vy} metastable epiallele. *Epigenetics* 5(7):637–644, 2010. PMID: 20671424

Downing, C.; Johnson, T.E.; Larson, C.; et al. Subtle decreases in DNA methylation and gene expression at the mouse Igf2 locus following prenatal alcohol exposure: Effects of a methyl-supplemented diet. *Alcohol* 45(1):65–71, 2011. PMID: 20705422

Eichner, E.R.; Pierce, H.I.; and Hillman, R.S. Folate balance in dietary-induced megaloblastic anemia. *New England Journal of Medicine* 284(17):933–938, 1971. PMID: 5551802

Esteller, M. Epigenetics in cancer. *New England Journal of Medicine* 358(11):1148–1159, 2008. PMID: 18337604

Finkelstein, J.D. Methionine metabolism in mammals: The biochemical basis for homocystinuria. *Metabolism: Clinical and Experimental* 23(4):387–398, 1974. PMID: 4593752

Friedler, G. Paternal exposures: Impact on reproductive and developmental outcome. *An overview. Pharmacology, Biochemistry, and Behavior* 55(4):691–700, 1996. PMID: 8981601

Friso, S.; Choi, S.W.; Girelli, D.; et al. A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. *Proceedings of the National Academy of Sciences of the United States of America* 99(8):5606–5611, 2002. PMID: 11929966

Garro, A.J.; McBeth, D.L.; Lima, V.; and Lieber, C.S. Ethanol consumption inhibits fetal DNA methylation in mice: Implications for the fetal alcohol syndrome. *Alcoholism: Clinical and Experimental Research* 15(3):395–398, 1991. PMID: 1877725

Gemma, S.; Vichi, S.; and Testai, E. Metabolic and genetic factors contributing to alcohol induced effects and fetal alcohol syndrome. *Neuroscience and Biobehavioral Reviews* 31(2):221–229, 2007. PMID: 16908065

Gilliam, D.M.; Kotch, L.E.; Dudek, B.C.; and Riley, E.P. Ethanol teratogenesis in selectivity bred long-sleep and short-sleep mice: A comparison to inbred C57BL/6J mice. *Alcoholism: Clinical and Experimental Research* 13(5):667–672, 1989. PMID: 2688466

Guo, W.; Crossey, E.L.; Zhang, L.; et al. Alcohol exposure decreases CREB binding protein expression and histone acetylation in the developing cerebellum. *PLoS One* 6(5):e19351, 2011. PMID: 21655322

Hackett, J.A., and Surani, M.A. DNA methylation dynamics during the mammalian life cycle. Philosophical transactions of the Royal Society of London. Series B, Biological *Sciences* 368(1609):20110328, 2013. PMID: 23166392

Halsted, C.H., and Medici, V. Aberrant hepatic methionine metabolism and gene methylation in the pathogenesis and treatment of alcoholic steatohepatitis. *International Journal of Hepatology* 2012:959746, 2012. PMID: 22007317

Hard, M.L.; Abdolell, M.; Robinson, B.H.; and Koren, G. Gene-expression analysis after alcohol exposure in the developing mouse. *Journal of Laboratory and Clinical Medicine* 145(1):47–54, 2005. PMID: 15668661

Hashimoto-Torii, K.; Kawasawa, Y.I.; Kuhn, A.; and Rakic, P. Combined transcriptome analysis of fetal human and mouse cerebral cortex exposed to alcohol. *Proceedings of the National Academy of Sciences of the United States of America* 108(10):4212–4217, 2011. PMID: 21368140

Haycock, P.C., and Ramsay, M. Exposure of mouse embryos to ethanol during preimplantation development: Effect on DNA methylation in the H19 imprinting control region. *Biology of Reproduction* 81(4):618–627, 2009. PMID: 19279321

Ito, S.; Shen, L.; Dai, Q.; et al. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science* 333(6047):1300–1303, 2011. PMID: 21778364

Jamerson, P.A.; Wulser, M.J.; and Kimler, B.F. Neurobehavioral effects in rat pups whose sires were exposed to alcohol. Brain Research. *Developmental Brain Research* 149(2):103–111, 2004. PMID: 15063090

Jones, K.L.; Smith, D.W.; Ulleland, C.N.; and Streissguth, P. Pattern of malformation in offspring of chronic alcoholic mothers. *Lancet* 1(7815):1267–1271, 1973. PMID: 4126070

Kaminen-Ahola, N.; Ahola, A.; Maga, M.; et al. Maternal ethanol consumption alters the epigenotype and the phenotype of offspring in a mouse model. *PLoS Genetics* 6(1):e1000811, 2010. PMID: 20084100

Kim, J.S., and Shukla, S.D. Histone H3 modifications in rat hepatic stellate cells by ethanol. *Alcohol and Alcoholism* 40(5):367–372, 2005. PMID: 15939707

Kim, J.S., and Shukla, S.D. Acute in vivo effect of ethanol (binge drinking) on histone H3 modifications in rat tissues. *Alcohol and Alcoholism* 41(2):126–132, 2006. PMID: 16314425

Kleiber, M.L.; Laufer, B.I.; Wright, E.; et al. Long-term alterations to the brain transcriptome in a maternal voluntary consumption model of fetal alcohol spectrum disorders. *Brain Research* 1458:18–33, 2012. PMID: 22560501

Klein de Licona, H.; Karacay, B.; Mahoney, J.; et al. A single exposure to alcohol during brain development induces microencephaly and neuronal losses in genetically susceptible mice, but not in wild type mice. *NeuroToxicology* 30(3):459–470, 2009. PMID: 19442832

Knezovich, J.G., and Ramsay, M. The effect of preconception paternal alcohol exposure on epigenetic remodeling of the H19 and Rasgrf1 imprinting control regions in mouse offspring. *Frontiers in Genetics* 3:10, 2012. PMID: 22371710

Kouzarides, T. Chromatin modifications and their function. *Cell* 128(4):693–705, 2007. PMID: 17320507

Lemoine, P.; Harousseau, H.; Borteyru, J.P.; and Menuet, J.C. Les enfants des parents alcoholiques: Anomolies observees a propos de 127 cas (The children of alcoholic parents: Anomalies observed in 127 cases). *Quest Medical* 25:476–482, 1968.

Li, E.; Bestor, T.H.; and Jaenisch, R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 69(6):915–926, 1992. PMID: 1606615

Liu, Y.; Balaraman, Y.; Wang, G.; et al. Alcohol exposure alters DNA methylation profiles in mouse embryos at early neurulation. *Epigenetics* 4(7):500–511, 2009. PMID: 20009564

May, P.A., and Gossage, J. P. Estimating the prevalence of fetal alcohol syndrome. A summary. *Alcohol Research & Health* 25(3):159–167, 2001. PMID: 11810953

May, P.A.; Gossage, J.P.; Marais, A.S.; et al. The epidemiology of fetal alcohol syndrome and partial FAS in a South African community. *Drug and Alcohol Dependence* 88(2–3):259–271, 2007. PMID: 17127017

McGivern, R.F. Low birthweight in rats induced by prenatal exposure to testosterone combined with alcohol, pair-feeding, or stress. *Teratology* 40(4):335–338, 1989. PMID: 2814895

Miranda, R.C.; Pietrzykowski, A.Z.; Tang, Y.; et al. MicroRNAs: Master regulators of ethanol abuse and toxicity? *Alcoholism: Clinical and Experimental Research* 34(4):575–587, 2010. PMID: 20102566

O'Leary, C. M. Fetal alcohol syndrome: Diagnosis, epidemiology, and developmental outcomes. *Journal of Paediatrics and Child Health* 40(1-2):2–7, 2004. PMID: 14717994

Ogawa, T.; Kuwagata, M.; Ruiz, J.; and Zhou, F.C. Differential teratogenic effect of alcohol on embryonic development between C57BL/6 and DBA/2 mice: A new view. *Alcoholism: Clinical and Experimental Research* 29(5):855–863, 2005. PMID: 15897731

Ooi, S.L., and Henikoff, S. Germline histone dynamics and epigenetics. *Current Opinion in Cell Biology* 19(3): 257–265, 2007. PMID: 17467256

Padmanabhan, R., and Hameed, M.S. Effects of acute doses of ethanol administered at pre-implantation stages on fetal development in the mouse. *Drug and Alcohol Dependence* 22(1–2):91–100, 1988. PMID: 3234238

Pal-Bhadra, M.; Bhadra, U.; Jackson, D.E.; et al. Distinct methylation patterns in histone H3 at Lys-4 and Lys-9 correlate with up- & down-regulation of genes by ethanol in hepatocytes. *Life Sciences* 81(12):979–987, 2007. PMID: 17826801

Pandey, S.C.; Ugale, R.; Zhang, H.; et al. Brain chromatin remodeling: A novel mechanism of alcoholism. *Journal of Neuroscience* 28(14):3729–3737, 2008. PMID: 18385331

Park, P.H.; Miller, R.; and Shukla, S.D. Acetylation of histone H3 at lysine 9 by ethanol in rat hepatocytes. *Biochemical and Biophysical Research Communications* 306(2): 501–504, 2003. PMID: 12804592

Parnell, S.E.; O'Leary-Moore, S.K.; Godin, E.A., et al. Magnetic resonance microscopy defines ethanol-induced brain abnormalities in prenatal mice: Effects of acute insult on gestational day 8. *Alcoholism: Clinical and Experimental Research* 33(6):1001–1011, 2009. PMID: 19302087

Pierce, D.R., and West, J.R. Blood alcohol concentration: A critical factor for producing fetal alcohol effects. *Alcohol* 3(4):269–272, 1986. PMID: 3638973

Ramsay, M. Genetic and epigenetic insights into fetal alcohol spectrum disorders. *Genome Medicine* 2(4):27, 2010. PMID: 20423530

Reik, W.; Dean, W.; and Walter, J. Epigenetic reprogramming in mammalian development. *Science* 293(5532): 1089–1093, 2001. PMID: 11498579

Rodenhiser, D., and Mann, M. Epigenetics and human disease: Translating basic biology into clinical applications. *CMAJ: Canadian Medical Association Journal* 174(3): 341–348, 2006. PMID: 16446478

Sampson, P.D.; Streissguth, A.P.; Bookstein, F.L.; et al. Incidence of fetal alcohol syndrome and prevalence of alcohol-related neurodevelopmental disorder. *Teratology* 56(5):317–326, 1997. PMID: 9451756

Sathyan, P.; Golden, H.B.; and Miranda, R. C. Competing interactions between micro-RNAs determine neural progenitor survival and proliferation after ethanol exposure: Evidence from an ex vivo model of the fetal cerebral cortical neuroepithelium. *Journal of Neuroscience* 27(32): 8546–8557, 2007. PMID: 17687032

Schalinske, K.L., and Nieman, K.M. Disruption of methyl group metabolism by ethanol. *Nutrition Reviews* 63(11):387–391, 2005. PMID: 16370223

Schneider, R., and Grosschedl, R. Dynamics and interplay of nuclear architecture, genome organization, and gene expression. *Genes & Development* 21(23):3027–3043, 2007. PMID: 18056419

Shukla, S.D.; Velaquez, J.; French, S.W.; et al. Emerging role of epigenetics in the actions of alcohol. *Alcoholism: Clinical and Experimental Research* 32(9):1525–1534, 2008. PMID: 18616668

Skinner, M.K. What is an epigenetic transgenerational phenotype? F3 or F2. *Reproductive Toxicology* 25(1): 2–6, 2008. PMID: 17949945

Smallwood, S.A., and Kelsey, G. De novo DNA methylation: A germ cell perspective. *Trends in Genetics* 28(1):33–42, 2012. PMID: 22019337

Soscia, S.J.; Tong, M.; Xu, X.J.; et al. Chronic gestational exposure to ethanol causes insulin and IGF resistance and impairs acetylcholine homeostasis in the brain. *Cellular and Molecular Life Sciences* 63(17):2039–2056, 2006. PMID: 16909201

Stockard, C.R. The effect on the offspring of intoxicating the male parent and the transmission of the defects to subsequent generations. *The American Naturalist* 47(563):641–682, 1913.

Stockard, C.R., and Papanicolaou, G. A further analysis of the hereditary transmission of degeneracy and deformaties by the descendants of alcoholized mammals. *The American Naturalist* 50(590):65–88, 1916.

Stouder, C., and Paoloni-Giacobino, A. Transgenerational effects of the endocrine disruptor vinclozolin on the methylation pattern of imprinted genes in the mouse sperm. *Reproduction* 139(2):373–379, 2010. PMID: 19887539

Stouder, C.; Somm, E.; and Paoloni-Giacobino, A. Prenatal exposure to ethanol: A specific effect on the H19 gene in sperm. *Reproductive Toxicology* 31(4):507–512, 2011. PMID: 21382472

Streissguth, A.P., and Dehaene, P. Fetal alcohol syndrome in twins of alcoholic mothers: Concordance of diagnosis and IQ. *American Journal of Medical Genetics* 47(6): 857–861, 1993. PMID: 8279483

Sulik, K.K. Critical periods for alcohol teratogenesis in mice, with special reference to the gastrulation stage of embryogenesis. *Ciba Foundation Symposium* 105:124–141, 1984. PMID: 6563984

Tahiliani, M.; Koh, K.P.; Shen, Y.; et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* 324(5929): 930–935, 2009. PMID: 19372391

Trimble, K.C.; Molloy, A.M.; Scott, J.M.; and Weir, D.G. The effect of ethanol on one-carbon metabolism: Increased methionine catabolism and lipotrope methyl-group wastage. *Hepatology* 18(4):984–989, 1993. PMID: 7691709

Veazey, K.J.; Carnahan, M.N.; Muller, D.; et al. Alcohol-induced epigenetic alterations to developmentally crucial genes regulating neural stemness and differentiation. *Alcoholism: Clinical and Experimental Research doi:* 10.1111/acer.12080 [Epub ahead of print], 2013. PMID: 23488822

Waddington, C.H. The epigenotype. 1942. *International Journal of Epidemiology* 41(1):10–13, 2012. PMID: 22186258

Wang, L.L.; Zhang, Z.; Li, Q.; et al. Ethanol exposure induces differential microRNA and target gene expression and teratogenic effects which can be suppressed by folic acid supplementation. *Human Reproduction* 24(3):562–579, 2009. PMID: 19091803

Wang, X.; Gomutputra, P.; Wolgemuth, D.J.; and Baxi, L.V. Acute alcohol exposure induces apoptosis and increases histone H3K9/18 acetylation in the mid-gestation mouse lung. *Reproductive Sciences* 17(4):384–390, 2010. PMID: 20124552

Warren, K.R., and Li, T.K. Genetic polymorphisms: Impact on the risk of fetal alcohol spectrum disorders. *Birth Defects Research. Part A, Clinical and Molecular Teratology* 73(4):195–203, 2005. PMID: 15786496

Wu, H.; D'Alessio, A.C.; Ito, S.; et al. Genome-wide analysis of 5-hydroxymethylcytosine distribution reveals its dual function in transcriptional regulation in mouse embryonic stem cells. *Genes & Development* 25(7): 679–684, 2011. PMID: 21460036

Wu, S., and Zhang, Y. Active DNA demethylation: Many roads lead to Rome. *Nature Reviews. Molecular Cell Biology* 11(9):607–620, 2010. PMID: 20683471

Yao, X.H., and Nyomba, B.L. Hepatic insulin resistance induced by prenatal alcohol exposure is associated with reduced PTEN and TRB3 acetylation in adult rat offspring. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* 294(6): R1797–1806, 2008. PMID: 18385463

Zhong, L.; Zhu, J.; Lv, T.; et al. Ethanol and its metabolites induce histone lysine 9 acetylation and an alteration of the expression of heart development-related genes in cardiac progenitor cells. *Cardiovascular Toxicology* 10(4):268–274, 2010. PMID: 20811785

Alcohol, DNA Methylation, and Cancer

Marta Varela-Rey, Ph.D.; Ashwin Woodhoo, Ph.D.; Maria-Luz Martinez-Chantar, Ph.D.; José M. Mato, Ph.D.; and Shelly C. Lu, M.D.

Marta Varela-Rey, Ph.D., *is a CIBERehd postdoctoral fellow,* **Ashwin Woodhoo, Ph.D.,** *is a Ramón y Cajal postdoctoral fellow, and* **Maria-Luz Martinez-Chantar, Ph.D.,** *is group leader, all at the Centro de Investigación Cooperative en Biosciencias (CIC bioGUNE), Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Technology Park of Bizkaia, Derio, Spain.*

José M. Mato, Ph.D., *is director of CIC bioGUNE and of the Center for Cooperative Research in Biomaterials (CIC bioMAGUNE), as well as research professor and group leader at CIC bioGUNE, (CIBERehd), Technology Park of Bizkaia, Derio, Spain.*

Shelly C. Lu, M.D., *is professor of medicine, associate chief of the Division of Gastrointestinal and Liver Diseases, and associate director of the USC Research Center for Liver Diseases, Keck School of Medicine, University of Southern California, Los Angeles, California.*

Cancer is one of the most significant diseases associated with chronic alcohol consumption, and chronic drinking is a strong risk factor for cancer, particularly of the upper aerodigestive tract, liver, colorectum, and breast. Several factors contribute to alcohol-induced cancer development (i.e., carcinogenesis), including the actions of acetaldehyde, the first and primary metabolite of ethanol, and oxidative stress. However, increasing evidence suggests that aberrant patterns of DNA methylation, an important epigenetic mechanism of transcriptional control, also could be part of the pathogenetic mechanisms that lead to alcohol-induced cancer development. The effects of alcohol on global and local DNA methylation patterns likely are mediated by its ability to interfere with the availability of the principal biological methyl donor, S-adenosylmethionine (SAMe), as well as pathways related to it. Several mechanisms may mediate the effects of alcohol on DNA methylation, including reduced folate levels and inhibition of key enzymes in one-carbon metabolism that ultimately lead to lower SAMe levels, as well as inhibition of activity and expression of enzymes involved in DNA methylation (i.e., DNA methyltransferases). Finally, variations (i.e., polymorphisms) of several genes involved in one-carbon metabolism also modulate the risk of alcohol-associated carcinogenesis. **KEY WORDS: Alcohol consumption; chronic alcohol use; alcohol use disorders; alcohol-induced cancer development; risk factors; epigenetics; epigenetic mechanisms; cancer; upper aerodigestive tract cancer; colorectum cancer; breast cancer; carcinogenesis; DNA methylation**

According to the World Health Organization (WHO) Global Burden of Disease Project, alcohol accounts for approximately 3.2 percent of all deaths per year worldwide (corresponding to 1.8 million people) and is causally related to more than 60 different medical conditions (Rehm et al. 2004). Cancer formation (i.e., carcinogenesis) is one of the most significant consequences attributed to alcohol consumption, and approximately 3.6 percent of all cancer-related cases (5.2 percent in men and 1.7 percent in women) worldwide, as well as 3.5 percent of all cancer-related deaths are related to chronic alcohol drinking (Boffetta et al. 2006). Based on available epidemiological data, an international group of epidemiologists and alcohol researchers concluded that alcohol induces carcinogenesis in numerous organs, including the upper aerodigestive tract, liver, colorectum, and female breast (Baan et al. 2007).

Several pathogenic mechanisms contribute to alcohol-induced carcinogenesis in each type of cancer. The most commonly cited mechanisms include the effect of acetaldehyde—the first metabolite of ethanol oxidation—and oxidative stress (Seitz and Stickel 2007). Increasing evidence shows that alcohol also can induce epigenetic alterations, for example, in pathological conditions such as fetal alcohol spectrum disorders (Kobor and Weinberg 2011). Epigenetic alterations also are a hallmark of cancer development in general (Esteller 2008). Therefore, this article reviews the available evidence that such changes also could be an important contributory factor to alcohol-induced carcinogenesis. In particular, it discusses the role of DNA

methylation in carcinogenesis and how alcohol may affect the pathways that regulate the availability of S-adenosylmethionine (SAMe), the principal biological methyl donor for methylation reactions. (For a list of abbreviations of the names for genes, proteins, and other compounds as well as their functions, see table).

DNA Methylation and Cancer

DNA methylation plays a critical role in the control of gene activity. This methylation almost exclusively involves the addition of a methyl group to carbon 5 of cytosine nucleotides, and specifically those cytosines that precede guanines (i.e., are part of CpG dinucleotides). CpG dinucleotides tend to cluster either in regions called CpG islands, which are located in approximately 60 percent of human gene promoters, or in regions that contain large repetitive DNA sequences (e.g., centromeres and retrotransposons). In the former case, the CpG dinucleotides generally tend to remain unmethylated, whereas in the latter case they mostly are methylated to prevent chromosome instability (Rodriguez-Paredes and Esteller 2011) (figure 1). DNA methylation also occurs in CpG island shores—that is, regions of lower CpG density that are located close to CpG islands (i.e., within 2 kb). In these three cases, DNA methylation typically is associated with repression of gene expression (i.e., transcription). In a few cases, however, DNA methylation also can enable gene transcription, namely when the methylation occurs in the body (i.e., coding sequences) of the gene rather than the promoter (Portela and Esteller 2010).

DNA methylation is mediated (i.e., catalyzed) by three main enzymes called DNA methyltransferases (DNMTs), all of which transfer a methyl group from SAMe to DNA:

- DNMT1, which also is referred to as the maintenance DNMT, completes the methylation of the partially methylated (i.e., hemimethylated) DNA that is present in the cell after cell division and the accompanying DNA replication.
- DNMT 3A and DNMT 3B are referred to as de novo methyltransferases because they target unmethylated CpGs.

Aberrant epigenetic regulation, including altered DNA methylation, characterizes a wide range of diseases, including cancer (Portela and Esteller

Table 1 Abbreviations of Gene Names, Protein Names, and Other Molecules

Abbreviation	Spelled-out Name
Genes	
APC-1A	Adenomatous polyposis coli
BRCA1	Breast cancer 1, early onset
CHRNA3	Cholinergic receptor, nicotinic, alpha 3
CDKN2A	Cyclin-dependent kinase inhibitor 2A
p14ARF	Cyclin-dependent kinase inhibitor 2A, isoform 4
CDKN2B, p15INK4b	Cyclin-dependent kinase 4 inhibitor B
CDKN2A, p16Ink4A	Cyclin-dependent kinase inhibitor 2A
ER-α	Estrogen receptor
GATA2	GATA binding protein 2
GSTP1	Glutathione S-transferase pi 1
HOX2A	homeobox B5
MGMT	O-6-Methylguanine-DNA methyltransferase
hMLH1	MutL homolog 1
RASSF1A	Ras association (RalGDS/AF-6) domain family member 1
RB	Retinoblastoma
Proteins	
BHMT	Betaine homocysteine methyltransferase
DHFR	Dihydrofolate reductase
DNMTs	DNA methyltransferases
LINE family member L1, LINE-1	Long interspersed nucleotide element-1
MAT	Methionine adenosyltransferase
MTR	Methionine synthase
MTHFR	methylenetetrahydrofolate reductase
MT	Methyltransferase
SFRP1	Secreted frizzled-related protein 1
TS	Thymidylate synthase
Other Compunds	
DHF	Dihydrofolate
dTMP	Deoxythymidine monophosphate
dUMP	Deoxyuridine monophosphate
Hcy	Homocysteine
Met	Methionine
5,10-MTHF	5,10-Methylenetetrahydrofolate
5-MTHF	5-Methyltetrahydrofolate
SAH	S-adenosylhomocysteine
SAMe	S-adenosylmethionine
THF	Tetrahydrofolate

2010). Compared with normal cells, the epigenome of cancer cells shows profound changes in DNA methylation patterns as well as histone modifications patterns (Rodriguez-Paredes and Esteller 2011), including the following:

- Genome-wide hypomethylation (about 20 to 60 percent less overall 5-methyl-cytosine compared with normal cells); global loss of DNA methylation occurs at many genomic regions, such as repetitive elements and retrotranposons, resulting in chromosomal instability, and activation of transposable elements and endoparasitic sequences (e.g., LINE family member L1) (figure 1).
- Hypomethylation at specific promoters, resulting in aberrant activation of cancer-inducing genes (i.e., oncogenes) (e.g., *SERPINB5*) and inducing loss of imprinting at other DNA sites (e.g., *IGF2*).
- Hypermethylation of CpG island promoters of genes involved in central cellular pathways, such as DNA repair (e.g., at genes called *hMLH1, MGMT,* and *BRCA1*) and cell cycle control (e.g., at genes called *p16INK4a, p15INK4b,* and *RB*), resulting in the silencing by is the most recognized epigenetic disruption in human tumors.
- Aberrant DNA methylation at CpG island shores (e.g., in genes called *HOX2A* and *GATA2*) (Portela and Esteller 2010; Rodriguez-Paredes and Esteller 2011).

One-Carbon Metabolism

Persuasive evidence indicates that several dietary factors, such as alcohol, can modulate DNA methylation patterns and increase susceptibility to disease, including cancer, by altering one-carbon metabolism (Choi and Friso 2010). The term one-carbon metabolism refers to a network of biochemical reactions in which a chemical unit containing one carbon atom (e.g., a methyl group) is transferred through several steps from a donor to another compound, such as DNA (figure 2). The first step in this chain of events is the transfer of the one-carbon group to tetrahydrofolate (THF), resulting in the formation of 5,10-MTHF. This molecule then can be used in the synthesis of the nucleotide thymidine in a

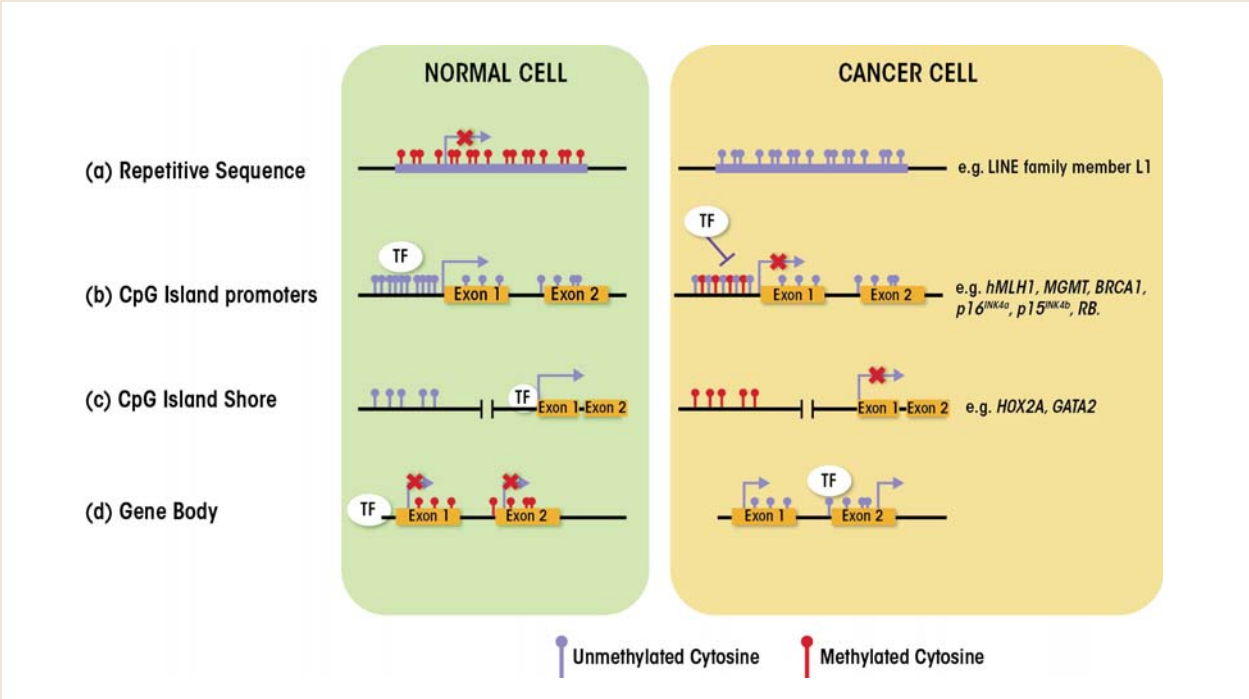


Figure 1 DNA methylation patterns in normal and cancer cells. (a) Repetitive sequences generally are methylated at cytosine nucleotides in normal cells. Global loss of methylation in cancer cells leads to chromosomal instability and activation of endoparasitic sequences. (b) CpG islands in promoter sequences typically are unmethylated in normal cells whereas they can become hypermethylated in cancer cells, leading to transcriptional repression. Examples of genes affected are shown on the right. (c) Similar patterns are seen in CpG island shores, located in front (i.e., upstream) of promoters. (d) CpGs located in gene bodies frequently are methylated in normal cells; this pattern is reversed in cancer cells, leading to initiation of transcription at several incorrect sites.

reaction catalyzed by the enzyme thymidylate synthase (TS). In this reaction, one carbon group of the 5,10-MTHF is transferred to a molecule called deoxyuridine monophosphate (dUMP), resulting in the formation of deoxythymidine monophosphate (dTMP) and dihydrofolate (DHF). This reaction is considered to be a limiting step in DNA synthesis and, more importantly, reduces dUMP levels, which can lead to breaks in both strands of the DNA molecules. Finally, the enzyme dihydrofolate reductase (DHFR) catalyzes the reduction of DHF back into THF (Mato et al. 2008; Van der Put et al. 2001).

In an alternative set of reactions, 5,10-MTHF also can be converted to 5-methyltetrahydrofolate (5-MTHF) in a reaction catalyzed by the enzyme methylenetetrahydrofolate reductase (MTHFR). The 5-MTHF generated then can be used in the remethylation of homocysteine (Hcy) to methionine, which is catalyzed by methionine synthase (MTR). In the liver and kidney, Hcy also can be remethylated into methionine through a folate-independent reaction, catalyzed by betaine homocysteine methyltransferase (BHMT). BHMT requires betaine, a break-down product (i.e., metabolite) of choline for its activity.

Methionine, in turn, is an essential amino acid that, together with ATP, participates in the formation of SAMe, in a reaction catalyzed by methionine adenosyltransferase (MAT). SAMe is the principal methyl donor for numerous reactions, including protein, RNA, DNA, and histone methylation. The formation of SAMe is a limiting step in DNA methylation. After transfer of its activated methyl group in the methylation reactions, SAMe is converted into S-adenosylhomocysteine (SAH). This compound also can affect DNA methylation because it is a potent competitive inhibitor of methyltransferases, including DNMTs. Both an increase in SAH levels and a decrease in the SAMe-to-SAH ratio can inhibit transmethylation reactions (Lu 2000).

Polymorphisms of One-Carbon Metabolism Enzymes

Several studies have shown that variations (i.e., polymorphisms) in several of the genes encoding enzymes involved in one-carbon metabolism and, consequently, in the resulting

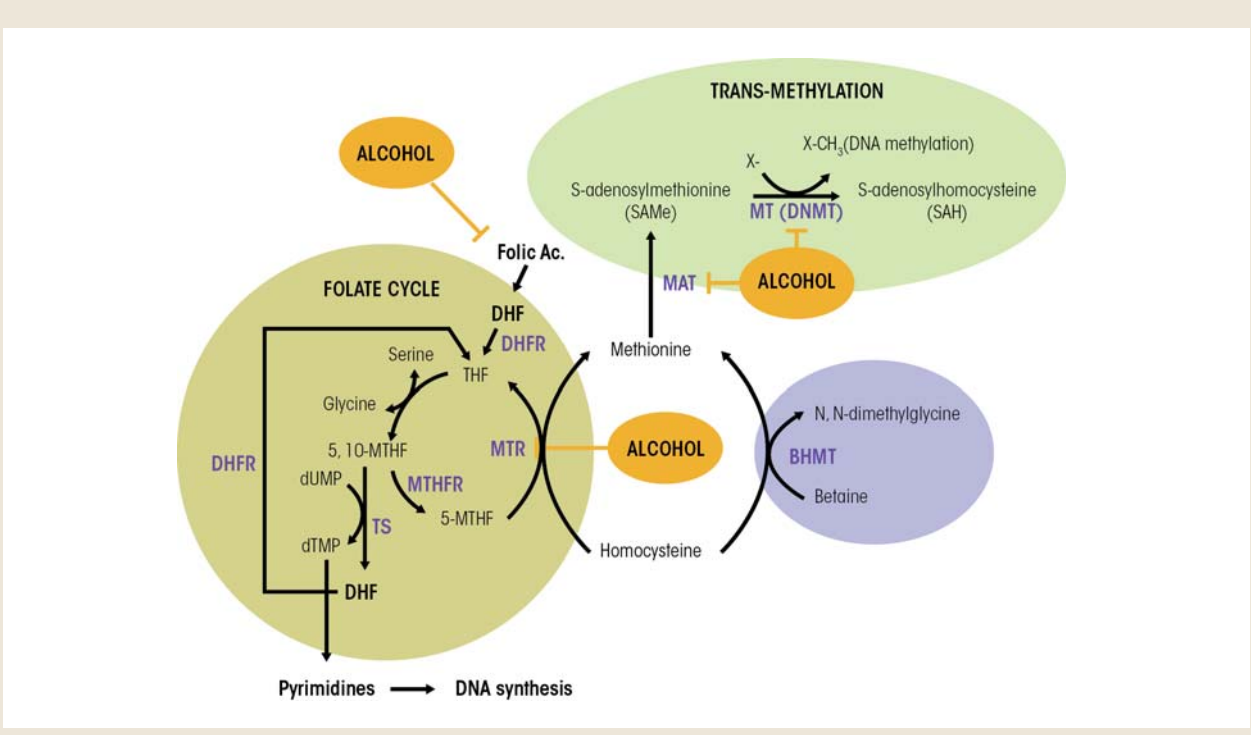


Figure 2 One-carbon metabolism with a schematic representation of the role of methionine in folate metabolism and transmethylation reactions and steps that are inhibited by alcohol. BHMT: betaine homocysteine methyltransferase; DHF: dihydrofolate; DHFR: dihydrofolate reductase; DNMT: DNA methyltransferase; dTMP: deoxythymidine monophosphate; dUMP: deoxyuridine monophosphate; Hcy: homocysteine; MAT: methionine adenosyl transferase; Met: methionine; MT: methyltransferase; 5-MTHF: 5-methyltetrahydrofolate; 5,10-MTHF: 5,10-methylenetetrahydrofolate; MTHFR: methylenetetrahydrofolate reductase; MTR: methionine synthase; SAH: S-adenosylhomocysteine; SAMe: S-adenosylmethionine; THF: tetrahydrofolate; TS: thymidylate synthase.

enzymes can impact the levels of various metabolites generated during these reactions. For some of these metabolites, altered levels in the body can be associated with either increased or decreased cancer susceptibility.

Polymorphisms in the Gene Encoding MTHFR. As mentioned above, MTHFR plays a central role in methionine formation by mediating the synthesis of 5-MTHF, which serves as a substrate for the remethylation of Hcy to methionine. Several polymorphisms in the *MTHFR* gene have been reported; two of these, known as the *C677T*[1] and *A1298C MTHFR* variants, in particular have been investigated in relation to cancer susceptibility. The *C677T* variant occurs in 8 to 15 percent of the population and results in a reduction of *MTHFR* activity. Thus, compared with people carrying two copies of the normal *MTHFR* gene (i.e., homozygous for *MTHFR C* [*CC*]), people carrying one normal gene copy and one variant gene copy (i.e., heterozygous with one *MTHFR C* and one *MTHFR T* [*CT*]) show a 45 percent reduction in *MTHFR* activity, and those homozygous for the *MTHFR T* variant (*TT*) show a 70 percent decrease of *MTHFR* activity (Motulsky 1996; Van der Put et al. 1995). Persons with the TT genotype have low levels of folate and vitamin B12 in the blood, as well as increased Hcy levels. Studies of the plasma folate and Hcy levels in people carrying one or two copies of the *A1298C MTHFR* polymorphism, however, have yielded inconsistent results (Sharp and Little 2004).

Polymorphisms in the Gene Encoding TS. TS catalyzes the conversion of dUMP into dTMP using 5,10-MTHF. Because dUMP can be misincorporated into new DNA strands during DNA replication, which can cause breaks in the double-stranded DNA molecules, the actions of TS can reduce the occurrence of DNA double-strand breaks. Two polymorphisms in the *TS* gene may be associated with an increased cancer risk:

- The presence of a repeated sequence of 28 nucleotides (i.e., a 28-bp tandem repeat) at the beginning of the *TS* gene (i.e., in the *TS* 5'-untranslated enhanced region); this variant is referred to as *TSER*;
- A 6-bp deletion/insertion at the end of the *TS* gene (i.e., in the *TS* 3'-untranslated region); this variant is known as *TS1494del6*.

Polymorphisms in the Gene Encoding MTR. MTR catalyzes the remethylation of Hcy to methionine; its activity is essential to maintain adequate folate pools. Most studies suggest that plasma Hcy levels are lower in carriers of a variant of the MTR gene called A2756G. To function properly, MTR must be maintained in an active form; this is achieved by another enzyme called MTR reductase (MTRR), for which polymorphisms also exist. For example, people homozygous for the A66G MTRR polymorphism had elevated levels of Hcy, indicating that the resulting MTRR enzyme led to higher-than-normal MTR activity (Van der Put et al. 2001).

Alcohol and DNA Methylation

As mentioned above, several pathogenetic mechanisms for alcohol-induced carcinogenesis have been described, including the effects of acetaldehyde and oxidative stress (Seitz and Stickel 2007). More recently, increasing evidence indicates that alcohol may induce epigenetic alterations, in particular aberrant DNA methylation patterns, which also could be important contributory factors to alcohol-induced carcinogenesis. For example, excessive alcohol use is associated with increased risk of colon cancer, which is characterized by global DNA hypomethylation as well as hypermethylation of certain genes (see below). Several mechanisms have been described to date that could contribute to these DNA methylation changes in cancer. These generally are associated with modulation of the pathways that regulate the availability of SAMe.

Alcohol and Lipotropes

The term lipotropes denotes compounds that help catalyze the breakdown of fat molecules in the body. Lipotropic nutrients (e.g., methionine, choline, folate, and betaine) are important dietary methyl donors and cofactors that play key roles in one-carbon metabolism. Dietary lipotropes influence the availability of SAMe and, consequently, may influence genomic DNA methylation patterns and the expression of multiple cancer-related genes. For example, methyl-deficient diets can induce the development of liver tumors (i.e., hepatocarcinogenesis) in rats by causing global and gene-specific hypomethylation (for a review, see Ross and Poirier 2002). Chronic alcoholics frequently suffer from malnutrition that results in depletion of lipotropes (Seitz and Stickel 2007). The lack of these nutrients in heavy drinkers could possibly result in an altered SAMe production, leading to changes in DNA methylation.

Similarly, malnutrition in alcoholics leads to a severe deficiency of other cofactors of one-carbon metabolism, such as folate (see below), vitamin B6, and vitamin B12. Long-term dietary intake of vitamin B6 is inversely correlated with the risk of developing colorectal cancer in women—that is, inadequate vitamin B6 intake increases the risk of this cancer. This effect is aggravated by chronic consumption of alcohol (Larsson et al. 2005).

Alcohol and Folate Status

Epidemiological studies have suggested that reduced folate levels in the body

[1]The name of the variant indicates that the amino acid cysteine (abbreviated as C), which normally is found at position 677 in the MTHFR molecule, is replaced by a threonine (abbreviated as T). The names of other gene variants can be interpreted similarly.

increase the risk of several types of cancer, including those of the upper aerodigestive tract, colon/rectum, and breast (Kim 2005). For example, heavy drinkers with low methionine and folate levels have a significantly increased relative risk (RR) for colorectal cancer compared with occasional drinkers with normal methionine and folate intake (Giovannucci et al. 1995). In chronic alcoholics, serum folate levels are significantly reduced compared with healthy subjects (Cravo et al. 1996), likely because folate absorption is reduced in these patients (Halsted et al. 1971). Polymorphisms of the *MTHFR* genes also lead to reduced folate levels, but their contribution to carcinogenesis is tissue-dependent and often contradictory. This is discussed in more detail below.

Two main mechanisms have been described that may explain the cancer-promoting effects of limited folate levels: increased DNA instability and aberrant DNA methylation patterns (Hamid 2012). Folate deficiency alters the balance of the pool of nucleotides needed for the synthesis of new DNA molecules, leading to dUMP accumulation. As a result, dUMP is misincorporated into new DNA molecules; this and the subsequent repair processes can lead to double-strand breaks in the DNA and chromosomal damage, ultimately resulting in cancer. The aberrant DNA methylation patterns associated with folate deficiency are the result of folate's role in one-carbon metabolism (see figure 2). As mentioned above, 5-MTHF can be used in the remethylation of Hcy to methionine, which in turn generates SAMe. Many studies have shown that folate deficiency reduces SAMe levels and the SAMe-to-SAH ratio as well as increases SAH concentrations (Kim 2005), all of which might contribute to carcinogenesis.

Alcohol and MTR Activity

Alcohol also can affect SAMe, SAH, and Hcy levels by reducing MTR activity, which in turn results in decreased SAMe levels and enhanced generation of Hcy and SAH (Barak et al. 1996) (see figure 2). To compensate for this decrease in MTR activity, the activity of BHMT is induced after alcohol ingestion. However, after extended periods of alcohol exposure this alternate pathway cannot be maintained. This results in a decrease in the hepatocyte level of SAMe, increases in SAH and Hcy levels, and a reduced SAMe-to-SAH ratio. These effects may contribute to the reduced hepatic SAMe levels observed in patients hospitalized for alcoholic hepatitis (Lee et al. 2004).

The major risk factors for developing hepatocellular carcinoma are viral infection, chronic alcoholism, and exposure to toxic substances called aflatoxins.

Alcohol and MAT Activity

As mentioned earlier, alcohol also contributes to the generation of oxidative stress through various mechanisms (for a review, see Seitz and Stickel 2007). Increased oxidative stress, in turn, can, at least in the liver, inactivate the MAT I/III enzymes that convert methionine to SAMe (see figure 2). This inactivation results from the covalent modification of a critical cysteine residue at position 121 by nitric oxide and hydroxyl radicals. MAT inhibition causes decreased SAMe levels, leading to reduced methylation reactions. The relevance of this pathway was demonstrated by findings that patients with alcoholic cirrhosis exhibit decreased hepatic MAT activity and SAMe formation (Lu and Mato 2008). In addition, patients with alcoholic hepatitis also show reduced expression of the *MAT1A* gene that encodes the MAT isoenzymes in normal liver, thereby contributing to lower hepatic SAMe levels (Lee et al. 2004).

Alcohol and DNMT Activity

Finally, both ethanol and its first breakdown product (i.e., acetaldehyde) can impact methylation patterns by altering DNMT activity. Thus, studies found that acetaldehyde can inhibit DNMT activity in vitro (Garro et al. 1991) and that alcohol reduced *DNMT* mRNA levels in rats treated with alcohol for 9 weeks (Bielawski et al. 2002). Similarly, studies in humans found that *DNMT3a* and *DNMT3b* mRNA levels were significantly reduced in patients with chronic alcoholism compared with healthy control subjects (Bonsch et al. 2006).

Alcohol, DNA Methylation, and Cancer

As described above, alcohol can interfere with one-carbon metabolism in several ways, thereby potentially generating aberrant DNA methylation patterns. The following sections review the currently available evidence indicating that alcohol-mediated changes in DNA methylation profiles contribute to the four main alcohol-associated cancers.

Liver Cancer

Liver cancer (i.e., hepatocellular carcinoma [HCC]) is a major cause of cancer-related death worldwide. The major risk factors for developing HCC are viral infection (i.e., with the hepatitis B or C viruses), chronic alcoholism, and exposure to toxic substances called aflatoxins. Alcohol remains the major cause of liver-related disease and deaths in the United States (Moghe et al. 2011). Alcohol-induced aberrant DNA methylation has been well characterized as a pathogenetic mechanism contributing to liver disease. Significant global DNA hypomethylation is associated with HCC with several etiologies, including chronic alcoholism, which may result in malignant transformation through mechanisms such as loss of imprinting and chromosomal instability, as described above (Calvisi

et al. 2007; Hernandez-Vargas et al. 2010). Similar to other types of cancer, global DNA hypomethylation in HCC is accompanied by greater-than-normal methylation levels (i.e., hypermethylation) at certain CpG sites. For example, a study analyzing 1,505 CpG sites in the promoter regions of 807 cancer-related genes in HCC tissues with different underlying causes, including chronic alcohol consumption, demonstrated an altered methylation pattern in 94 genes. Furthermore, for specific subsets of genes significant associations existed between methylation patterns and tumor progression (i.e., stage of the tumor and grade of differentiation) and background (i.e., cirrhotic versus non-cirrhotic surrounding tissue) (Hernandez-Vargas et al. 2010). Genes exhibiting hypermethylation included *RASSF1, APC,* and *CDKN2A,* all of which have important functions in the liver (Tischoff and Tannapfel 2008). A more recent study (Lampert et al. 2011) also detected aberrant hypermethylation of several genes, including *RASSF1, GSTP1, MGMT,* and *CHRNA3,* in alcohol-associated HCC. Finally, mRNA expression of RB1, an important cell cycle regulator, is decreased as a result of promoter methylation (Edamoto et al. 2003).

The link between alcohol and the mechanisms leading to aberrant methylation has been well elucidated in HCC. Chronic alcoholic patients have reduced hepatic MAT activity resulting from both decreased expression of the *MAT1A* gene and inactivation of the MAT I/III proteins. Reduced MAT activity, in turn, leads to decreased SAMe biosynthesis (Tsukamoto and Lu 2001), which may contribute to the severe loss of global DNA methylation in HCC. Similarly, micropigs fed ethanol show reduced hepatic MTR activity and SAMe/SAH ratio (Halsted et al. 1996) and rats fed ethanol exhibit decreased SAMe levels and global DNA hypomethylation.

Patients heterozygous or homozygous for the previously mentioned C677T *MTHFR* polymorphism have been shown to have a lower risk of developing alcohol-related HCC, but not of HCC with other etiologies (Saffroy et al. 2004). Similarly, people carrying variant alleles of both *MTHFR* and *TS* genes had a statistically significant reduced risk of developing HCC (Yuan et al. 2007). However, these findings sharply contradict another study showing that male patients with alcoholic cirrhosis who were homozygous for the *C677T MTHFR* polymorphism had an increased risk of developing HCC (Fabris et al. 2009). How can these conflicting reports be explained? In principle, the *C677T MTHFR* polymorphism has both protective and cancer-promoting effects. On the one hand, it can have a protective effect because it leads to an increase in the supply of 5,10-MTHF, which can serve as a substrate in the conversion of dUMP to dTMP. This would reduce misincorporation of dUMP into DNA, preventing DNA double-strand breaks and chromosomal instability. On the other hand, the reduced activity of the MTHFR enzyme encoded by the *C677T MTHFR* polymorphism would lead to lower levels of 5-MTHF that is used for the remethylation of Hcy to methionine, which in turn generates SAMe. Thus, the polymorphism would result in lower SAMe levels and SAMe/SAH ratio as well as increased SAH concentrations, which would then contribute to carcinogenesis.

The overall effects of the *C677T MTHFR* polymorphism can be determined when folate levels are taken into account. In situations of adequate folate supply, the levels of 5,10-MTHF increase, leading to a protective effect as described above, possibly counteracting the effects of reduced SAMe. In folate-deficiency situations, however, the levels of both 5,10-MTHF and SAMe would be reduced, leading to chromosomal instability that would be exacerbated by DNA hypomethylation induced by reduced SAMe. This model is consistent with a study in colon cancer showing that patients with the CC genotype have significantly reduced risk of cancer development in situations of adequate folate supply, but that this protection is absent in folate deficiency (Ma et al. 1997). It also can reconcile the contradictory findings of the two studies mentioned above. In the study by Saffron and colleagues (2004), folate levels were similar between healthy and alcoholic patients, thus favoring the protective role of the polymorphism. In the study by Fabris and colleagues (2009), conversely, increased risk of HCC in people with the polymorphisms was only found in males, and not in females. Although the investigators did not measure folate levels, it is plausible that these levels would be lower in men, who tend to be heavier and more regular drinkers compared with women.

Colorectal Cancer

Colorectal cancer is the third most common cancer and the second leading cause of cancer deaths for both sexes (Marin et al. 2012). Alcohol is a likely etiologic factor for this cancer (Baan et al. 2007). Several studies have indicated that epigenetic processes play a role in alcohol-related colorectal carcinogenesis. For example, rats chronically fed alcohol show genomic DNA hypomethylation but a normal pattern of methylation of the gene *TRP53,* which encodes a protein called p53, in the colonic mucosa (Choi et al. 1999). In a cohort of 609 patients, excessive alcohol use was associated with increased risk of colon cancer with global DNA hypomethylation (Schernhammer et al. 2010). In addition, people with low folate intake/high alcohol intake show a higher frequency of promoter methylation of genes involved in colorectal cancer carcinogenesis (e.g., *APC-1A, p14ARF, p16INK4a, hMLH1, O6-MGMT,* and *RASSF1A*) compared with people with high folate intake/low alcohol intake (Van Engeland et al. 2003). Many of these genes have fundamental roles in many cellular pathways, including DNA repair (e.g., *hMLH1, O6-MGMT*) and cell cycle

control (e.g., *p16INK4a*) (Portela and Esteller 2010).

The association between *MTHFR* polymorphisms and colon cancer has been studied extensively, and several factors have been found to influence the relation between the *MTHFR* variants and cancer risk (for a review, see Sharp and Little 2004). For example, one study (Iacopetta et al. 2009) has shown that colon cancer risk in homozygous carriers was dependent on the location of the cancer in the colon. The investigators observed that the TT genotype was associated with an increased risk for of cancer in colon regions closer to the small intestine (i.e., proximal colon cancer) (adjusted odds ratio (AOR) = 1.29) but with a decreased risk for cancer in colon regions closer to the rectum (i.e., distal cancers) (AOR = 0.87). More importantly, the increased risk for proximal cancers was especially pronounced in individuals with high alcohol consumption (AOR = 1.90). Microsatellite instability also has been related with colon cancer risk in people with the TT genotype (Shannon et al. 2002). Finally, the dietary intake of folate and alcohol also has been associated with colon cancer risk in people carrying the *MTHFR* C variant. Thus, Kim and colleagues (2012) observed that low folate intake together with high alcohol intake increased the risk of colon cancer in people with either the CC or the CT genotype.

A subset of colorectal cancers exhibit promoter methylation in multiple genes; these tumors are referred to as the CpG island methylator phenotype (CIMP) (Toyota et al. 1999). The frequency of these tumors depends on the location of the cancer. Thus, 30 to 40 percent of sporadic proximal colon cancers are CIMP+, compared with 3 to 12 percent of distal colon and rectal cancers (Curtin et al. 2011). The presence of the A1298C *MTHFR* polymorphism, interacting with diet, may be involved in the development of highly CpG-methylated colon cancers. Homozygous and heterozygous genotypes in conjunction with a high-risk dietary pattern (i.e., low folate and methionine intake and high alcohol use) were associated with CIMP+ phenotype (Curtin et al. 2007).

Breast Cancer

Breast cancer is the second leading cause of cancer death among women (DeSantis et al. 2011). Low doses of alcohol consumption (i.e., ≤1 drink/day) increase the risk of breast cancer by about 4 percent (Hamajima et al. 2002), whereas heavy alcohol consumption (i.e., ≥ 3 drink/day) is associated with an increase in risk of 40 to 50 percent (Pelucchi et al. 2011; Seitz et al. 2012). In addition, high frequency of alcohol consumption is associated with increased breast cancer mortality (Allemani et al. 2011). The role of epigenetic mechanisms in alcohol-related breast cancer also has been investigated. In a recent study of the methylation profiles of 1,413 CpG sites, Christensen and colleagues (2010) showed a strong trend toward decreased DNA methylation with increasing alcohol intake, and a trend toward increased methylation with increasing dietary folate. Other studies have shown altered methylation patterns for several genes associated with alcohol consumption, including hypermethylation of the *ER-a* (Zhu et al. 2003) and *E-cadherin* genes (Tao et al. 2011) and hypomethylation of *p16* (Tao et al. 2011).

As with other cancers, the person's genotype for the *C677T MTHFR* variant modulates the effect of alcohol consumption on breast cancer risk. Thus, women with the TT genotype are at a higher risk of breast cancer than those with other genotypes. In postmenopausal women, the breast cancer risk was increased in women with the *C677T MTHFR* variant who had high lifetime daily alcohol intake, suggesting that folate metabolism has an impact on cancer development (Platek et al. 2009). As mentioned earlier, chronic alcohol abuse can cause folate deficiency, which is a well-documented risk factor for breast cancer (Sellers et al. 2001). Why this risk is only observed in post-menopausal women is not clear, but the levels of estrogen in the woman's body may play a role. Alcohol can interfere with estrogen pathways and increase the levels of estrogen in the blood (Dumitrescu and Shields 2005). Higher estrogen exposure, in turn, can induce aberrant DNA methylation associated with breast carcinogenesis both in vivo and in vitro (Fernandez and Russo 2010). These observations suggest another possible mechanism of alcohol-induced carcinogenesis, at least in breast cancer.[2]

Tobacco and alcohol are the major risk factors of upper aerodigestive tract cancers, or head and neck cancers, including cancers of the oral cavity, pharynx, larynx, and esophagus.

Upper Aerodigestive Tract Cancer

Tobacco and alcohol are the major risk factors of upper aerodigestive tract cancers, or head and neck cancers, including cancers of the oral cavity, pharynx, larynx, and esophagus. Each year, about 125,000 new cases of upper aerodigestive tract cancers are diagnosed across Europe, and more than half of the patients die from the disease (Anantharaman et al. 2011).

Several studies have shown an association between alcohol and aberrant DNA methylation in head and neck cancers and that the degree of DNA

[2] A woman's risk of breast cancer depends at least in part on her overall exposure to estrogen across her life span. Thus, women who begin menstruating earlier and reach menopause later have a higher cumulative estrogen exposure and, consequently, breast cancer risk than women with a shorter "menstrual life." Similarly, postmenopausal alcoholic women have higher estrogen levels than women who are not alcoholic, increasing their overall estrogen exposure, which may in turn increase breast cancer risk.

hypomethylation is associated with alcohol use in these cancers. A study examining the DNA methylation profiles of 1,413 CpG loci from 773 genes in head and neck squamous cell carcinomas showed that significant associations existed between methylation profiles and alcohol consumption (Marsit et al. 2009). Other small-scale studies also have shown alcohol-associated promoter hypermethylation for several genes, including *E-cadherin* and *p16INKa* (Hasegawa et al. 2002), p15 (Chang et al. 2004), *MGMT* (Puri et al. 2005), *p14ARF* (Ishida et al. 2005), *SFRP1* (Marsit et al. 2006), and *Fusel 18* and *Septin 9* (Bennett et al. 2010). The genotype at the *C677T MTHFR* variant also plays a role in the risk of alcohol-related upper aerodigestive tract cancers. Thus, heavy-drinking individuals with the TT genotype have an increased risk of oral cancer compared with the CC genotype (Supic et al. 2011) but a decreased risk of esophageal cancer (Yang et al. 2005). Thus, the effect of the *MTHFR* polymorphism appears to differ substantially depending on the type of cancer.

Conclusions and Outlook

Aberrant DNA methylation is a hallmark of cancer development, and many studies have shown its contribution to tumor initiation and progression. In fact, methylation patterns nowadays are used as markers for cancer detection, tumor prognosis, and prediction of treatment responses. At the moment, evidence suggests that alcohol use is associated with aberrant DNA methylation patterns in several types of cancer. However, most studies have relied on looking at individual genes or a limited number of CpG loci. Genome-wide DNA methylation analyses may yield comprehensive maps of DNA methylation changes in alcohol-associated carcinogenesis, which could be important for use in pharmacoepigenetics, serving as additional markers for cancer detection, prognosis, and treatment response. Furthermore, despite all the progress that has been made in elucidating how alcohol consumption might lead to altered DNA methylation patterns, the molecular mechanisms that lead to these alterations have to be better characterized so that effective therapies could be devised. ■

Acknowledgements

This work is supported by NIH grants RO1–AT–1576, RO1–AT–004896, and R01–DK–51719 (to S.C.L., M.L.M-C., and J.M.M.); SAF 2011–29851 (to J.M.M); ETORTEK–2011, Sanidad Gobierno Vasco 2008, Educación Gobierno Vasco 2011 (PI2011/29), and FIS (PI11/01588) (to M.L.M.-C); FIS (PS09/ 00094), Fundación Científica de la Asociación Española Contra el Cáncer (Cancer Infantil), and the Program Ramón y Cajal, Spanish Ministry of Science and Innovation (to A.W.); and Sanidad Gobierno Vasco 2011 (to M.V.R.). CIBERehd is funded by the Instituto de Salud Carlos III.

Financial Disclosure

The authors declare that they have no competing financial interests.

References

Allemani, C.; Berrino, F.; Krogh, V.; et al. Do pre-diagnostic drinking habits influence breast cancer survival? *Tumori* 97(2):142–148, 2011. PMID: 21617706

Anantharaman, D.; Marron, M.; Lagiou, P.; et al. Population attributable risk of tobacco and alcohol for upper aerodigestive tract cancer. *Oral Oncology* 47(8):725–731, 2011. PMID: 21684805

Baan, R.; Straif, K.; Grosse, Y.; et al. Carcinogenicity of alcoholic beverages. *Lancet Oncology* 8(4):292–293, 2007. PMID: 17431955

Barak, A.J.; Beckenhauer, H.C.; and Tuma D.J. Betaine effects on hepatic methionine metabolism elicited by short-term ethanol feeding. *Alcohol* 13(5):483–486, 1996. PMID: 8888945

Bennett, K.L.; Lee, W.; Lamarre, E.; et al. HPV status-independent association of alcohol and tobacco exposure or prior radiation therapy with promoter methylation of FUSSEL18, EBF3, IRX1, and SEPT9, but not SLC5A8, in head and neck squamous cell carcinomas. *Genes, Chromosomes & Cancer* 49(4):319–326, 2010, PMID: 20029986

Bielawski, D.M.; Zaher, F.M.; Svinarich, D.M.; and Abel, E.L. Paternal alcohol exposure affects sperm cytosine methyltransferase messenger RNA levels. *Alcoholism: Clinical and Experimental Research* 26(3):347–351, 2002. PMID: 11923587

Boffetta, P.; Hashibe, M.; La Vecchia, C.; et al. The burden of cancer attributable to alcohol drinking. International *Journal of Cancer* 119(4):884–887, 2006. PMID: 16557583

Bönsch, D.; Lenz, B.; Fiszer, R.; et al. Lowered DNA methyltransferase (DNMT-3b) mRNA expression is associated with genomic DNA hypermethylation in patients with chronic alcoholism. *Journal of Neural Transmission* 113(9):1299–1304, 2006. PMID: 16463117

Calvisi, D.F.; Ladu, S.; Gorden, A.; et al. Mechanistic and prognostic significance of aberrant methylation in the molecular pathogenesis of human hepatocellular carcinoma. *Journal of Clinical Investigation* 117(9): 2713–2722. PMID: 17717605

Chang, H.W.; Ling, G.S.; Wei, W.I.; and Yuen, A.P. Smoking and drinking can induce p15 methylation in the upper aerodigestive tract of healthy individuals and patients with head and neck squamous cell carcinoma. *Cancer* 101(1):125–132, 2004, PMID: 15221997

Choi, S.W., and Friso, S. Interactions between folate and aging for carcinogenesis. *Clinical Chemistry and Laboratory Medicine* 43(10):1151–1157, 2005. PMID: 16197313

Choi, S.W.; Stickel, F.; Baik, H.W.; et al. Chronic alcohol consumption induces genomic but not p53-specific DNA hypomethylation in rat colon. *Journal of Nutrition* 129(11):1945–50, 1999. PMID: 10539767

Christensen, B.C.; Kelsey, K.T.; Zheng, S.; et al. Breast cancer DNA methylation profiles are associated with tumor size and alcohol and folate intake. *PLoS Genetics* 6(7):e1001043, 2010. PMID: 20686660

Cravo, M.L.; Glória, L.M.; Selhub, J.; et al. Hyperhomocysteinemia in chronic alcoholism: Correlation with folate, vitamin B-12, and vitamin B-6 status. *American Journal of Clinical Nutrition* 63(2): 220–224, 1996. PMID: 8561063

Curtin, K.; Slattery, M.L.; and Samowitz, W.S. CpG island methylation in colorectal cancer: Past, present and future. *Pathology Research International* 2011:902674, 2011. PMID: 21559209

Curtin, K.; Slattery, M.L.; Ulrich, C.M.; et al. Genetic polymorphisms in one-carbon metabolism: Associations with CpG island methylator phenotype (CIMP) in colon cancer and the modifying effects of diet. *Carcinogenesis* 28(8):1672–1679, 2007 PMID: 17449906

DeSantis, C.; Siegel, R.; Bandi, P.; and Jemal, A. Breast cancer statistics, 2011. CA: *A Cancer Journal for Clinicians* 61(6):409–418, 2011. PMID: 21969133

Dumitrescu, R.G., and Shields, P.G. The etiology of alcohol-induced breast cancer. *Alcohol* 35(3):213–25, 2005. PMID: 16054983.

Edamoto, Y.; Hara, A.; Biernat, W.; et al. Alterations of RB1, p53 and Wnt pathways in hepatocellular carcino-

mas associated with hepatitis C, hepatitis B and alcoholic liver cirrhosis. *International Journal of Cancer* 106(3):334–341, 2003. PMID: 12845670

Esteller, M. Epigenetics in cancer. *New England Journal of Medicine* 358(11):1148–1159, 2008. PMID: 18337604

Fabris, C.; Toniutto, P.; Falleti, E.; et al. MTHFR C677T polymorphism and risk of HCC in patients with liver cirrhosis: Role of male gender and alcohol consumption. *Alcoholism: Clinical and Experimental Research* 33(1):102–107, 2009. PMID: 18945219

Fernandez, S.V., and Russo, J. Estrogen and xenoestrogens in breast cancer. *Toxicologic Pathology* 38(1): 110–122, 2010. PMID: 19933552

Garro, A.J.; McBeth, D.L.; Lima, V.; and Lieber, C.S. Ethanol consumption inhibits fetal DNA methylation in mice: Implications for the fetal alcohol syndrome. *Alcoholism: Clinical and Experimental Research* 15(3):395–398, 1991. PMID: 1877725

Giovannucci, E.; Rimm, E.B.; Ascherio, A.; et al. Alcohol, low-methionine—low-folate diets, and risk of colon cancer in men. *Journal of the National Cancer Institute* 87(4):265–273, 1995. PMID: 7707417

Halsted, C.H.; Robles, E.A.; and Mezey, E. Decreased jejunal uptake of labeled folic acid (3H-PGA) in alcoholic patients: Roles of alcohol and nutrition. *New England Journal of Medicine* 285(13):701–706, 1971. PMID: 5571128

Halsted, C.H.; Villanueva, J.; Chandler, C.J.; et al. Ethanol feeding of micropigs alters methionine metabolism and increases hepatocellular apoptosis and proliferation. *Hepatology* 23(3):497–505.1996. PMID: 8617429

Hamajima, N.; Hirose, K.; Tajima, K.; et al. Alcohol, tobacco and breast cancer—Collaborative reanalysis of individual data from 53 epidemiological studies, including 58,515 women with breast cancer and 95,067 women without the disease. *British Journal of Cancer* 87(11):1234–1245, 2002. PMID: 12439712

Hamid, A. Folate malabsorption and its influence on DNA methylation during cancer development. *DNA and Cell Biology* April 2, 2012. [Epub ahead of print]. PMID: 22468673

Hasegawa, M.; Nelson, H.H.; Peters, E.; et al. Patterns of gene promoter methylation in squamous cell cancer of the head and neck. *Oncogene* 21(27):4231–4236, 2002. PMID: 12082610

Hernandez-Vargas, H.; Lambert, M.P.; Le Calvez-Kelm, F.; et al. Hepatocellular carcinoma displays distinct DNA methylation signatures with potential as clinical predictors. *PLoS One* 5(3):e9749, 2010. PMID: 20305825

Iacopetta, B.; Heyworth, J.; Girschik, J.; et al. The MTHFR C677T and DeltaDNMT3B C-149T polymorphisms confer different risks for right- and left-sided colorectal cancer. *International Journal of Cancer* 125(1):84–90, 2009. PMID: 19326430

Ishida, E.; Nakamura, M.; Ikuta, M.; et al. Promotor hypermethylation of p14ARF is a key alteration for progression of oral squamous cell carcinoma. *Oral Oncology* 41(6):614–622, 2005. PMID: 15975525

Kim, J.; Cho, Y.A.; Kim, D.H.; et al. Dietary intake of folate and alcohol, MTHFR C677T polymorphism, and colorectal cancer risk in Korea. *American Journal Clinical* Nutrition 95(2):405–412, 2012. PMID: 22218157

Kim, Y.I. Nutritional epigenetics: Impact of folate deficiency on DNA methylation and colon cancer susceptibility. *Journal of Nutrition* 135(11):2703–2709, 2005, PMID: 16251634

Kobor, M.S., and Weinberg, J. Epigenetics and fetal alcohol spectrum disorders. *Alcohol Research & Health* 34(1):29–37.

Lambert, M.P.; Paliwal, A.; Vaissière, T.; et al. Aberrant DNA methylation distinguishes hepatocellular carcinoma associated with HBV and HCV infection and alcohol intake. J*ournal of Hepatology* 54(4):705–715, 2011. PMID: 21146512

Larsson, S.C.; Giovannucci, E.; and Wolk, A. Vitamin B6 intake, alcohol consumption, and colorectal cancer: A longitudinal population-based cohort of women. *Gastroenterology* 128(7):1830–1837, 2005. PMID: 15940618

Lee, T.D.; Sadda, M.E.; Mendler, M.H.; et al. Abnormal hepatic methionine and glutathione metabolism in patients with alcoholic hepatitis. *Alcoholism: Clinical and Experimental Research* 28(1):173–181, 2004. PMID: 14745316

Lu, S.C. S-Adenosylmethionine. International *Journal of Biochemistry & Cell Biology* 32(4):391–395, 2000. PMID: 10762064

Lu, S.C., and Mato, J.M. S-Adenosylmethionine in cell growth, apoptosis and liver cancer. *Journal of Gastroenterology and Hepatology* 23(Suppl. 1):S73–S77, 2008. PMID: 18336669

Ma, J.; Stampfer, M.J.; Giovannucci, E.; et al. Methylenetetrahydrofolate reductase polymorphism, dietary interactions, and risk of colorectal cancer. *Cancer Research* 57(6):1098–1102, 1997. PMID: 9067278

Marin, J.J.; Sanchez de Medina, F.; Castaño, B.; et al. Chemoprevention, chemotherapy, and chemoresistance in colorectal cancer. *Drug Metabolism Reviews* 44(2): 148–172, 2012. PMID: 22497631

Marsit, C.J.; Christensen, B.C.; Houseman, E.A.; et al. Epigenetic profiling reveals etiologically distinct patterns of DNA methylation in head and neck squamous cell carcinoma. *Carcinogenesis* 30(3):416–422, 2009. PMID: 19126652

Marsit, C.J.; McClean, M.D.; Furniss, C.S.; and Kelsey, K.T. Epigenetic inactivation of the SFRP genes is associated with drinking, smoking and HPV in head and neck squamous cell carcinoma. *International Journal of Cancer* 119(8):1761–1766, 2006. PMID: 16708382

Mato, J.M.; Martínez-Chantar, M.L.; and Lu, S.C. Methionine metabolism and liver disease. *Annual Review of Nutrition* 28:273–293, 2008, PMID: 18331185

Moghe, A.; Joshi-Barve, S.; Ghare, S.; et al. Histone modifications and alcohol-induced liver disease: Are altered nutrients the missing link? *World Journal of Gastroenterology* 17(20):2465–2472, 2011. PMID: 21633651

Motulsky, A.G. Nutritional ecogenetics: Homocysteine-related arteriosclerotic vascular disease, neural tube defects, and folic acid. *American Journal Human Genetics* 58(1):17–20, 1996. PMID: 8554053

Pelucchi, C.; Tramacere, I.; Boffetta, P.; et al. Alcohol consumption and cancer risk. *Nutrition and Cancer* 63(7):983–990, 2011. PMID: 21864055

Platek, M.E.; Shields, P.G.; Marian, C.; et al. Alcohol consumption and genetic variation in methylenetetrahydrofolate reductase and 5-methyltetrahydrofolate-homocysteine methyltransferase in relation to breast cancer risk. *Cancer Epidemiology, Biomarkers & Prevention* 18(9):2453–2459, 2009. PMID: 19706843

Portela, A., and Esteller, M. Epigenetic modifications and human disease. *Nature Biotechnology* 28(10):1057–1068, 2010. PMID: 20944598

Puri, S.K.; Si, L.; Fan, C.Y.; and Hanna, E. Aberrant promoter hypermethylation of multiple genes in head and neck squamous cell carcinoma. *American Journal of Otolaryngology* 26(1):12–17, 2005. PMID: 15635575

Rehm, J.; Room, R.; Monteiro, M.; et al. Alcohol use. In: Ezzati, M.; Murray, C.; Lopez, A.D.; and Rodgers, A., Eds. *Comparative Quantification of Health Risks: Global and Regional Burden of Disease Attributable to Selected Major Risk Factors.* Geneva, Switzerland: World Health Organization, 2004, pp. 959–1108.

Rodríguez-Paredes, M., and Esteller M. Cancer epigenetics reaches mainstream oncology. *Nature Medicine* 17(3):330–339, 2011. PMID: 21386836

Ross, S.A., and Poirier, L. Proceedings of the Trans-HHS Workshop: Diet, DNA methylation processes and health. *Journal of Nutrition* 132(Suppl. 8):2329S–2332S, 2002. PMID: 12163686

Saffroy, R.; Pham, P.; Chiappini, F.; et al. The MTHFR 677C > T polymorphism is associated with an increased risk of hepatocellular carcinoma in patients with alcoholic cirrhosis. *Carcinogenesis* 25(8):1443–1448, 2004. PMID: 15033905

Schernhammer, E.S.; Giovannucci, E.; Kawasaki, T.; et al. Dietary folate, alcohol and B vitamins in relation to LINE-1 hypomethylation in colon cancer. *Gut* 59(6):794–799, 2010. PMID: 19828464

Seitz, H.K.; Pelucchi, C.; Bagnardi, V.; and La Vecchia, C. Epidemiology and pathophysiology of alcohol and breast cancer: Update 2012. *Alcohol and Alcoholism* 47(3):204–212, 2012. PMID: 22459019

Seitz, H.K., and Stickel, F. Molecular mechanisms of alcohol-mediated carcinogenesis. *Nature Reviews. Cancer* 7(8):599–612, 2007. PMID: 17646865

Sellers, T.A.; Kushi, L.H.; Cerhan, J.R.; et al. Dietary folate intake, alcohol, and risk of breast cancer in a prospective study of postmenopausal women. *Epidemiology* 12(4):420–428, 2001. PMID: 11416780

Shannon, B.; Gnanasampanthan, S.; Beilby, J.; and Iacopetta, B. A polymorphism in the methylenetetrahydrofolate reductase gene predisposes to colorectal cancers with microsatellite instability. *Gut* 50(4):520-524, 2002. PMID: 11889073

Sharp, L., and Little, J. Polymorphisms in genes involved in folate metabolism and colorectal neoplasia: A HuGE review. *American Journal of Epidemiology* 159(5):423–443, 2004. PMID: 14977639

Supic, G.; Jovic, N.; Kozomara, R.; et al. Interaction between the MTHFR C677T polymorphism and alcohol—Impact on oral cancer risk and multiple DNA methylation of tumor-related genes. *Journal of Dental Research* 90(1):65–70, 2011. PMID: 20940365

Tao, M.H.; Marian, C.; Shields, P.G.; et al. Alcohol consumption in relation to aberrant DNA methylation in breast tumors. *Alcohol* 45(7):689–699, 2011. PMID: 21168302

Tischoff, I., and Tannapfe, A. DNA methylation in hepatocellular carcinoma. *World Journal of Gastroenterology* 14(11):1741–1748, 2008. PMID: 18350605

Toyota, M., and Issa J.P. CpG island methylator phenotypes in aging and cancer. *Seminars in Cancer Biology* 9(5):349–357, 1999. PMID: 10547343

Tsukamoto, H., and Lu, S.C. Current concepts in the pathogenesis of alcoholic liver injury. *FASEB Journal* 15(8):1335–1349, 2001. PMID: 11387231

van der Put, N.M.; Steegers-Theunissen, R.P.; Frosst, P.; et al. Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida. *Lancet* 346(8982):1070–1071, 1995. PMID: 7564788

van der Put, N.M.; van Straaten, H.W.; Trijbels, F.J.; and Blom, H.J. Folate, homocysteine and neural tube defects: An overview. *Experimental Biology and Medicine* (Maywood) 226(4):243–270, 2001. PMID: 11368417

Van Engeland, M.; Weijenberg, M.P.; Roemen, G.M.; et al. Effects of dietary folate and alcohol intake on promoter methylation in sporadic colorectal cancer: The Netherlands cohort study on diet and cancer. *Cancer Research* 63(12):3133–3137, 2003. PMID: 12810640

Yang, C.X.; Matsuo, K.; Ito, H.; et al. Gene-environment interactions between alcohol drinking and the MTHFR C677T polymorphism impact on esophageal cancer risk: Results of a case-control study in Japan. *Carcinogenesis* 26(7):1285–1290, 2005. PMID: 15790587

Yuan, J.M.; Lu. S.C.; Van Den Berg, D.; et al. Genetic polymorphisms in the methylenetetrahydrofolate reductase and thymidylate synthase genes and risk of hepatocellular carcinoma. *Hepatology* 46(3):749–758, 2007. PMID: 17659576

Zhu, K.; Davidson, N.E.; Hunter, S.; et al. Methyl-group dietary intake and risk of breast cancer among African-American women: A case-control study by methylation status of the estrogen receptor alpha genes. *Cancer Causes & Control* 14(9):827–836, 2003. PMID: 14682440

Dysregulation of microRNA Expression and Function Contributes to the Etiology of Fetal Alcohol Spectrum Disorders

Sridevi Balaraman, Ph.D.; Joseph D. Tingling; Pai-Chi Tsai; and Rajesh C. Miranda, Ph.D.

Sridevi Balaraman, Ph.D., *is a postdoctoral research associate;* **Joseph D. Tingling,** *is a graduate research assistant;* **Pai-Chi Tsai,** *is a graduate research assistant; and* **Rajesh C. Miranda, Ph.D.,** *is a professor in the Department of Neuroscience and Experimental Therapeutics, Texas A&M Health Science Center, Bryan, Texas.*

MicroRNAs (miRNAs) are members of a large class of non–protein-coding RNA (ncRNA) molecules that represent a significant, but until recently unappreciated, layer of cellular regulation. Assessment of the generation and function of miRNAs suggests that these ncRNAs are vulnerable to interference from genetic, epigenetic, and environmental factors. A small but rapidly expanding body of studies using a variety of animal- and cell culture–based experimental models also has shown that miRNAs are important targets of alcohol during fetal development and that their dysregulation likely plays a significant role in the etiology of fetal alcohol spectrum disorders (FASD). Accordingly, an analysis of the regulation and function of these miRNAs may yield important clues to the management of FASD. **Key words: fetal alcohol spectrum disorders; fetal development; microRNAs; cellular regulation; genetic factors; epigenetic factors; environmental factors; animal models; cell culture studies**

MicroRNAs (miRNAs) are members of a vast, evolutionarily ancient, but poorly understood class of regulatory RNA molecules, termed non–protein-coding RNAs (ncRNAs). This means that in contrast to RNA molecules generated during gene expression (i.e., messenger RNA [mRNA] molecules), they are not used as templates for the synthesis of proteins. ncRNAs are encoded within the genomes of both eukaryotic and prokaryotic organisms and represent a novel layer of cell regulation and function that rivals the diversity and function of protein-coding mRNAs (for review, see Mattick 2007).

In recent years, researchers have investigated whether, and how, miRNAs interact with beverage alcohol (i.e., ethanol) and/or mediate its effects. Initial studies (Sathyan et al. 2007) explored the ethanol–miRNA interactions in fetal neural stem cells. Since then, increasing evidence has indicated that miRNAs play a role in the etiology of alcoholism (Pietrzykowski et al. 2008) and potentially alcohol withdrawal (Guo et al. 2011), as well as in ethanol's effects on brain development (Guo et al. 2011; Tal et al. 2012; Wang et al. 2009), brain damage associated with adult alcoholism (Lewohl et al. 2011), and liver damage (i.e., hepatotoxicity) (Dolganiuc et al. 2009; Tang et al. 2008). Other drugs of abuse such as nicotine are also known to influence miRNA expression (Huang 2009); furthermore, ethanol and nicotine collaborate to regulate the expression of miRNAs in neural tissues (Balaraman et al. 2012). These data collectively suggest that miRNAs are an important, but as yet poorly understood, component of alcoholism and ethanol-associated toxicology and damage to the developing fetus (i.e., teratology). This review specifically focuses on the association between miRNAs and the developmental effects of ethanol exposure, examining both the current data and future potential for research in this field of ncRNA biology to promote a coherent understanding of teratology associated with alcohol exposure.

Fetal Alcohol Spectrum Disorders

Maternal alcohol consumption during pregnancy can lead to a constellation of brain, face, cardiovascular, and skeletal defects of varying severity that collectively have been termed fetal alcohol spectrum disorders (FASD). At the extreme end of the spectrum of severity is fetal alcohol syndrome (FAS) (Clarren 1986), which is characterized by craniofacial abnormalities (e.g., small openings of the eyes, thin upper lip, flattened area above the upper lip),

motor dysfunction, impaired coordination of muscle movements (i.e., ataxia), behavioral disturbances, and cognitive deficits as well as growth retardation (Jones et al. 1973). According to the Centers for Disease Control and Prevention, the incidence of FAS is 1 to 3 per 1,000 live births, and these rates increase to 10 to 15 per 1,000 in at-risk groups, such as the foster care population (May and Gossage 2001). More recent estimates suggest that the prevalence of FASD in school-aged children in the United States is between 2 and 5 percent (May et al. 2009). FASD imposes significant socioeconomic costs on families and society. The lifetime cost of caring for a child with FASD was estimated at about $2 million, and the total annual cost of FASD in the United States was estimated at $4 billion in 2004 (Lupton et al. 2004); these costs may be significantly higher today.

Although the facial characteristics seen in patients with FASD are the most obvious signs of fetal alcohol exposure, the most devastating consequences of prenatal alcohol exposure are brain defects that result in cognitive, affective, and motor deficits (Sampson et al. 2000). Therefore, understanding the diverse effects of alcohol on the developing brain during pregnancy may provide researchers with the key to developing therapies for managing both fetal and adult effects of alcohol exposure during pregnancy. This review focuses on an emerging body of data from animal and cell-culture studies that implicates miRNA dysregulation in the etiology of FASD.

Focus on miRNAs

miRNAs are a class of ncRNAs that posttranscriptionally regulate the expression of protein-coding genes. When protein-coding genes are expressed (i.e., the encoded protein is produced), first an mRNA copy of the corresponding DNA sequence is generated in a process called transcription. This mRNA molecule consists of three parts: a noncoding start region (i.e., the 5'-end), the sequence actually containing the information for the encoded protein (i.e., the open reading frame), and a noncoding tail region (i.e., the 3'-end). miRNAs mainly act by binding to the 3'-untranslated region of their mRNA targets (Ambros et al. 2003; Bartel 2004; Ghildiyal and Zamore 2009), although that is not the only function attributable to these molecules. Many microRNAs are evolutionally conserved across species. They initially were discovered in the roundworm *Caenorhabditis elegans* (Lee et al. 1993), but since then they also have been found in plants, invertebrates, mammals, and humans (Bartel 2009). miRNAs play crucial roles in development, stem-cell self-renewal, programmed cell death (i.e., apoptosis), and cell-cycle regulation but also feature prominently in human disease, including cancers and neurodegenerative and metabolic diseases (Ambros 2004; Bartel 2004). miRNAs are abundant in the central nervous system (CNS) (Krichevsky et al. 2003; Vreugdenhil 2010), and brain miRNAs are crucial for regulating nerve cell generation (i.e., neurogenesis); neuronal degeneration; and maintaining normal neuronal functions associated with memory formation, neuronal differentiation, and synaptic plasticity (Li 2010; Schratt 2009; Smalheiser 2009).

miRNA Biogenesis

miRNAs are encoded within the genome either as independent genes or in gene clusters; however, they also can be encoded within introns[1] of protein-coding genes, or even within introns and exons of another type of ncRNA called long intergenic non-(protein)-coding RNAs (lincRNAs). The generation of mature miRNAs from these coding sequences is a multistep process, as follows (see figure 1A):

- A normal transcription process, which is mediated by an enzyme called RNA-polymerase II, generates a longer primary transcript termed pri-miRNA. Like mRNA, the pri-miRNA transcripts can have certain modifications at their ends (i.e., a "cap" at the 5'-end and multiple adenosine units [i.e., a poly-A tail] at the 3'-end) and can be spliced (Cai et al. 2004). Furthermore, the pri-miRNAs typically are folded into a double-stranded, hairpin–loop structure several hundred base pairs in length.

- Most pri-miRNA transcripts are processed within the nucleus by a protein complex called the DiGeorge syndrome critical region-8 (Drosha/DGCR8) "microprocessor" complex to generate stem-loop structures termed pre-miRNAs that are approximately 70 nucleotides in length (Han et al. 2006; Lee et al. 2003).

- The pre-miRNAs are moved from the nucleus to the cytoplasm by a chaperone protein called exportin-5 (Bohnsack et al. 2004).

- Within the cytoplasm, a protein complex known as Dicer enzyme further processes pre-miRNAs into mature double-stranded miRNA molecules (Hutvagner et al. 2001; Zhang et al. 2002). This process, and thus miRNA formation in general, is crucial for embryonic development because mutations in the Dicer proteins, which are exclusively part of the miRNA processing machinery, cause death of the embryo (Bernstein et al. 2003).

- Once the Dicer complex is cut off to release the mature miRNA, one strand of the double-stranded molecule, termed the guide strand miRNA, preferentially attaches to another protein complex called

[1] Genes encoding specific proteins typically comprise not only the DNA sequences that contain the building instructions for the resulting protein, but also noncoding sequences (i.e., introns). These introns are interspersed with the coding sequences (i.e., exons). During transcription, first an RNA copy of the entire gene, including introns and exons, is generated. This transcript is then processed by cutting out the intron sequences, to generate the final mRNA molecule. This process is known as splicing.

RNA-induced silencing complex (RISC). This results in a microribonucleoprotein (miRNP) complex that can either destabilize mRNA transcripts or repress the next step of gene expression in protein-coding genes (i.e., translation) (Mourelatos et al. 2002; Williams 2008). The second, complementary strand, known as passenger strand or miRNA* (see figure 2) has been thought to be quickly degraded (Matranga et al. 2005). However, as discussed later in this article, recent studies indicate that passenger-strand miRNAs can be retained by cells and exhibit independent biological functions.

- Finally, the mature miRNA can be degraded by an enzyme called 5'-3' exoribonuclease (XRN2) (Bail et al. 2010).

Role of miRNAs in Ethanol's Teratologic Effects

In 2007, Sathyan and colleagues (2007) showed for the first time that miRNAs could mediate the effects of ethanol or indeed other teratogens. Using isolated tissue from the nervous system (i.e., neuroepithelium) of second-trimester fetuses, the investigators demonstrated that ethanol suppressed the expression of four miRNAs—miR-9, miR-21, miR-153, and miR-335—in fetal neural stem cells (NSCs) and neural progenitor cells (NPCs). The simultaneous suppression of miR-21 and miR-335 accounted for earlier observations (Prock and Miranda 2007; Santillano et al. 2005) that ethanol-exposed NSCs/NPCs are resistant to apoptosis, whereas the suppression of miR-335 explained the increase in NSC/NPC proliferation. Three of the four suppressed miRNAs target the mRNAs for two proteins called Jagged-1 and ELAVL2/HuB;[2] accordingly, by suppressing the miRNAs, ethanol induced the expression of both target mRNAs. ELAVL2/HuB overexpression promotes neuronal differentiation (Akamatsu et al. 1999), and Jagged-1–induced proliferation establishes neuronal identity (Salero and Hatten 2007). These data collectively suggest that by interfering with miRNA function, ethanol may deplete the fetal pool of NSCs/NPCs and promote premature neuronal differentiation. More recently, Tal and colleagues (2012), using a zebrafish model, also showed that ethanol exposure during embryonic development suppressed the expression of miR-9 and miR-153. Importantly, these investigators demonstrated both behavioral and anatomical consequences of miRNA depletion. In particular, miR-153 depletion resulted in significantly increased locomotor activity in juvenile zebrafish, reminiscent of increased hyperactivity observed in children with FASD.

Other developmental ethanol exposure models also have indicated that ethanol alters the expression of several miRNAs. For example, Wang and colleagues (2009) showed that ethanol exposure during a period bracketing the end of the first trimester to the middle of the second trimester resulted in altered miRNA expression in brain tissue sampled near the end of the second trimester. In that study, ethanol induced a significant increase in the expression of two miRNAs (i.e., miR-10a and miR-10b), resulting in down-regulated expression of a protein called Hoxa1 in fetal brains. Other analyses had indicated that loss of Hoxa1 function (e.g., from familial Hoxa1 mutations) is associated with a variety of cranial defects and mental retardation (Bosley et al. 2007). This suggests that by suppressing translation of Hoxa1 and related genes, ethanol-mediated induction of miR10a/b may lead to similar defects.

Although the miRNAs identified by Wang and colleagues (2009) do not overlap with those identified by Sathyan and colleagues (2007) in NSCs/NPCs, miR-10a/b upregulation may have similar consequences for premature NSC differentiation. For example, miR-10a/b promotes the differentiation of cells from a type of nerve cell tumor (i.e., neuroblastoma cells) by suppressing translation of a protein called nuclear receptor corepressor-2 (NCOR2) (Foley et al. 2011). This effect is similar to the induction of Elavl2/HuB and Jagged-1.

Finally, Guo and colleagues (2011) assessed the effects of chronic intermittent ethanol exposure on cultured neuronal cells obtained from mouse cerebral cortex at gestational day 15, which is equivalent to the middle of the second trimester. The investigators found that ethanol induced several miRNAs in these cells. Interestingly, a prolonged period of withdrawal following the ethanol exposure resulted in a more than fourfold increase in the number of significantly regulated miRNAs, suggesting that withdrawal itself also may have a significant damaging effect on neuronal maturation in the developing fetal brain. Although these data were obtained from a cell-culture model, the implications of maternal binge drinking–withdrawal cycles on fetal miRNAs and their control over neural differentiation need further investigation.

Effects of Coexposure to Ethanol and Other Drugs on miRNA Levels

Pregnant women who abuse ethanol also are likely to coabuse other drugs, such as nicotine (Substance Abuse and Mental Health Administration 2009). These other drugs also can affect miRNA levels. For example, Huang and colleagues (2009) demonstrated that nicotine induced expression of miR140* in a developmental model using the rat PC12 cell line. These effects may enhance or oppose those of ethanol. Thus, a recent study showed that ethanol and nicotine behaved as functional antagonists—that is, miRNAs that were suppressed by ethanol in fetal NSCs/NPCs were induced by nicotine exposure (Balaraman et al. 2012). Moreover, nicotine prevented the ethanol-mediated decrease in these miRNAs; this effect was pharmacologically mediated

[2] Jagged-1 binds to (i.e., is a ligand of) Notch receptor and ELAVL2/HuB is a neuron-specific RNA-binding protein.

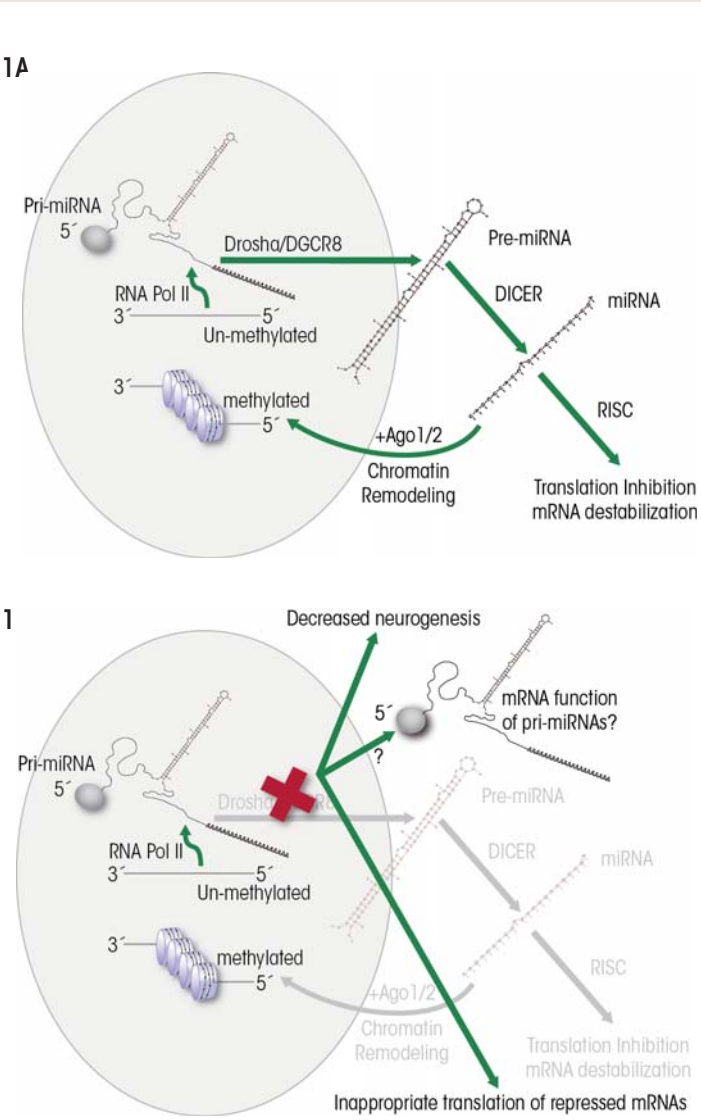


Figure 1 Models for standard (i.e., canonical) and disturbed (i.e., noncanonical) modes of miRNA biogenesis and function. **(A)** miRNAs often are generated (i.e., transcribed) from miRNA genes, as long mRNA-like transcripts, with a "cap" at the start (i.e., 5′-end) and several adenosine units at the end (i.e., 3′- polyA tail). The initial primary miRNA transcripts (pri-miRNAs) are processed to shorter, hairpin-shaped premature miRNAs (pre-miRNAs) by a protein complex called the DiGeorge syndrome critical region-8 (Drosha/DGCR8) complex. The pre-miRNAs then are transported to the cytoplasm for final processing into mature miRNAs by the Dicer complex. Mature miRNAs attain their function by being integrated into RNA-induced silencing complexes (RISC) that can degrade target mRNAs or silence translation. Processed miRNAs also can relocate to the nucleus to influence chromatin remodeling. **(B)** Disturbances in Drosha/DGCR8 processing (e.g., because of a mutation in the genes encoding these enzymes) may reveal alternate, mRNA-like functions of unprocessed pri-miRNAs and result in disrupted stem cell maturation.

by a certain type of nicotine receptor (i.e., the nicotinic acetylcholine receptor). There is little generalized evidence as yet that drugs of abuse interact at the level of miRNAs to regulate cell function. Nevertheless these findings suggest that such an interaction is a real possibility, and the consequences for the teratologic effects of the drugs are likely to be significant.

Teratogenic Implications of Altered miRNA Biogenesis, Cellular Localization, and Function

The data cited above show that ethanol alters the expression of several miRNAs at different developmental stages and that these alterations have consequences for fetal neural development and behavior. miRNA dysregulation is likely to influence teratogenesis by destabilizing the mRNAs of individual genes or gene networks. However, emerging evidence indicates that miRNA function also can be altered at several stages in the miRNA biogenesis pathway. Although to date such alterations are poorly understood, they may have important implications for teratology. The following represent four intriguing possibilities.

First, the presence of a 5' cap and a 3'-polyA tail indicates that primary miRNA transcripts may have characteristics and function like regular mRNAs, and indeed evidence has been found for such a role (Cai et al. 2004). Although the conditions that permit the appearance of mRNA-like functionality are unclear, it is likely that interference with Dicer/DGCR8, which is essential for miRNA processing, can lead to the emergence of alternate functionality associated with pri-miRNA transcripts (see figure 1B). In this context, it is interesting to note that disruption of the DGCR8 locus is associated with mental retardation and that DGCR8 deletion interferes with the maturation of embryonic stem cells, causing them to aberrantly retain their ability to differentiate into differ-

ent cell types (i.e., their pluripotency) while initiating differentiation (Wang et al. 2007). In this instance, the biology of stem cells seems to be intimately linked with the development of normal brain function.

Second, until recently, the complementary miRNA* strands (Hutvagner et al. 2001) were thought to be quickly degraded following Dicer cleavage of the double-stranded pre-miRNA molecule (Matranga et al. 2005). However, recent evidence (Ghildiyal et al. 2010; Okamura et al. 2009; Tyler et al. 2008) shows that these passenger strands also can be functional, acting on their own binding sites and regulating expression of their own sets of targets (figure 2). Thus, both strands of a pre-miRNA can be functional, each with a specific set of targets. The ratio of functional guide versus passenger strand miRNAs is regulated by an as-yet-unknown biology. Guo and colleagues (2011) identified several ethanol-sensitive miRNA* species that mainly were induced following ethanol exposure. Furthermore, other drugs of abuse, such as nicotine, also have been shown to induce the expression of a miRNA* (i.e., miR140*) (Huang and Li 2009). Alterations in the miRNA-to-miRNA*s ratio are likely to yield alternate biological outcomes that are particularly relevant to teratogenesis, as has been demonstrated in an analysis of the established ethanol-sensitive miRNA, miR-9. Tal and colleagues (2012) showed in their developmental zebrafish model that in addition to decreasing miR-9 (which now is also called miR-9-5p [www.mirbase.org, miRBAse Release 19]), ethanol produced a more modest decrease in the expression of miR-9* (now called miR-9-3p). The ratio of miR-9 to miR-9* is important for development and teratogenesis because these two miRNAs work together to regulate two molecules controlling neuronal differentiation. Thus, miR-9 levels influence the levels of a neuronal differentiation inhibitor called RE1 silencing transcription factor/neuron-restrictive silencer factor (REST), whereas miR-9* regulates its cofactor, coREST (Packer et al. 2008). Therefore, the simultaneous suppression of miR-9 and miR-9* may be expected to result in derepression of the REST/coREST complex and, consequently, inhibition of neuronal differentiation. On the other hand, preferential suppression of either miR-9 or miR-9* would be predicted to alter the ratio of REST to coREST, which has important and complex consequences for neural stem-cell renewal and altered lineage specification (Abrajano et al. 2009, 2010). Clearly, the involvement of passenger-strand miRNA biology in teratogenesis needs further investigation.

Third, in humans, about 6 percent of mature miRNAs undergo editing by the enzyme adenosine deaminase (Blow et al. 2006), resulting in alterations in either miRNA processing or miRNA efficiency (Kawahara et al. 2007; Yang et al. 2005). Furthermore, evidence suggests that edited miRNAs may exhibit different target specificity compared with their nonedited counterparts (Ekdahl et al. 2012). miRNA editing increases during brain development and may permit the emergence of new biological functions (e.g., a novel translational control of the development of nerve cell extensions [i.e., dendritogenesis]) (Ekdahl et al. 2012). These data collectively suggest that the role of miRNA editing in ethanol teratology warrants further exploration.

Finally, emerging evidence indicates that some mature miRNAs are transported back to the nucleus, where they mediate the formation of heterochromatin[3] (Gonzalez et al. 2008). This observation suggests that miRNAs can directly influence the epigenetic landscape (figure 1A). Ethanol also alters

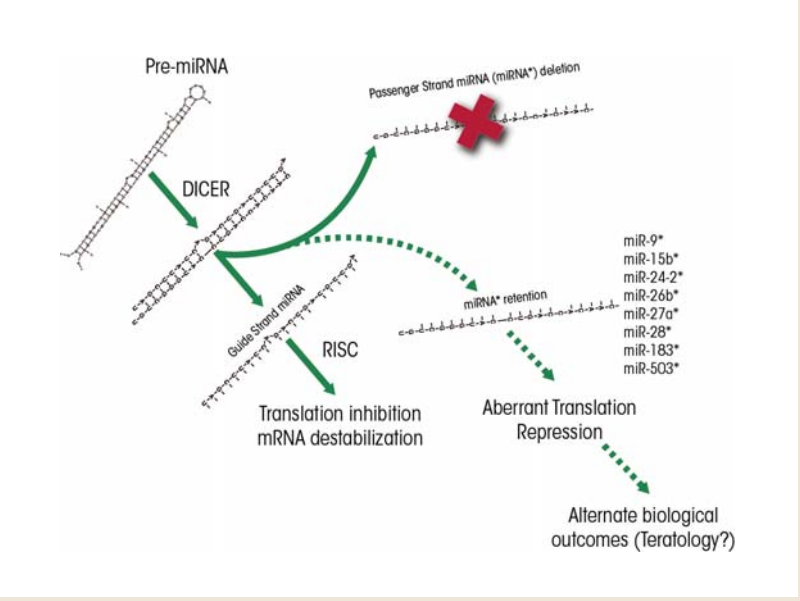


Figure 2 Model for the activity of the two strands of the processed pre-miRNA molecules (i.e., the guide strand [miRNA] and passenger strand [miRNA*]). Dicer processing of pre-miRNAs typically results in the formation of a guide stand miRNA that binds to the RNA-induced silencing complex (RISC). This guide strand can be derived from either the 3′- (termed –3p) or 5′- (termed –5p) end of the pre-miRNA. The complementary passenger strand typically is degraded. However, under various conditions, including ethanol exposure, miRNA* strands may be retained or otherwise differentially regulated, resulting the emergence of alternate biological end points.

SOURCE: Guo et al. 2012, Acer 2012, Tal et al. 2012.

the epigenetic landscape in differentiating fetal NSCs (Zhou et al. 2011), and the contributory role of nuclear miRNAs to this process is unknown. All of these modifications to miRNA biology represent novel and uninvestigated layers of regulatory processes that may have important consequences for cell and tissue differentiation and, consequently, teratogenesis.

Implications for the Management of FASD

Despite strong evidence that maternal alcohol consumption during pregnancy leads to harmful effects on the fetus, a significant number of women continue to report drinking even into the third trimester of pregnancy. Therefore, early detection and management of fetal alcohol exposure remains an urgent public health concern, as does the development of approaches to ameliorate or prevent ethanol's detrimental effects. The identification of miRNAs as ethanol targets presents one hope for the development of novel therapeutic programs. miRNAs have coevolved with their mRNA targets to orchestrate development. It is possible that miRNA-like drugs may be used to mitigate the effects of fetal ethanol exposure on the development of specific organs. The challenge will be to identify tissue-specific miRNAs that can be used to reprogram development. In this context, miRNAs such as miR-9 make intriguing therapeutic targets because they are fairly specific to neuronal cells (Leucht et al. 2008; Shibata et al. 2011; Smirnova et al. 2005). Evidence that ethanol-sensitive miRNAs also are sensitive to nicotine (Balaraman et al. 2012) suggests a promising and alternative, pharmacological approach to reprogramming fetal development following maternal ethanol exposure. Recent evidence suggests that pharmacologic approaches can indeed be used successfully in human populations, for example, to normalize cellular miRNA levels in neurological diseases such as multiple sclerosis (Waschbisch et al. 2011). Such an approach therefore may be similarly efficacious with FASD. Finally, Guo and colleagues (2011) have implicated DNA methylation as a mechanism for miRNA regulation, and Wang and colleagues (2009) demonstrated that folic acid administration could reverse ethanol's effects on miRNAs. These data suggest that nutritional supplementation programs also may be an effective means towards ameliorating the effects of miRNA dysregulation. Research into miRNA involvement in fetal alcohol teratology is in its infancy. However, this research has significant potential for both uncovering principles underlying alcohol's detrimental consequences and for developing novel strategies for the management of fetal alcohol effects. ■

[3] Heterochromatin is a tightly packed complex of DNA and proteins in the cell nucleus in which the DNA is not easily accessible to other proteins and therefore generally is not expressed. In contrast, a more lightly packed DNA–protein complex (i.e., euchromatin) is more easily accessible for transcription enzymes.

Acknowledgements

The preparation of this manuscript and some of the research reported here was supported by a grant from NIAAA (R01–AA–013440) to Dr. Miranda.

Financial Disclosure

The authors declare that they have no competing financial interests.

References

Abrajano, J.J.; Qureshi, I.A.; Gokhan, S. et al. Corepressor for element-1-silencing transcription factor preferentially mediates gene networks underlying neural stem cell fate decisions. *Proceedings of the National Academy of Sciences of the United States of America* 107(38): 16685–16690, 2010. PMID: 20823235

Abrajano, J.J.; Qureshi, I.A.; Gokhan, S.; et al. REST and CoREST modulate neuronal subtype specification, maturation and maintenance. *PLoS One* 4(12):e7936, 2009. PMID: 19997604

Akamatsu, W.; Okano, H.J.; Osumi, N.; et al. Mammalian ELAV-like neuronal RNA-binding proteins HuB and HuC promote neuronal development in both the central and the peripheral nervous systems. *Proceedings of the National Academy of Sciences of the United States of America* 96(17):9885–9890, 1999. PMID: 10449789

Ambros, V. The functions of animal microRNAs. *Nature* 431(7006):350–355, 2004. PMID: 15372042

Ambros, V.; Lee, R.C.; Lavanway, A.; et al. MicroRNAs and other tiny endogenous RNAs in C. elegans. *Current Biology* 13(10):807–818, 2003. PMID: 12747828

Bail, S.; Swerdel, M.; Liu, H.; et al. Differential regulation of microRNA stability. *RNA* 16(5):1032–1039, 2010. PMID: 20348442

Balaraman, S.; Winzer-Serhan, U.H.; and Miranda, R.C. Opposing actions of ethanol and nicotine on microRNAs are mediated by nicotinic acetylcholine receptors in fetal cerebral cortical-derived neural progenitor cells. *Alcoholism: Clinical and Experimental Research,* 36(10):1669-1677, 2012. PMID: 22458409

Bartel, D.P. MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* 116(2):281–297, 2004. PMID: 14744438

Bartel, D.P. MicroRNAs: Target recognition and regulatory functions. *Cell* 136(2):215–233, 2009. PMID: 19167326

Bernstein, E.; Kim, S.Y.; Carmell, M.A.; et al. Dicer is essential for mouse development. *Nature Genetics* 35(3):215–217, 2003. PMID: 14528307

Blow, M.J.; Grocock, R.J.; van Dongen, S.; et al. RNA editing of human microRNAs. *Genome Biology* 7(4):R27, 2006. PMID: 16594986

Bohnsack, M.T.; Czaplinski, K.; and Gorlich, D. Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *RNA* 10(2):185–191, 2004. PMID: 14730017

Bosley, T.M.; Salih, M.A.; Alorainy, I.A.; et al. Clinical characterization of the HOXA1 syndrome BSAS variant. *Neurology* 69(12):1245–1253, 2007. PMID: 17875913

Cai, X.; Hagedorn, C.H.; and Cullen, B.R. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *RNA* 10(12):1957–1966, 2004. PMID: 15525708

Clarren, S.K. Neuropathology in fetal alcohol syndrome. In: West, J.R., ed. *Alcohol and Brain Development.* New York: Oxford University Press, 1986, pp. 158–166.

Dolganiuc, A.; Petrasek, J.; Kodys, K.; et al. MicroRNA expression profile in Lieber-DeCarli diet-induced alcoholic and methionine choline deficient diet-induced nonalcoholic steatohepatitis models in mice. *Alcoholism: Clinical and Experimental Research* 33(10):1704–1710, 2009. PMID: 19572984

Ekdahl, Y.; Farahani, H.S.; Behm, M.; et al. A-to-I editing of microRNAs in the mammalian brain increases during development. *Genome Research* 22(8):1477–1487, 2012. PMID: 22645261

Foley, N.H.; Bray, I.; Watters, K.M.; et al. MicroRNAs 10a and 10b are potent inducers of neuroblastoma cell differentiation through targeting of nuclear receptor corepressor 2. *Cell Death and Differentiation* 18(7):1089–1098, 2011. PMID: 21212796

Ghildiyal, M.; Xu, J.; Seitz, H.; et al. Sorting of Drosophila small silencing RNAs partitions microRNA* strands into the RNA interference pathway. *RNA* 16(1):43–56, 2010. PMID: 19917635

Ghildiyal, M., and Zamore, P.D. Small silencing RNAs: An expanding universe. *Nature Reviews. Genetics* 10(2): 94–108, 2009. PMID: 19148191

Gonzalez, S.; Pisano, D.G.; and Serrano, M. Mechanistic principles of chromatin remodeling guided by siRNAs and miRNAs. *Cell Cycle* 7(16):2601–2608, 2008. PMID: 18719372

Guo, Y.; Chen, Y.; Carreon, S.; and Qiang, M. Chronic intermittent ethanol exposure and its removal induce a different miRNA expression pattern in primary cortical neuronal cultures. *Alcoholism: Clinical and Experimental Research* 36(6):1058-1066, 2012. PMID: 22141737

Han, J.; Lee, Y.; Yeom, K.H.; et al. Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. *Cell* 125(5):887–901, 2006. PMID: 16751099

Huang, W., and Li, M.D. Nicotine modulates expression of miR-140*, which targets the 3'-untranslated region of dynamin 1 gene (Dnm1). *International Journal of Neuropsychopharmacology* 12(4):537–546, 2009. PMID: 18845019

Hutvagner, G.; McLachlan, J.; Pasquinelli, A.E.; et al. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* 293(5531):834–838, 2001. PMID: 11452083

Jones, K.L.; Smith, D.W.; Ulleland, C.N.; and Streissguth, P. Pattern of malformation in offspring of chronic alcoholic mothers. *Lancet* 1(7815):1267–1271, 1973. PMID: 4126070

Kawahara, Y.; Zinshteyn, B.; Chendrimada, T.P.; et al. RNA editing of the microRNA-151 precursor blocks cleavage by the Dicer-TRBP complex. *EMBO Reports* 8(8):763–769, 2007. PMID: 17599088

Krichevsky, A.M.; King, K.S.; Donahue, C.P.; et al. A microRNA array reveals extensive regulation of microRNAs during brain development. *RNA* 9(10):1274–1281, 2003. PMID: 13130141

Lee, R.C.; Feinbaum, R.L.; and Ambros, V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 75(5):843–854, 1993. PMID: 8252621

Lee, Y.; Ahn, C.; Han, J.; et al. The nuclear RNase III Drosha initiates microRNA processing. *Nature* 425(6956):415–419, 2003. PMID: 14508493

Leucht, C.; Stigloher, C.; Wizenmann, A.; et al. MicroRNA-9 directs late organizer activity of the midbrain-hindbrain boundary. *Nature Neuroscience* 11(6):641–648, 2008. PMID: 18454145

Lewohl, J.M.; Nunez, Y.O.; Dodd, P.R.; et al. Up-regulation of microRNAs in brain of human alcoholics. *Alcoholism: Clinical and Experimental Research* 35(11):1928–1937, 2011. PMID: 21651580

Li, X., and Jin, P. Roles of small regulatory RNAs in determining neuronal identity. *Nature Reviews. Neuroscience* 11(5):329–338, 2010. PMID: 20354535

Lupton, C.; Burd, L.; and Harwood, R. Cost of fetal alcohol spectrum disorders. *American Journal of Medical Genetics. Part C: Seminars in Medical Genetics* 127C(1):42–50, 2004. PMID: 15095471

Matranga, C.; Tomari, Y.; Shin, C.; et al. Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell* 123(4):607–620, 2005. PMID: 16271386

Mattick, J.S. A new paradigm for developmental biology. *Journal of Experimental Biology* 210(Pt 9):1526–1547, 2007. PMID: 17449818

May, P.A., and Gossage, J.P. Estimating the prevalence of fetal alcohol syndrome. A summary. *Alcohol Research & Health* 25(3):159–167, 2001. PMID: 11810953

May, P.A.; Gossage, J.P.; Kalberg, W.O.; et al. Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies. *Developmental Disability Research Reviews* 15(3):176–192, 2009. PMID: 19731384

Mourelatos, Z.; Dostie, J.; Paushkin, S.; et al. miRNPs: A novel class of ribonucleoproteins containing numerous microRNAs. *Genes & Development* 16(6):720–728, 2002. PMID: 11914277

Okamura, K.; Liu, N.; and Lai, E.C. Distinct mechanisms for microRNA strand selection by Drosophila Argonautes. *Molecular Cell* 36(3):431–444, 2009. PMID: 19917251

Packer, A.N.; Xing, Y.; Harper, S.Q.; et al. The bifunctional microRNA miR-9/miR-9* regulates REST and CoREST and is downregulated in Huntington's disease. *Journal of Neuroscience* 28(53):14341–14346, 2008. PMID: 19118166

Pietrzykowski, A.Z.; Friesen, R.M.; Martin, G.E.; et al. Posttranscriptional regulation of BK channel splice variant stability by miR-9 underlies neuroadaptation to alcohol. *Neuron* 59(2):274–287, 2008. PMID: 18667155

Prock, T.L., and Miranda, R.C. Embryonic cerebral cortical progenitors are resistant to apoptosis, but increase expression of suicide receptor DISC-complex genes and suppress autophagy following ethanol exposure. *Alcoholism: Clinical and Experimental Research* 31(4):694–703, 2007. PMID: 17374049

Salero, E., and Hatten, M.E. Differentiation of ES cells into cerebellar neurons. *Proceedings of the National Academy of Sciences of the United States of America* 104(8):2997–3002, 2007. PMID: 17293457

Substance Abuse and Mental Health Services Administration (SAMHSA). *The NSDUH Report: Substance Use among Women During Pregnancy and Following Childbirth.* Pub. No. NSDUH09–0521. Rockville, MD: SAMHSA, Office of Applied Studies, 2009.

Sampson, P.D.; Streissguth, A.P.; Bookstein, F.L.; and Barr, H.M. On categorizations in analyses of alcohol teratogenesis. *Environmental Health Perspectives* 108(Suppl. 3):421–428, 2000. PMID: 10852839

Santillano, D.R.; Kumar, L.S.; Prock, T.L.; et al. Ethanol induces cell-cycle activity and reduces stem cell diversity to alter both regenerative capacity and differentiation potential of cerebral cortical neuroepithelial precursors. *BMC Neuroscience* 6:59, 2005. PMID: 16159388

Sathyan, P.; Golden, H.B.; and Miranda, R.C. Competing interactions between micro-RNAs determine neural progenitor survival and proliferation after ethanol exposure: Evidence from an ex vivo model of the fetal cerebral cortical neuroepithelium. *Journal of Neuroscience* 27(32):8546–8557, 2007. PMID: 17687032

Schratt, G. Fine-tuning neural gene expression with microRNAs. *Current Opinion in Neurobiology* 19(2):213–219, 2009. PMID: 19539460

Shibata, M.; Nakao, H.; Kiyonari, H.; et al. MicroRNA-9 regulates neurogenesis in mouse telencephalon by targeting multiple transcription factors. *Journal of Neuroscience* 31(9):3407–3422, 2011. PMID: 21368052

Smalheiser, N.R., and Lugli, G. microRNA regulation of synaptic plasticity. *Neuromolecular Medicine* 11(3): 133–140, 2009. PMID: 19458942

Smirnova, L.; Grafe, A.; Seiler, A.; et al. Regulation of miRNA expression during neural cell specification. *European Journal of Neuroscience* 21(6):1469–1477, 2005. PMID: 15845075

Tal, T.L.; Franzosa, J.A.; Tilton, S.C.; et al. MicroRNAs control neurobehavioral development and function in zebrafish. *FASEB Journal* 26(4):1452-1461, 2012. PMID: 22253472

Tang, Y.; Banan, A.; Forsyth, C.B.; et al. Effect of alcohol on miR-212 expression in intestinal epithelial cells and its potential role in alcoholic liver disease. *Alcoholism: Clinical and Experimental Research* 32(2):355–364, 2008. PMID: 18162065

Tyler, D.M.; Okamura, K.; Chung, W.J.; et al. Functionally distinct regulatory RNAs generated by bidirectional transcription and processing of microRNA loci. *Genes & Development* 22(1):26–36, 2008. PMID: 18172163

Vreugdenhil, E., and Berezikov, E. Fine-tuning the brain: MicroRNAs. *Frontiers in Neuroendocrinology* 31(2):128–133, 2010. PMID: 19683017

Wang, L.L.; Zhang, Z.; Li, Q.; et al. Ethanol exposure induces differential microRNA and target gene expression and teratogenic effects which can be suppressed by folic acid supplementation. *Human Reproduction* 24(3):562–579, 2009. PMID: 19091803

Wang, Y.; Medvid, R.; Melton, C.; et al. DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. *Nature Genetics* 39(3):380–385, 2007. PMID: 17259983

Waschbisch, A.; Atiya, M.; Linker, R.A.; et al. Glatiramer acetate treatment normalizes deregulated microRNA expression in relapsing remitting multiple sclerosis. *PLoS One* 6(9):e24604, 2011. PMID: 21949733

Williams, A.E. Functional aspects of animal microRNAs. *Cellular and Molecular Life Sciences* 65(4):545–562, 2008. PMID: 17965831

Yang, W.; Wang, Q.; Howell, K.L.; et al. ADAR1 RNA deaminase limits short interfering RNA efficacy in mammalian cells. *Journal of Biological Chemistry* 280(5):3946–3953, 2005. PMID: 15556947

Zhang, H.; Kolb, F.A.; Brondani, V.; et al. Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. *EMBO Journal* 21(21):5875–5885, 2002. PMID: 12411505

Zhou, F.C.; Balaraman, Y.; Teng, M.; et al. Alcohol alters DNA methylation patterns and inhibits neural stem cell differentiation. *Alcoholism: Clinical and Experimental Research* 35(4):735-746, 2011. PMID: 21223309

Alcohol Metabolism and Epigenetics Changes

Samir Zakhari, Ph.D.

Samir Zakhari, Ph.D., *former director of the Division of Metabolism and Health Effects at the National Institute on Alcohol Abuse and Alcoholism, is Senior Vice President of Science, the Distilled Spirits Council of the United States (DISCUS), Washington, DC.*

Metabolites, including those generated during ethanol metabolism, can impact disease states by binding to transcription factors and/or modifying chromatin structure, thereby altering gene expression patterns. For example, the activities of enzymes involved in epigenetic modifications such as DNA and histone methylation and histone acetylation, are influenced by the levels of metabolites such as nicotinamide adenine dinucleotide (NAD), adenosine triphosphate (ATP), and S-adenosylmethionine (SAM). Chronic alcohol consumption leads to significant reductions in SAM levels, thereby contributing to DNA hypomethylation. Similarly, ethanol metabolism alters the ratio of NAD^+ to reduced NAD (NADH) and promotes the formation of reactive oxygen species and acetate, all of which impact epigenetic regulatory mechanisms. In addition to altered carbohydrate metabolism, induction of cell death, and changes in mitochondrial permeability transition, these metabolism-related changes can lead to modulation of epigenetic regulation of gene expression. Understanding the nature of these epigenetic changes will help researchers design novel medications to treat or at least ameliorate alcohol-induced organ damage. **KEY WORDS: Alcohol consumption; alcohol metabolism; ethanol metabolism; alcohol-induced organ damage; disease; epigenetics; epigenetic mechanisms; epigenetic modifications; gene expression; DNA; DNA methylation; histone modification; histone acetylation**

The concept that only DNA and proteins can impact disease states is an oversimplification. It does not take into account different metabolic pathways in which key metabolites bind to transcription factors and alter gene expression patterns that contribute to the observable characteristics (the phenotype) of a given disease. Simple metabolites dictate the actions of specific transcription factors that sense the minute-to-minute cellular environment to determine which parts of, and the extent to which, the genetic code will be transcribed.

An important mechanism in the regulation of gene expression, particularly its first step (transcription), is chromatin remodeling. The human genome is packaged into a dynamic complex of DNA, histone proteins, and non-histone proteins (i.e., chromatin). This chromatin can be packaged more or less densely, and the degree of compactness, which is influenced by histone modifications, determines the DNA's accessibility to the transcription machinery. In general, condensed chromatin (heterochromatin), which is associated with the removal of acetyl groups (i.e., deacetylation) from histones, mediates transcriptional repression. Conversely, transcriptionally active genes are found in open chromatin areas (i.e., euchromatin).

An example of how simple metabolites affect gene transcription is demonstrated in caloric restriction (CR) studies. Research in yeast and rodents has shown that limiting their caloric intake increases their life span (Anderson and Weindruch 2012). This effect is achieved through internal "sensors" that recognize food scarcity and regulate energy expenditure. One such sensor, the protein encoded by the *silent information regulator2* (*SIR2*) gene in yeast (and its mammalian orthologue *SIRT1*), mediates transcriptional silencing through its nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase (HDAC) activity. Entire sets of genes may be silenced by NAD-dependent HDACs, thus influencing obesity and longevity following CR. In fact, NAD^+, reduced NAD (NADH), and the ratio between both compounds (i.e., the NADH/NAD^+ ratio) all serve important regulatory functions, promoting or preventing numerous biochemical reactions, including gene transcription. Ethanol metabolism can drastically change the NADH/NAD^+ ratio, providing an example of a metabolic factor that controls gene transcription and thus may influence gene silencing or activation, leading to diseased phenotype.

Many studies suggest that gene expression is not solely determined by the DNA code itself. Rather, gene expression also depends on a host of

epigenetic phenomena—that is, gene-regulating activity that does not involve changes to the DNA code. Although the genetic code is the same in all cells, each cell in the body has a unique epigenome that can change over time and which regulates gene expression, thereby determining cellular identity in health. Complex human diseases, which involve feedback between many genes and cells, likely are driven by epigenetic changes and responses as well as by allelic variations. Also, progression of disease often may be better explained by epigenetic alterations than by mutations. Perturbation of cellular epigenetic status (Slomko et al. 2012), for example by alcohol metabolism, can result in the loss of tissue identity or activation of anomalous signaling pathways that lead to developmental defects (e.g., fetal alcohol spectrum disorders [FASD]) or disease states such as liver cirrhosis and cancer. Unlike genetic defects, epigenetic alterations can be reversed by epigenetic therapy.

The best known epigenetic signal is DNA methylation, which tags cytosine, one of the four chemical bases that make up the genetic code, with a methyl group. Cytosine methylation is a major contributor to the generation of disease-causing germline mutations and somatic mutations that cause cancer. Also, abnormal methylation of the promoters of regulatory genes causes their silencing or overexpression—an important pathway to tissue pathology, including cancer. The ability of alcohol to perturb normal patterns of DNA methylation is of considerable interest with regard to its cancer-promoting effects. Global hypomethylation of DNA is a consistent feature of neoplastic transformation.

In addition to DNA methylation, gene expression is influenced by post-translational modifications of histone proteins, such as acetylation, methylation, phosphorylation, ubiquitination, and crotonylation. These modifications determine the genome's accessibility to transcription factors (Tan et al. 2011). Furthermore, non-coding RNAs (ncRNAs)—including microRNAs (miRNAs), small interfering RNAs (siRNAs), long non-coding RNAs (lncRNAs), promoter-associated RNAs (PARs), and enhancer RNAs (eRNAs)—have emerged as critical factors that control gene expression, cell-cycle regulation, energy metabolism, chromatin architecture, transcription, and RNA splicing (Collins et al. 2010). Dysregulation of ncRNAs, therefore, contributes to various pathological conditions. These epigenetic alterations may substantially change the transcriptional potential of a cell, thereby altering gene expression with outcomes relevant to particular disease phenotypes.

The role of epigenetics in alcohol's actions has been reviewed by Shukla and colleagues (2008); their publication comprises an extensive background to this article and is highly recommended to the reader. More recently, Mandrekar (2011) discussed the role of epigenetic regulation in alcoholic liver disease. This article focuses on the perturbation of the folate/methionine cycles that influence DNA and histone methylation, as well as on epigenetic processes that are intertwined with alcohol metabolism, namely the increase in NADH/NAD^+ ratio and the formation of reactive oxygen species (ROS) and acetate, which affects histone acetylation. Alcohol's epigenetic effect via a host of ncRNAs is beyond the scope of this article. Understanding how alcohol affects these epigenetic processes and how that leads to tissue damage, including FASD and carcinogenesis, is of paramount importance in drug design for the treatment of alcohol-induced disorders.

Metabolism and Epigenetics

Metabolism produces the energy necessary for various cellular processes. An imbalance between energy intake and expenditure results in the accumulation of nutrients and metabolites, ultimately leading to metabolic diseases. To avoid this, cells constantly are adjusting their metabolic state based on nutrient availability, using extracellular signaling driven by growth factors, hormones, or cytokines. An important feature of metabolic control is the transcriptional regulation of rate-limiting metabolic enzymes, which usually involves epigenetic mechanisms. The ad hoc levels of metabolites, such as NAD^+, adenine triphosphate (ATP), acetyl-CoA, and S-adenosylmethionine (SAM), and of metabolic hormones such as insulin and leptin, contribute to the temporal control of gene expression. The activities of enzymes involved in epigenetic modifications, such as DNA methyltransferases (DNMTs), histone acetyltransferases (HATs), HDACs, histone methyltransferases (HMTs), and histone demethylases (HDMs), are regulated, in part, by the concentrations of their required substrates and cofactors (Lee and Workman 2007). Thus, the cell's metabolic state is tightly integrated in the epigenome and transcriptional regulation.

An example of the close integration of metabolism and epigenetics is CR, in which food intake is reduced by 30 to 50 percent. CR decreases NADH/NAD^+ ratio and increases the AMP/ATP ratio. These changes are sensed by SIRT1 (which has HDAC activity) and AMP-activated protein kinase (AMPK), respectively. One of SIRT1's functions is to deacetylate and activate two proteins called forkhead box protein O1 (FOXO1) and peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), a key regulator of energy metabolism. Both of these result in gene expression favoring the synthesis of glucose in the body (i.e., gluconeogenesis) (Vaquero and Reinberg 2009). AMPK directly phosphorylates PGC-1α, which enables SIRT1 to deacetylate and activate PGC-1α (Cantó and Auwerx 2011).

Recent research suggests that chromatin structure is determined, to a great extent, by metabolic signals, and cells' decision to proliferate, differentiate, migrate, or be quiescent is determined by their micro-environment (Lu and Thompson 2012). Studies in cancer development have revealed a tight link between metabolism and epigenetics. The molecular connections between metabolism and epigenetic modifica-

tions of chromatin (Lu and Thompson 2012) and the role of histones as metabolic sensors that convert changes in metabolism into stable patterns of gene expression (Katada et al. 2012) have been described. The relationship between epigenetic modifications and metabolism is complex and bidirectional. On the one hand, epigenetic changes can influence metabolism by regulating the expression of metabolic enzymes (Wolf et al. 2011). On the other hand, metabolism can disturb epigenetic status, resulting in changes in gene expression or chromatin structure (Wellen and Thompson 2012). In fact, all epigenetic enzymes depend on various substrates or cofactors that are intermediates of cell metabolism (Locasale and Cantley 2011).

The molecular machinery that senses changes in the micro-environment of cells and translates them into epigenome modulations of DNA or histone tails, thereby influencing gene expression, is comprised of various kinases, acetyltransferases, and methyltransferases. These enzymes need appropriate levels of phosphate, acetyl, and methyl groups, respectively, to elicit these modifications (Lee and Workman 2007). As a result, chromatin-remodeling enzymes consume key metabolites such as SAM for methylation, ATP for phosphorylation, and acetyl-CoA, NAD^+, NADH, and acetyl-ADP-ribose for acetylation. These enzymes differ significantly in their affinities for their cofactors, which together with fluctuations in the cofactors' concentrations and their subcellular distribution (e.g., as a result of circadian rhythmicity, oxygen tension, or nutritional status) influence enzymes' ability to perform their functions (Albaugh et al. 2011). At least two groups of chromatin regulators—the sirtuins that act as class III histone deacetylases and the poly-ADP ribose polymerases (PARPs)—depend on NAD^+ levels, which are regulated in a circadian manner linked to energy metabolism (Nakahata et al. 2009). Many signaling pathways, such as Notch and TGFβ, in conjunction with downstream transcription factors, can express or recruit enzymes that modify chromatin (Mohammad and Baylin 2010).

DNA Methylation

The cytosine moiety of cytosine–guanine (CpG) dinucleotides in mammalian DNA can be methylated at carbon 5 to form 5′ methyl-cytosine (figure 1). The methyl group for this chemical modification of the DNA is donated by SAM. This reaction is catalyzed by a family of DNMTs. Of these, DNMT3A and 3B primarily perform de novo methyl transfer, whereas DNMT1 mainly acts as maintenance DNMT with greater affinity for partially methylated (i.e., hemi-methylated) DNA. Methylation of cytosine in CpG-rich regions (i.e., CpG islands) located in or near gene promoters results in gene silencing (Ulrey et al. 2005).

SAM, a methyl donor for reactions catalyzed by DNMT, is generated by adding ATP to methionine by the enzyme methionine adenosyl trans-

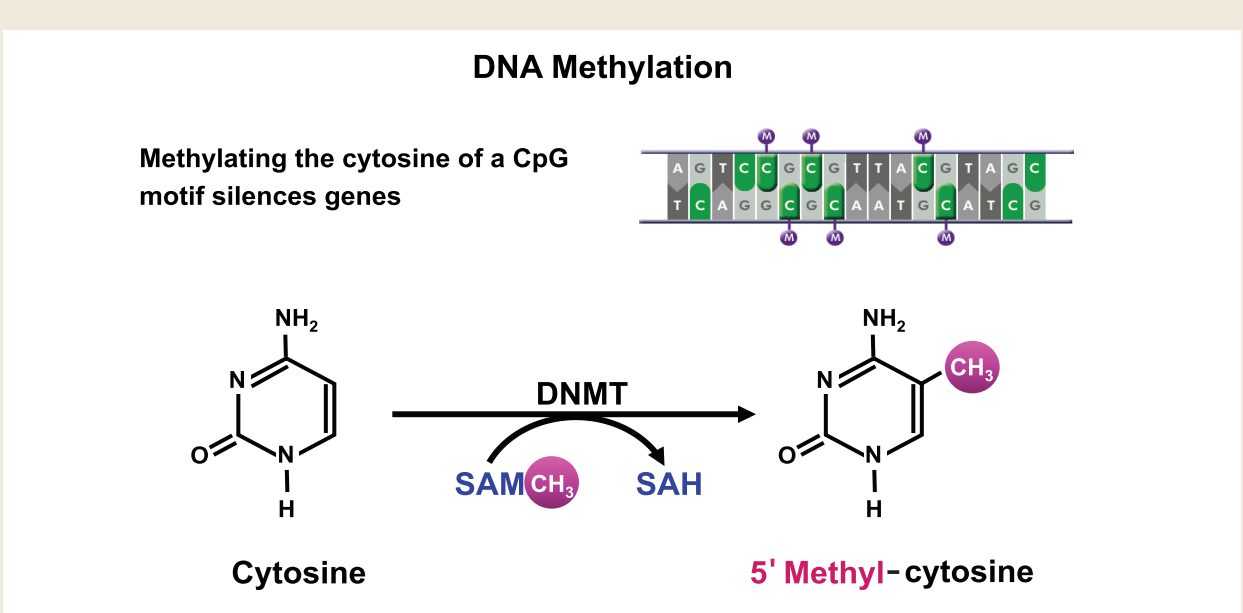


Figure 1 Schematic representation of DNA methylation, which converts cytosine to 5′methyl-cytosine via the actions of DNA methyltransferase (DNMT). DNA methylation typically occurs at cytosines that are followed by a guanine (i.e., CpG motifs).

NOTES: SAM = S-adenosylmethionine; SAH = S-adenosylhomocysteine.

ferase (MAT) (figure 2). After the methyl transfer reaction, SAM forms a byproduct, S-adenosyl homocysteine (SAH), which acts as a potent inhibitor of DNMT and HMTs. SAH then is broken down by SAH hydrolase (SAHH) to form homocysteine, which can either enter a set of reactions called the transsulfuration pathway to form glutathione (GSH) or be remethylated to form methionine (Grillo and Colombatto 2008). For remethylation of homocysteine, a methyl group can be transferred either from N5-methyl tetrahydrofolate (THF) by methionine synthase, or from betaine by betaine homocysteine methyl transferase (BHMT). Excessive ROS formation, which can occur during ethanol metabolism, acutely can deplete GSH. This could promote the transsulfuration of homocysteine to generate new GSH and thus divert the reactions from producing methionine and SAM, thereby decreasing DNA methylation.

Chronic alcohol consumption leads to substantial DNA hypomethylation as a result of significant reduction in tissue SAM. Rats fed alcohol for nine weeks showed a decrease in hepatic concentrations of SAM, methionine, and GSH as well as about 40-percent reduction in methylation (Lu et al. 2000). In addition, the investigators observed increased expression of a gene called *c-myc* and increased accumulation of breaks in the DNA strand, both of which predispose to hepatocellular carcinoma (HCC). In fact, chronic hepatic SAM deficiency in a certain mouse strain (i.e., Mat1a knockout mice) resulted in spontaneous development of HCC (Martinez-Chantar et al. 2002). Additionally, alcohol perturbs the folate cycle that is involved in methionine production and the generation of DNA building blocks (i.e., purines and pyrimidines) and which is integral to supplying the methyl groups necessary for DNA methylation.

Altered methionine metabolism and the subsequent hypomethylation is one mechanism by which alcohol produces alcoholic liver disease and HCC (Medici and Halsted 2013). In addition, alcohol-induced degradation of DNMTs and hypomethylation supports a potential epigenetic mechanism for FASD (Chen et al. 2013; Mukhopadhyay et al. 2013).

Histone Modification

Histone modification plays an important part in epigenetics, affecting transcription, DNA repair, and DNA replication (Esteller 2008). As mentioned above,

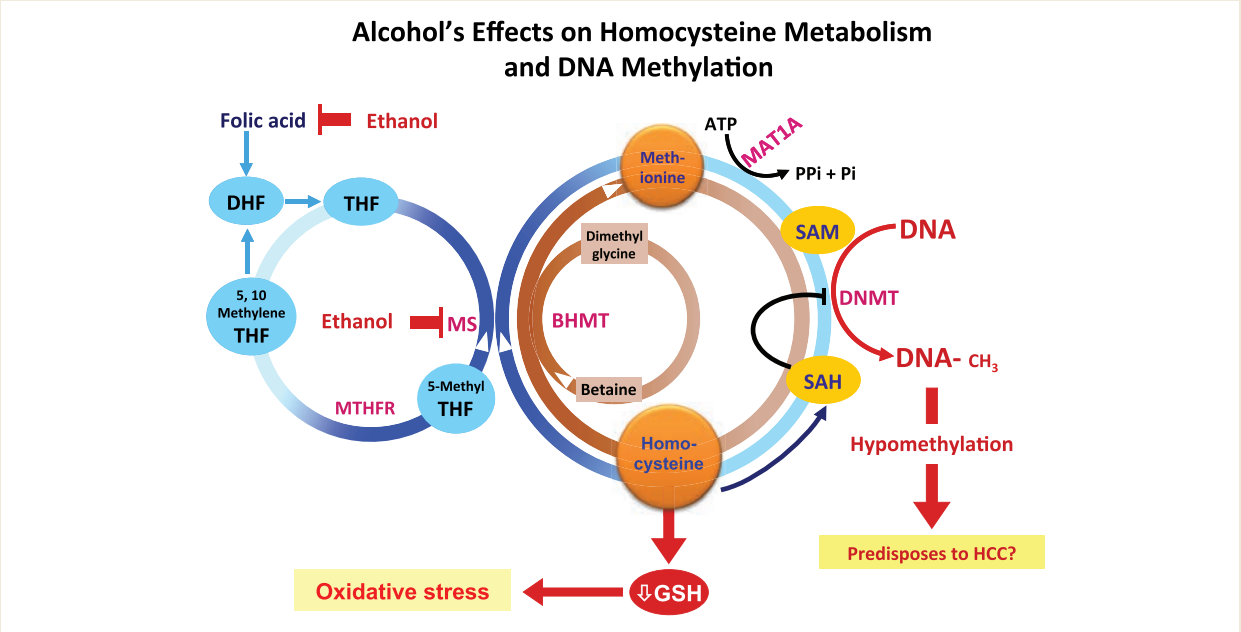


Figure 2 Alcohol's effects on homocysteine/methionine metabolism and DNA methylation. Methionine, which is formed by methylation of homocysteine (using either 5-methyl tetrahydrofolate [5-methyl THF] or betaine as methyl donors), is essential for the production of S-adenosylmethionine (SAM), which in turn is used to methylate DNA. Chronic heavy drinking reduces folate levels and inhibits methionine synthase (MS), resulting in the reduction of methionine and SAM and the concurrent increase in homocysteine and S-adenosylhomocysteine (SAH). SAH further inhibits DNA methyltransferases (DNMTs), ultimately resulting in global hypomethylation of DNA.

NOTES: MTHFR = methylene tetrahydrofolate reductase; MAT = methionine adenosyltransferase; HCC = hepatocellular carcinoma; BHMT = betaine homocysteine methyltransferases; GSH = glutathione; ATP = adenosine triphosphate; Pi = inorganic phosphate.

histone modifications include a plethora of post-translational modifications. This review, however, focuses only on histone acetylation and methylation.

Histone acetylation is regulated mainly by the opposing activities of two families of enzymes—the HATs that acetylate histones and the HDACs (Shahbazian and Grunstein 2007). HATs, which transfer acetyl groups from acetyl-CoA to lysine residues, include three main subfamilies that are functionally distinct—GCN5-related N-acetyltransferase (GNAT), MYST histone acetyltransferase, and p300/CBP. HDACs, in contrast, remove acetyl groups from histones; they comprise four groups (classes I–IV) (Zhang and Dent 2005), some of which are dependent on $Zn2^+$ (Haberland et al. 2009). Class III HDACs, known as sirtuins, however, require NAD^+ as a cofactor. In general, histone acetylation results in transcriptional activation, whereas deacetylation is associated with gene silencing (Lane and Chabner 2009).

Histone methylation is achieved by HMTs. They can be classified into three groups: SET domain and non-SET domain lysine methyltransferases, and arginine methyltransferases. All of these use SAM as a coenzyme to transfer methyl groups to lysine or arginine residues of substrate proteins. There are three distinct states of lysine methylation (i.e., mono-, di-, and trimethylated) (Varier and Timmers 2011). Histone methylation can result in transcriptional activation or repression, depending on the position of the lysine that is modified (Berger 2007). For example, methylation of H3K4,[1] H3K36, and H3K791 is associated with active transcription, whereas methylation of H3K9, H3K27, and H4K20 generally indicates silenced chromatin. Histone demethylation is achieved by a group of enzymes collectively known as HDMs.

The effects of alcohol metabolism on histone acetylation have been demonstrated in animal experiments, including studies of obese mice.[2] Alcohol administration to these animals was associated with exacerbation of fatty liver, which resulted from an impairment of the hepatic lipid metabolism pathways, mainly those mediated by SIRT1 and AMPK (Everitt et al. 2012). The development of alcohol-induced fatty liver could be prevented by administering rosiglitazone, an anti-diabetic medication that binds to certain receptors in fat cells and makes them more sensitive to insulin. The protective effect of rosiglitazone was attributed to its enhancement of the hepatic adiponectin–SIRT1–AMPK signaling pathway (Shen et al. 2010). Other studies found that chronic alcohol consumption can result in protein hyperacetylation within cell components called mitochondria. Most proteins in the mitochondria normally are deacetylated through SIRT3-dependent mechanisms (Fritz et al. 2012). Ethanol-induced suppression of SIRT3 and the concomitant increase of another acetylation pathway (i.e., cyclophilin-D acetylation) could be prevented by AMPK activation (Shulga and Pasorino 2010). The role of alcohol metabolism in histone acetylation is shown in figure 3.

Histone acetylation is regulated mainly by the opposing activities of two families of enzymes—the HATs that acetylate histones and the HDACs.

Alcohol Metabolism and Its Effects on Epigenetic Mechanisms

To appreciate the effects of alcohol on histone acetylation and changes in redox state that result in epigenetic modifications of gene expression, a brief overview of alcohol metabolism is warranted.

Alcohol is metabolized mainly by two pathways—oxidative pathways that take place mainly in the liver and non-oxidative pathways that occur mainly in extrahepatic tissues. The following discussion will focus only on oxidative pathways.

Oxidative ethanol metabolism mainly occurs in the liver via a major pathway in which the enzyme cytosolic alcohol dehydrogenase (ADH) produces acetaldehyde, a highly reactive and toxic molecule. This oxidation is accompanied by the reduction of NAD^+ to NADH. Through this pathway, ethanol oxidation generates a highly reduced cytosolic environment, predominantly in liver cells (i.e., hepatocytes).

In addition to ADH, a group of enzymes known as the cytochrome P450 isozymes, including CYP2E1, 1A2, and 3A4, also contribute to ethanol oxidation to acetaldehyde in the liver. These enzymes, which are present predominantly in a cell component called the endoplasmic reticulum (ER), become involved particularly after chronic ethanol intake. CYP2E1 is induced by chronic ethanol consumption and assumes an important role in metabolizing ethanol to acetaldehyde at elevated alcohol concentrations.[3] Alcohol metabolism by CYP2E1 also produces highly reactive ROS, including hydroxyethyl, superoxide anions, and hydroxyl radicals. Finally, another enzyme called catalase, which is located in cell components called peroxisomes, also can oxidize ethanol (figure 4); however, quantitatively this is considered a minor pathway of ethanol oxidation. All of these oxidative pathways generate acetaldehyde, which then is rapidly metabolized further. This is done mainly by mitochondrial aldehyde dehydrogenase (ALDH2) to form acetate and NADH. Mitochondrial

[1] Histone modifications are denoted by the number of the histone protein affected (e.g., histone 3 is noted as H3) as well as the lysine residue affected (e.g., the fourth lysine in the molecule is denoted as K4).

[2] The animals had inherited two gene copies predisposing them to obesity (i.e., had an ob/ob genotype).

[3] In other words, CYP2E1 has a high Km for ethanol of 8–10 mM, compared with a Km of 0.05–40 mM for hepatic ADH.

NADH is oxidized by the electron transport chain.

Epigenetics-Relevant Consequences of Oxidative Alcohol Metabolism

Oxidative alcohol metabolism can exert epigenetic effects through several mechanisms, including increase in the NADH/NAD$^+$ ratio and the formation of ROS and acetate.

Increases in NADH/NAD$^+$ Ratio and Their Consequences

The ratio of NADH to NAD$^+$ fluctuates in response to changes in metabolism. Alcohol metabolism produces a significant increase in the hepatic NADH/NAD$^+$ ratio in the cytoplasm and mitochondria of hepatocytes, as evidenced by changes in the levels of several other molecules in those cell compartments (i.e., an increase in the lactate/pyruvate ratio in the cytoplasm and in the γ-hydroxybutyrate/acetoacetate ratio in the mitochondria) (Cunningham and Bailey 2001). The resulting shift of the redox potential of the hepatocytes causes a marked alteration in various reversible metabolic pathways (Krebs and Veech 1970). As a result, ethanol oxidation vastly increases the availability of oxidizable NADH to the electron transport chain in the mitochondria. NAD$^+$ influences many important cellular reactions.

NAD$^+$ and NADH mainly are used by enzymes that catalyze substrate oxidation involving energy metabolism, histone deacetylation, and cell death. Under normal physiological conditions, the ratio of cytosolic free NAD$^+$ to NADH is approximately 700:1, whereas that of the mitochondria has been reported to be 7–8 to 1 (Stubbs et al. 1972; Veech et al. 1969). Increased NADH/NAD$^+$ ratios (e.g., because of depletion of NAD$^+$) in both the cytosol and mitochondria of hepatocytes influence the direction of several reversible reactions, leading to alterations in hepatic lipid, carbohydrate, protein, lactate, and uric acid metabolism. The increase in the ratio of NADH/NAD$^+$ also results in derangement of carbohydrate metabolism, cell death, modulation of the mitochondrial permeability transition (MPT) opening, and modulation of gene expression.

Derangement of Carbohydrate Metabolism. NAD$^+$ mediates cytosolic energy metabolism by influencing the breakdown of glucose molecules (i.e., glycolysis) and by modulating the lactate–pyruvate conversion by lactate dehydrogenase (LDH). An increase in the NADH/NAD$^+$ ratio interferes with both of

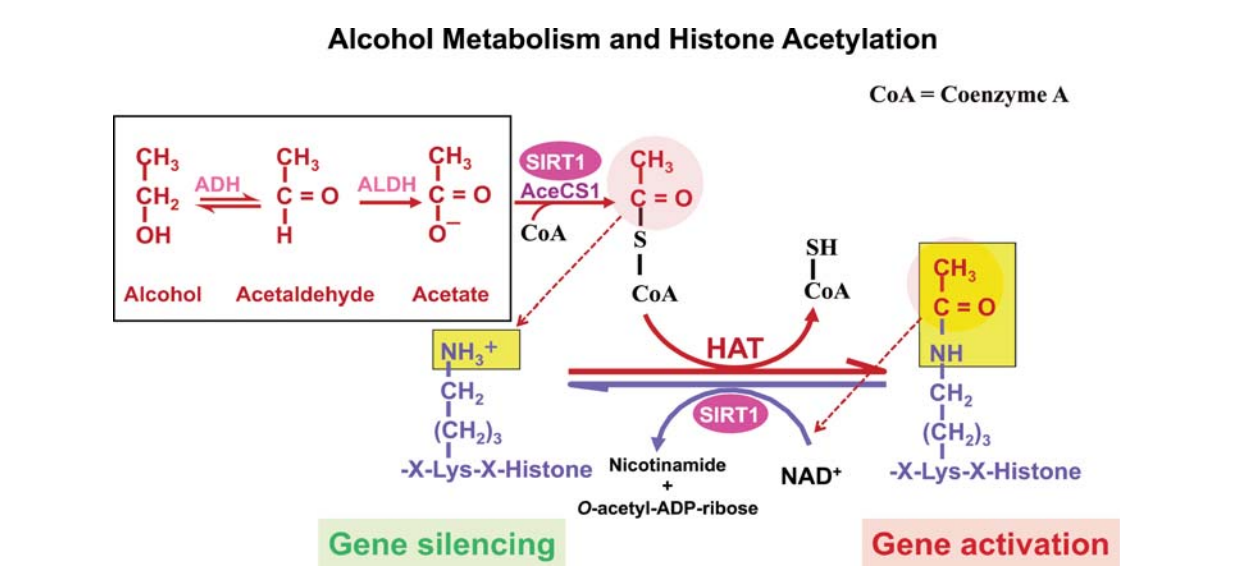


Figure 3 Alcohol metabolism and histone acetylation. Acetyl-coenzyme A (acetyl-CoA) synthetase (AceCS), an enzyme that converts acetate to acetyl-CoA, is activated by SIRT1. Acetyl-CoA is used by histone acetyltransferase (HAT) to acetylate the lysine residues in histone proteins. This neutralizes the positive charge and allows the chromatin to assume an open conformation, thus resulting in gene activation. SIRT1 also deaceytlates acetylated histones, resulting in gene silencing. Thus, SIRT1 is a sensor that balances gene activation and silencing in the cell based on the cell's energy status. Alcohol metabolism results in acetate formation, which is used in extrahepatic tissues to produce acetyl-CoA.

NOTES: AceCS1 = Acetyl-CoA synthase 1; ADH = alcohol dehydrogenase; ALDH = Aldehyde dehydrogenase.

these processes. NAD^+ depletion also causes inhibition of the later steps of glucose metabolism (i.e., the tricarboxylic acid [TCA]) cycle. As NADH accumulates, NAD^+ becomes depleted. As a result, oxidation of acetyl-CoA by the TCA cycle is inhibited because of a lack of oxidized coenzymes. In addition, NADH accumulation inhibits pyruvate dehydrogenase (PDH), thus decreasing the conversion of pyruvate to acetyl-CoA. Instead, NADH accumulation in the cytosol favors the conversion of pyruvate to lactate by LDH. This lowers the concentration of pyruvate, which in turn decreases the pyruvate carboxylase reaction, one of the rate limiting steps of gluconeogenesis (Krebs et al. 1969). Collectively, the increase in NADH results in the inhibition of gluconeogenesis and, during starvation, can cause clinically significant hypoglycemia.

Cell Death. Mitochondria play important roles in the regulation of cell death (i.e., apoptosis and necrosis). They release pro-apoptotic factors such as cytochrome c and apoptosis-inducing factor (AIF), which activate caspase-dependent and caspase-independent cell death, respectively. Another enzyme called poly(ADP-ribose) polymerase 1 (PARP-1), a mediator of programmed necrosis activated by oxidative stress, is an important activator of caspase-independent cell death (Zhang et al. 1994). Overactivation of PARP-1 can induce NAD^+ depletion (Ying et al. 2003), leading to the inhibition of SIRT1 as well as inhibition of glycolysis, which in turn would reduce pyruvate supply to the TCA cycle and cause ATP depletion (Ying et al. 2002). The NADH/NAD^+ ratio also affects MPT (Alano et al. 2004), which results in the translocation of AIF from mitochondria to the nucleus (Churbanova and Sevrioukova 2008; Yu et al. 2002), ultimately resulting in apoptosis. Thus, an increased NADH/NAD^+ ratio caused by alcohol metabolism can influence pro-death and pro-survival signals in the PARP-1–mediated cell-death program.

Modulation of the MPT Opening. MPT is defined as an increase in the ability of the membranes surrounding the mitochondria to allow passage of molecules of a certain size (i.e., increase in membrane permeability). This increase in permeability, which results from the opening of specific pores, typically occurs in response to certain pathological conditions and can lead to mitochondrial swelling and cell death through apoptosis or necrosis. Increases in NADH/NAD^+

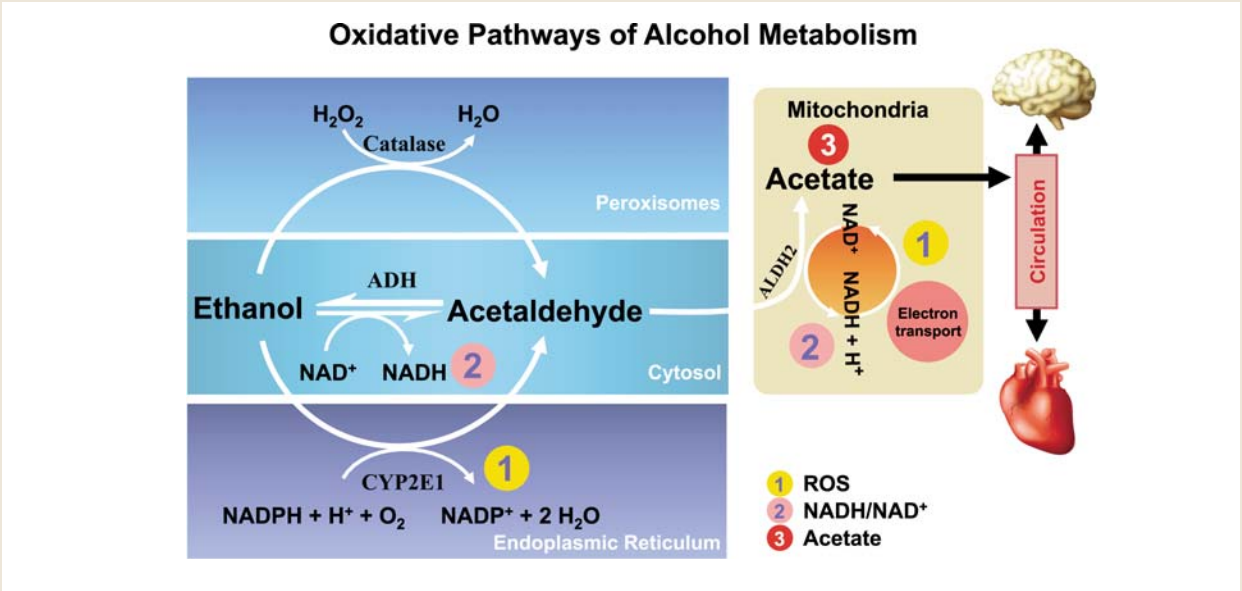


Figure 4 Oxidative pathways of alcohol metabolism. Alcohol is metabolized mainly in the cytosol by alcohol dehydrogenase (ADH) to produce acetaldehyde. At high levels of alcohol consumption, an enzyme in the endoplasmic reticulum, cytochrome P450 IIE1 (CYP2E1), becomes involved in metabolizing alcohol to acetaldehyde; this enzyme also is induced by chronic drinking. A catalase-mediated reaction in the peroxisomes is considered a minor metabolic pathway of alcohol metabolism. Acetaldehyde is further metabolized to acetate in the mitochondria. Alcohol metabolism results in the formation of NADH and thus changes the redox state of hepatocytes (i.e., increases the ratio of NADH/NAD^+). Both alcohol metabolism by CYP2E1 and the re-oxidation of NADH via the electron transport chain in the mitochondria results in the formation of reactive oxygen species (ROS).

ratio resulting from ethanol metabolism lead to an increase in MPT opening, which significantly influences mitochondrial membrane potential (Zoratti and Szabo 1995). In addition, NADH can have other effects, such as increased release of calcium ions (Ca^{++}) release from certain channels on the ER, as demonstrated in a cell line called PC12 cells (Kaplin et al. 1996), and inhibition of ryanodine receptors of cardiac muscles (Zima et al. 2003).

Modulation of Gene Expression. The NADH/NAD^+ ratio influences gene expression through several pathways. The following discussion focuses on three NAD^+-dependent enzymes: carboxyl-terminal binding protein (CtBP); silent information regulator (Sir2); and the heterodimeric Clock/NPAS2 transcriptional regulator, whose activities may play a role in ethanol-induced injury.

CtBP is a regulatory factor mediating transcriptional repression that is important for cell cycle regulation and development; it acts as a NAD^+-dependent D2-hydroxyacid dehydrogenase (Kumar 2002). Studies on mice carrying altered *Ctbp* genes suggest that two mutant genes (*Ctbp1* and *Ctbp2*) play unique regulatory roles during development (Chinnadurai 2003). Mice lacking a functional *Ctbp1* gene (i.e., *Ctbp1*-null mice) are about 30 percent smaller than wild type mice, whereas *Ctbp2*-null mice exhibit defects in heart morphogenesis and neural structures. NAD^+ enhances the interaction of CtBP with target transcription factors (Zhang et al. 2002). Furthermore, CtBP is a metabolic sensor that has been implicated in regulating adipogenesis (Jack et al. 2011). The role of CtBP and changes in the redox state caused by alcohol metabolism in ethanol-induced teratogenesis and effects on adipose tissue remains to be elucidated.

The Sir2 protein links cellular metabolism and transcriptional silencing through its NAD^+-dependent HDAC activity (Imai et al. 2000). This activity is essential for Sir2 functions, including gene silencing, regulation of the circadian clock, and its role in obesity and longevity (Rutter et al. 2001). The extraordinary requirement of NAD^+ in the deacetylation reaction suggests that Sir2 may function as a sensor for the energy status of cells, linking the energy level represented by available NAD^+ to the silencing of gene expression. The mammalian orthologue of Sir2, known as SIRT1, also is a NAD^+-dependent deacetylase (Imai et al. 2000) whose substrates include histones and the transcription factor p53 (Vaziri et al. 2001). NAD^+ activates Sir2 during CR, which not only extends the life span in a wide variety of organisms, but also reduces the incidence of age-related diseases such as diabetes, cancer, immune deficiencies, and cardiovascular disorders (Bordone and Guarente 2005).

Human and animal studies have demonstrated that both acute and chronic alcohol intake can affect many aspects of circadian rhythms.

The Clock protein is part of the transcriptional feedback system whose activity fluctuates with the light–dark cycle and which controls the circadian rhythm in mammals. Generally, humans work, eat, and exercise during the day and rest at night. Synchronization of these activities with metabolic reactions is achieved by the circadian clock. The circadian system comprises a central clock, which is regulated by light and located in a brain region called the suprachiasmatic nucleus (SCN) of the anterior hypothalamus, and peripheral clocks present in metabolic tissues that are entrained by the central clock via feeding/fasting cycles. The master regulators (transcriptional activators) of the central clock are two proteins called circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNTL-like protein 1 (BMAL1). Both of these are transcriptional factors that regulate the expression of cryptochrome (*CRY1* and *CRY2*) and period (*PER1, PER2, PER3*) genes. *PER* and *CRY* proteins bind to CLOCK/BMAL1 and inhibit their transcriptional activity (Bass and Takahashi 2010). Furthermore, using the nuclear receptor REV-ERB as a feedback loop, BMAL1 drives the transcription of *Rev-erb*α, which in turn inhibits *Bmal1* transcription (Preitner et al. 2002). In addition, the retinoid-related orphan receptors (RORα, β, γ) activate BMAL1 and REV-ERBα (Jetten 2009). Misalignment of these activities with the internal clock disrupts energy homeostasis and could result in metabolic diseases, such as those observed in shift workers (Wang et al. 2011).

Conversely, metabolic reactions and the resulting redox state of cells have been shown to play an important role in the function of the circadian rhythm. For example, the intracellular NADH/NAD^+ ratio, through the sensor SIRT1, influences BMAL1 and CLOCK in both central and peripheral clocks. In addition, AMP levels in the cell regulate the circadian clock by activating AMPK through inhibiting AMPK dephosphorylation (Um et al. 2011). Thus, whereas the central clock is regulated by an environmental cue to the SCN (i.e., light sensed by the retina), cellular metabolites influence the peripheral clocks, which are also entrained by the central clock.

Human and animal studies have demonstrated that both acute and chronic alcohol intake can affect many aspects of circadian rhythms, including physiological, endocrine, and behavioral functions. Alcohol intake and withdrawal have been shown to affect the circadian rhythm of body temperature in rats and to alter circadian melatonin secretion in both healthy and alcoholic people (Danel and Touitou 2006; Danel et al. 2009). In addition, alcohol alters the circadian expression

of *Per2* and *Per3* in the SCN, suggesting that alcohol may directly affect the central pacemaker and interfere with its circadian functioning (Chen et al. 2004). In rats, Farnell and colleagues (2008) have demonstrated that neonatal alcohol exposure during the brain growth spurt can alter clock gene oscillations in the liver, in addition to the SCN.

Formation of ROS and Oxidative Stress and Their Consequences

ROS, including superoxide ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), hypochlorite ion (OCl^-), and hydroxyl ($\bullet OH$) radicals, are generated by many reactions in multiple compartments in the cell, including NADPH oxidases, lipid metabolism within the peroxisomes; and various cytosolic cyclooxygenases. However, in most cells the vast majority of ROS result from electron transport by the mitochondria. The role of ROS in inducing epigenetic alterations in human carcinogenesis has been discussed (Ziech et al. 2011).

Acetate Formation and Its Consequences

Most of the acetate resulting from ethanol metabolism escapes the liver into the blood. In cells with mitochondria that contain enzymes capable of transforming acetate to acetyl CoA, such as heart, skeletal muscle, and brain, the acetate is eventually metabolized to CO_2 in the TCA cycle. As shown in figure 3, SIRT1 activates mammalian acetyl-CoA synthase through deacetylation, resulting in the formation of acetyl-CoA. The acetyl-CoA then is used to acetylate histones, resulting in gene activation. Subsequently, SIRT1 deacetylates the histones, resulting in gene silencing. Thus, SIRT1 can act as a sensor to regulate gene transcription.

Summary and Conclusions

Figure 5 summarizes the epigenetic effects of alcohol metabolism, which include the following:

- Global hypomethylation resulting from a reduction in SAM levels. SAM levels are reduced as a result of alcohol-induced reduction in folate and the inhibition of methionine synthase. At the same time, SAH levels increase, which inhibits DNMT.
- Histone modification that is associated with an increase in NADH levels caused by alcohol metabolism. The increase in NADH affects SIRT1 activity, leading to gene expression and/or silencing.
- Production of ROS, which affect the expression of inflammatory genes, and acetate, which is used

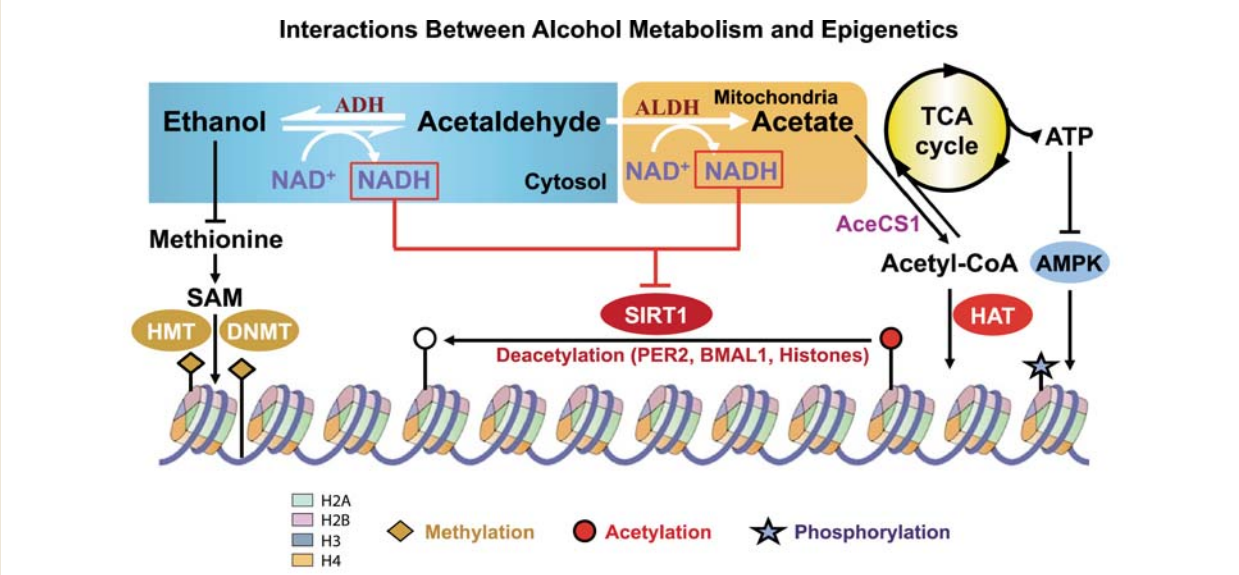


Figure 5 Interactions between alcohol metabolism and epigenetic mechanisms. Chronic alcohol consumption leads to lower-than-normal methylation (i.e., hypomethylation) by decreasing the levels of S-adenosylmethionine (SAM), which is used by DNA methyltransferases (DNMTs) and histone methyl transferases (HMTs) to methylate DNA and histones, respectively. Furthermore, alcohol metabolism increases the ratio of the reduced nicotinamide adenine dinucleotide (NADH) to the oxidized nicotinamide adenine dinucleotide (NAD^+); this inhibits SIRT1, thereby interfering with normal histone acetylation patterns.

NOTES: ATP = Adenosine triphosphate; AMPK = AMP-activated protein kinase; HAT = histone acetyl transferase; TCA = tricarboxylic acid cycle.

in extrahepatic tissues to produce acetyl-CoA. The latter then is used in histone acetylation by HATs.

These epigenetic changes resulting from chronic alcohol consumption can lead to organ pathology. Understanding the exact nature of the epigenetic changes will help design medication for the treatment or alleviation of alcohol-induced organ damage. ■

Financial Disclosure

The author declares that he has no competing financial interests.

References

Alano, C.C.; Ying, W.; and Swanson, R.A. Poly(ADP-ribose) polymerase-1-mediated cell death in astrocytes requires NAD+ depletion and mitochondrial permeability transition. *Journal of Biological Chemistry* 279:18895–18902, 2004. PMID: 14960594

Albaugh, B.N.; Arnold, K.M.; and Denu, J.M. KAT(ching) metabolism by the tail: Insight into the links between lysine acetyltransferases and metabolism. *ChemBioChem* 12:290–298, 2011. PMID: 21233716

Anderson, R.M., and Weindruch, R. The caloric restriction paradigm: Implications for healthy human aging. *American Journal of Human Biology* 24:101–106, 2012. PMID: 22290875

Bass, J., and Takahashi, J.S. Circadian integration of metabolism and energetics. *Science* 330:1349–1354, 2010. PMID: 21127246

Berger, S.L. The complex language of chromatin regulation during transcription. *Nature* 447:407–412. PMID: 17522673

Bordone, L., and Guarente, L. Calorie restriction, SIRT1 and metabolism: Understanding longevity. *Nature Reviews. Molecular Cell Biology* 6:298–305, 2005. PMID: 15768047

Cantó, C., and Auwerx, J. Calorie restriction: Is AMPK a key sensor and effector? *Physiology* 26:214–224, 2011. PMID: 21841070

Chen, C.P.; Kuhn, P.; Advis, J.P.; and Sarkar, D.K. Chronic ethanol consumption impairs the circadian rhythm of pro-opiomelanocortin and period genes mRNA expression in the hypothalamus of the male rat. *Journal of Neurochemistry* 88:1547–1554, 2004. PMID: 15009656

Chen, Y.; Ozturk, N.C.; and Zhou, F.C. DNA methylation program in developing hippocampus and its alteration by alcohol. *PlosOne* 8:e60503, 2013. PMID: 23544149

Chinnadurai, G. CtBP family proteins: More than transcriptional corepressors. *Bioessays* 25:9–12, 2003. PMID: 12508276

Churbanova, I.Y., and Sevrioukova, I.F. Redox-dependent changes in molecular properties of mitochondrial apoptosis-inducing factor. *Journal of Biological Chemistry* 283:5622–5631, 2008. PMID: 18167347

Collins, L.J.; Schönfeld, B.; and Chen, X.S. The epigenetics of non-coding RNA. In: Tollefsbol, T., Ed. *Handbook of Epigenetics: The New Molecular and Medical Genetics*. New York: Elsevier, pp. 49–61, 2010.

Cunningham, C.C., and Bailey, S.M. Ethanol consumption and liver mitochondria function. *Biological Signals and Receptors* 10:271–282, 2001. PMID: 11351133

Danel, T., and Touitou, Y. Alcohol consumption does not affect melatonin circadian synchronization in healthy men. *Alcohol and Alcoholism* 41:386–390, 2006. PMID: 16679342

Danel, T.; Cottencin, O.; Tisserand, L.; and Touitou, Y. Inversion of melatonin circadian rhythm in chronic alcoholic patients during withdrawal: Preliminary study on seven patients. *Alcohol and Alcoholism* 44:42–45, 2009. PMID: 19029096

Esteller, M. Epigenetics in cancer. *New England Journal of Medicine* 358:1148–1159, 2008. PMID: 18337604

Everitt, H.; Hu, M.; Ajmo, J.M.; et al. Ethanol administration exacerbates the abnormalities in hepatic lipid oxidation in genetically obese mice. *American Journal of Physiology. Gastrointestinal and Liver Physiology* 304:G38–G47, 2013. PMID: 23139221

Farnell, Y.Z.; Allen, G.C.; Nahm, S.S.; et al. Neonatal alcohol exposure differentially alters clock gene oscillations within the suprachiasmatic nucleus, cerebellum, and liver of adult rats. *Alcoholism: Clinical and Experimental Research* 32:544–552, 2008. PMID: 18215209

Fritz, K.S.; Galligan, J.J.; Hirschey, M.D.; et al. Mitochondrial acetylome analysis in a mouse model of alcohol-induced liver injury utilizing SIRT3 knockout mice. *Journal of Proteome Research* 11:1633–1643, 2012. PMID: 22309199

Grillo, M.A., and Colombatto, S. S-adenosylmethionine and its products. *Amino Acids* 34:187–193, 2008. PMID: 17334902

Haberland, M.; Montgomery, R.L.; and Olson, E.N. The many roles of histone deacetylases in development and physiology: Implications for disease and therapy. *Nature Reviews. Genetics* 10:32–42, 2009. PMID: 19065135

Hallows, W.C.; Lee, S.; and Denu, J.M. Sirtuins deacetylate and activate mammalian acetyl-CoA synthetases. *Proceedings of the National Academy of Sciences of the United States of America* 103:10230–10235, 2006. PMID: 16790548

Imai, S.; Armstrong, C.M.; Kaeberlein, M.; and Guarente, L. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* 403:795–800, 2000. PMID: 10693811

Jack, B.H.; Pearson, R.C.; and Crossley, M. C-terminal binding protein: A metabolic sensor implicated in regulating adipogenesis. *International Journal of Biochemistry & Cell Biology* 43:693–696, 2011. PMID: 21281737

Jetten, A.M. Retinoid-related orphan receptors (RORs): Critical roles in development, immunity, circadian rhythm, and cellular metabolism. *Nuclear Receptor Signaling* 7:e003, 2009. http://dx.doi.org/10.1621/nrs.07003 PMID: 19381306

Kaplin, A.I.; Snyder, S.H.; and Linden, D.J. Reduced nicotinamide adenine dinucleotide-selective stimulation of inositol 1,4,5-triphosphate receptors mediates hypoxic mobilization of calcium. *Journal of Neuroscience* 16:2002–2011, 1996. PMID: 8604044

Katada, S.; Imhof, A.; and Sassone-Corsi, P. Connecting threads: Epigenetics and metabolism. *Cell* 148:24–28, 2012. PMID: 22265398

Krebs, H.A.; Freeland, R.A.; Hems, R.; and Stubbs, M. Inhibition of hepatic gluconeogenesis by ethanol. *Biochemical Journal* 112:117–124, 1969. PMID: 5774487

Krebs, H.A., and Veech, R.L. Regulation of the redox state of the pyridine nucleotides in rat liver. In: Sund, H., Ed. *Pyridine Nucleotide-Dependent Dehydrogenases*. New York: Springer-Verlag, p. 413–438, 1970.

Kumar, V.; Carlson, J.E.; Ohgi, K.A.; et al. Transcription corepressor CtBP is an NAD+-regulated dehydrogenase. *Molecular Cell* 10:857–869, 2002. PMID: 12419229

Lane, A.A., and Chabner, B.A. Histone deacetylase inhibitors in cancer therapy. *Journal of Clinical Oncology* 27:5459–5468, 2009. PMID: 19826124

Lee, K.K., and Workman, J.L. Histone acetyltransferase complexes: One size doesn't fit all. *Nature Reviews. Molecular Cell Biology* 8:284–295, 2007. PMID: 17380162

Locasale, J.W., and Cantley, L.C. Metabolic flux and the regulation of mammalian cell growth. *Cell Metabolism* 14:443–451, 2011. PMID: 21982705

Lu, C., and Thompson, C.B. Metabolic regulation of epigenetics. *Cell Metabolism* 16:9–17, 2012. PMID: 22768835

Lu, S.C.; Huang, Z.Z.; Yang, H.; et al. Changes in methionine adenosyltransferase and S-adenosylmethionine homeostasis in the alcoholic rat liver. *American Journal of Physiology. Gastrointestinal and Liver Physiology* 279:G178–G185, 2000. PMID: 10898761

Mandrekar, P. Epigenetic regulation in alcoholic liver disease. *World Journal of Gastroenterology* 17(20):2456–2464, 2011. PMID: 21633650

Martínez-Chantar, M.L.; Corrales, F.J.; Martínez-Cruz, L.A.; et al. Spontaneous oxidative stress and liver tumors in mice lacking methionine adenosyltransferase 1A. *FASEB Journal* 16:1292–1294, 2002. PMID: 12060674

Medici, V., and Halsted, C.H. Folate, alcohol, and liver disease. *Molecular Nutrition & Food Research* 57:596–606, 2013. PMID: 23136133

Mohammad, H.P., and Baylin, S.B. Linking cell signaling and the epigenetic machinery. *Nature Biotechnology* 28:1033–1038, 2010. PMID: 20944593

Mukhopadhyay, P.; Rezzoug, F.; Kaikaus, J.; et al. Alcohol modulates expression of DNA methyltransferases and methyl CpG-/CpG domain-binding proteins in murine embryonic fibroblasts. *Reproductive Toxicology* 37:40–48, 2013. PMID: 23395981

Nakahata, Y.; Sahar, S.; Astarita, G.; et al. Circadian control of the NAD+ salvage pathway by CLOCK-SIRT1. *Science* 324:654–657, 2009. PMID: 19286518

Preitner, N.; Damiola, F.; Lopez-Molina, L.; et al. The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* 110:251–260, 2002. PMID: 12150932

Rutter, J.; Reick, M.; Wu, L.C.; and McKnight, S.L. Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors. *Science* 293:510–514, 2001. PMID: 11441146

Shahbazian, M.D., and Grunstein, M. Functions of site-specific histone acetylation and deacetylation. *Annual Review of Biochemistry* 76:75–100, 2007. PMID: 17362198

Shen, Z.; Liang, X.; Rogers, C.Q.; et al. Involvement of adiponectin-SIRT1-AMPK signaling in the protective action of rosiglitazone against alcoholic fatty liver in mice. *American Journal of Physiology. Gastrointestinal and Liver Physiology* 298:G364–G374, 2010. PMID: 20007851

Shukla, S.D.; Velazquez, J.; French, S.W.; et al. Emerging role of epigenetics in the actions of alcohol. *Alcoholism: Clinical and Experimental Research* 32:1525–1534, 2008. PMID: 18616668

Shulga, N., and Pastorino, J.G. Ethanol sensitizes mitochondria to the permeability transition by inhibiting deacetylation of cyclophilin-D mediated by sirtuin-3. *Journal of Cell Science* 123:4117–4127, 2010. PMID: 21062897

Slomko, H.; Heo, H.J.; and Einstein, F.H. Minireview: Epigenetics of obesity and diabetes in humans. *Endocrinology* 153:1025–1030, 2012. PMID: 22253427

Stubbs, M.; Veech, R.L.; and Krebs, H.A. Control of the redox state of the nicotinamide-adenine dinucleotide couple in rat liver cytoplasm. *Biochemical Journal* 126:59–65, 1972. PMID: 4342386

Tan, M.; Luo, H.; Lee, S.; et al. Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification. *Cell* 146:1016–1028, 2011. PMID: 21925322

Ulrey, C.L.; Liu, L.; Andrews, L.G.; and Tollefsbol, T.O. The impact of metabolism on DNA methylation. *Human Molecular Genetics* 14 Spec No 1:R139–R147, 2005. PMID: 15809266

Um, J.H.; Pendergast, J.S.; Springer, D.A.; et al. AMPK regulates circadian rhythms in a tissue-and isoform-specific manner. *PLoS One* 6:e18450, 2011. http://dx.doi.org/10.1371/journal.pone.0018450 PMID: 21483791

Vaquero, A., and Reinberg, D. Calorie restriction and the exercise of chromatin. *Genes & Development* 23:1849–1869, 2009. PMID: 19608767

Varier, R.A., and Timmers, H.T. Histone lysine methylation and demethylation pathways in cancer. *Biochimica et Biophysica Acta* 1815:75–89, 2011. PMID: 20951770

Vaziri, H.; Dessain, S.K.; Ng Eaton, E.; et al. hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell* 107:149–159, 2001. PMID: 11672523

Veech, R.L.; Eggleston, L.V.; and Krebs, H.A. The redox state of free nicotinamide-adenine dinucleotide phosphate in the cytoplasm of rat liver. *Biochemical Journal* 115:609–619, 1969. PMID: 4391039

Wang, X.S.; Armstrong, M.E.; Cairns, B.J.; et al. Shift work and chronic disease: The epidemiological evidence. *Occupational Medicine (London)* 61:78–89, 2011. PMID: 21355031

Wellen, K.E., and Thompson, C.B. A two-way street: Reciprocal regulation of metabolism and signalling. *Nature Reviews. Molecular Cell Biology* 13:270–276, 2012. PMID: 22395772

Wolf, A.; Agnihotri, S.; Munoz, D.; and Guha, A. Developmental profile and regulation of the glycolytic enzyme hexokinase 2 in normal brain and glioblastoma multiforme. *Neurobiology of Disease* 44:84–91, 2011. PMID: 21726646

Ying, W.; Chen, Y.; Alano, C.C.; and Swanson, R.A. Tricarboxylic acid cycle substrates prevent PARP-mediated death of neurons and astrocytes. *Journal of Cerebral Blood Flow and Metabolism* 22:774–779, 2002. PMID: 12142562

Ying, W.; Garnier, P.; and Swanson, R.A. NAD+ repletion prevents PARP-1-induced glycolytic blockade and cell death in cultured mouse astrocytes. *Biochemical and Biophysical Research Communications* 308:809–813, 2003. PMID: 12927790

Yu, S.W.; Wang, H.; Poitras, M.F.; et al. Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. *Science* 297:259–263, 2002. PMID: 12114629

Zhang, J.; Dawson, V.L.; Dawson, T.M.; and Snyder, S.H. Nitric oxide activation of poly(ADP-ribose) synthetase in neurotoxicity. *Science* 263:687–689, 1994. PMID: 8080500

Zhang, Q.; Piston, D.W.; and Goodman, R.H. Regulation of corepressor function by nuclear NADH. *Science* 295:1895–1897, 2002. PMID: 11847309

Zhang, K., and Dent, S.Y. Histone modifying enzymes and cancer: Going beyond histones. *Journal of Cellular Biochemistry* 96:1137–1148, 2005. PMID: 16173079

Ziech, D.; Franco, R.; Pappa, A.; and Panayiotidis, M.I. Reactive oxygen species (ROS)—Induced genetic and epigenetic alterations in human carcinogenesis. *Mutation Research* 711:167–173, 2011. PMID: 21419141

Zima, A.V.; Copello, J.A.; and Blatter, L.A. Differential modulation of cardiac and skeletal muscle ryanodine receptors by NADH. *FEBS Letters* 547:32–36, 2003. PMID: 12860382

Epigenetics—New Frontier for Alcohol Research

Shivendra D. Shukla, Ph.D., and Samir Zakhari, Ph.D.

Shivendra D. Shukla, Ph.D., *is Margaret Proctor Mulligan Professor in the Department of Medical Pharmacology & Physiology, School of Medicine, University of Missouri, Columbia, Missouri.*

Samir Zakhari, Ph.D., *former director of the Division of Metabolism and Health Effects at the National Institute on Alcohol Abuse and Alcoholism, is Senior Vice President of Science, the Distilled Spirits Council of the United States (DISCUS), Washington, DC.*

The term "epigenetics" is rapidly becoming one of the more important watchwords in the field of alcohol research. Put simply, epigenetics is the study of changes in gene function that occur without a change in the body's genetic code, instead relying on epigenetic markers on, among others, the DNA and certain nuclear proteins to turn genes "on" and "off." Epigenetic changes also are brought about by histone modifications, as well as by the role that noncoding RNA (ncRNA) plays. By acting on these epigenetic markers, environmental factors such as diet, stress, and prenatal nutrition can make an imprint on the genes that are active in different tissues and at various stages of life. Even more importantly, these alterations may be passed along from one generation to the next. The result is that the influences from harmful environmental factors can be extended beyond the individual and passed to his or her offspring.

This issue of *Alcohol Research: Current Reviews* explores the concept of epigenetics and the role it plays—not only in shaping the key characteristics that ensure normal functioning but also which may lead to disease. In particular, the issue looks at how epigenetics influences the body's response to alcohol and the development of alcohol use disorders and various disease states.

The concept of epigenetics is not new, originating in the 1940s (Waddington 1942). The idea was put forth to explain alterations in an organism's phenotype that could not be attributed to modifications in its genotype. Or, in more modern terms, epigenetic modifications are the reason that identical DNA sequences can lead to different gene expression profiles. The specific nature of these modifications, however, has come to light only in recent years. These findings have painted a fascinating and complex picture involving the coordinated interplay of numerous regulatory epigenetic mechanisms that help ensure the organism's normal development and function as well as adaptability to changes in environmental conditions. Importantly, epigenetic modifications of both DNA and histones are time- and tissue- or organ-specific; as a result, disruptions of the epigenome can have vastly diverse consequences, depending on the developmental stage and tissue or organ affected.

One crucial epigenetic mechanism involves methylation of the DNA, particularly in regulatory regions, which typically results in the silencing of genes. Other modifications center on the histone proteins that help package the DNA in the cell nucleus and which determine how accessible the DNA is to the proteins required for gene expression. Finally, several types of non–protein-coding RNA transcripts also can influence the epigenetic status of the cell. If any of these finely tuned mechanisms goes awry, changes in gene expression result that can increase susceptibility to disease (Shukla and colleagues 2008). In fact, epigenetic mechanisms

have been linked to numerous diseases, including cancer, autoimmune disease, and age-related and neurological disorders (Moss and Wallrath 2007; Rodenhiser and Mann 2006).

Environmental factors, including toxic agents and drugs, can exert some of their harmful effects by altering normal epigenetic patterns, leading to abnormal expression or silencing of essential genes and their encoded proteins. Alcohol is fast emerging as one of the chief agents to alter the epigenome of cells and tissues throughout the organism.

The precise regulatory mechanisms through which ethanol alters DNA methylation and histone modifications and, consequently, gene expression are only beginning to be elucidated. This issue features some of the latest discoveries in the field. The authors summarize what is currently known about epigenetic changes related to alcohol metabolism and explore the relationship between alcohol-related epigenetic disturbances and in utero development and the pathophysiology of fetal alcohol spectrum disorders (FASD). Other reviews demonstrate how far-reaching epigenetic influences can be, influencing all major body systems, including the liver and gastrointestinal system, the brain, and the immune system.

Clearly, epigenetic changes, whether transient or permanent, play a pivotal role in mediating alcohol's actions in a variety of cells and organ systems. Understanding the exact nature of alcohol's interactions with the epigenome will help scientists design better medications to treat or alleviate a wide range of alcohol-related disorders, including FASD, alcohol addiction, and organ damage. The articles in this journal issue are testament to the progress researchers have made in recent years toward this goal.

References

Moss, T.J., and Wallrath, L.L. Connections between epigenetic silencing and human disease. *Mutation Research* 618:163–174, 2007.

Rodenhiser, D., and Mann, M. Epigenetics and human disease: Translating basic biology into clinical applications. *Canadian Medical Association Journal* 174:341–348, 2006.

Shukla, S.D.; Valaquez, J.; French, S.W.; et al. Emerging role of epigenetics in the actions of alcohol. Alcoholism: *Clinical and Experimental Research* 32(9):1525–1534, 2008.

Waddington, C.H. Canalization of development and inheritance of acquired characters. *Nature* 150:563–565, 1942.

Stress, Epigenetics, and Alcoholism

Sachin Moonat, M.S., and Subhash C. Pandey, Ph.D.

Acute and chronic stressors have been associated with alterations in mood and increased anxiety that may eventually result in the development of stress-related psychiatric disorders. Stress and associated disorders, including anxiety, are key factors in the development of alcoholism because alcohol consumption can temporarily reduce the drinker's dysphoria. One molecule that may help mediate the relationship between stress and alcohol consumption is brain-derived neurotrophic factor (BDNF), a protein that regulates the structure and function of the sites where two nerve cells interact and exchange nerve signals (i.e., synapses) and which is involved in numerous physiological processes. Aberrant regulation of BDNF signaling and alterations in synapse activity (i.e., synaptic plasticity) have been associated with the pathophysiology of stress-related disorders and alcoholism. Mechanisms that contribute to the regulation of genetic information without modification of the DNA sequence (i.e., epigenetic mechanisms) may play a role in the complex control of BDNF signaling and synaptic plasticity—for example, by modifying the structure of the DNA–protein complexes (i.e., chromatin) that make up the chromosomes and thereby modulating the expression of certain genes. Studies regarding the epigenetic control of BDNF signaling and synaptic plasticity provide a promising direction to understand the mechanisms mediating the interaction between stress and alcoholism. **KEY WORDS: Alcoholism; alcohol consumption; genetic factors; epigenetics; acute stressors; anxiety disorders; stress-related psychiatric disorders; physiological response to stress; dysphoria; brain; brain-derived neurotrophic factor (BDNF); synaptic plasticity**

Alcoholism is a complex disorder characterized by compulsive alcohol seeking and consumption that also is impacted by related psychiatric states, such as anxiety (Koob 2003; Pandey 2003). Both environmental and genetic factors influence alcohol drinking patterns and may increase susceptibility to the development of alcohol addiction (Cloninger 1987; Crabbe 2002). The presence or development of comorbid stress-related psychiatric disorders, which typically are characterized by features such as altered mood and anxiety, often has been associated with an increased propensity for alcoholism (Bolton et al. 2009; Grant et al. 2004; Schuckit and Hesselbrock 1994). More specifically, alcohol consumption is thought to reduce negative symptoms such as depressed mood and anxiety (i.e., dysphoria) linked with stress-related disorders, which ultimately results in self-medication (Bolton et al. 2009; Robinson et al. 2009).

Acute and chronic stressors also may be important factors in regulating alcohol craving and may play a significant role in the relapse to alcohol and drug dependence (Breese et al. 2011; Self and Nestler 1998; Sinha 2007; Uhart and Wand 2009). Various forms of stress, including early-life stress; severe acute stress, such as that experienced in posttraumatic stress disorder (PTSD); and chronic stress, likely can be associated with an increased risk of alcohol and drug dependence (Gordon 2002; Sinha 2008; Uhart and Wand 2009). At the same time, early alcohol exposure and acute alcohol withdrawal may increase vulnerability to stress that may result in the development of negative affective states, such as anxiety or depression (Guerri and Pascual 2010; Hellemans et al. 2010; Koob 2003; Pandey 2003). Taken together, these findings delineate an intricate and complex relationship between stress and alcohol exposure and have stimulated various lines of research that attempt to identify the molecular mechanisms involved in the development of dysphoric symptoms related to the pathophysiology of alcoholism (Koob 2003; Moonat et al. 2010; Pandey 2003).

One focus of this research is the hypothalamus, a key brain region involved in the body's two main stress response systems: (1) the hormonal system known as the hypothalamic–pituitary–adrenal axis that culminates in the release of stress hormones from the adrenal glands to elicit responses throughout the body and (2) the brain's central stress response system that includes clusters of brain cells (i.e., nuclei) in the limbic system and autonomic centers of the brain stem (Koob 2008; Smith and Vale 2006). The neurocircuitry related to the central stress response comprises connections between various hypothalamic nuclei, the hippocampus, brain stem nuclei, and a system of interconnected nuclei in the basal forebrain, the extended amygdala (Koob 2008, 2009). These include the central nucleus of amygdala (CeA), medial nucleus of amygdala (MeA), bed nucleus of the stria terminalis, and the shell of the nucleus accumbens (NAc) (Alheid 2003; Koob 2003). Some regions of the extended amygdala, such as the CeA, also have been associated with the development of alcoholism and stress-related disorders such as anxiety,

Sachin Moonat, M.S., *is a doctoral student in the neuroscience graduate program at the University of Illinois at Chicago, Chicago, Illinois.*

Subhash C. Pandey, Ph.D., *is a professor and director of Neuroscience Alcoholism Research in the Department of Psychiatry and Department of Anatomy and Cell Biology at the University of Illinois at Chicago and a Veterans Affairs career scientist at the Jesse Brown Veterans Affairs Medical Center, both in Chicago, Illinois.*

suggesting that the extended amygdala is a neuroanatomical substrate for the interaction between stress and alcoholism (Koob and Volkow 2010; Pandey 2003, 2004).

One mechanism that may provide a link between stress-related psychiatric disorders and alcoholism is modification of synaptic plasticity via neuroadaptation (figure 1) (Moonat et al. 2010; Pandey et al. 2008*b*; Pittenger and Duman 2008). Studies found that ethanol exposure and related withdrawal symptoms can result in structural and functional modifications at the sites where two nerve cells (i.e., neurons) interact and transmit nerve signals (i.e., at the synapse). These modifications at the synaptic level have been observed in various brain regions as well as in neuronal cultures (Carpenter-Hyland and Chandler 2006; Pandey et al. 2008*b*; Roberto et al. 2002; Zhou et al. 2007). Chronic stress also is associated with changes in structural and functional plasticity in various brain regions, including the hippocampus, amygdala, and prefrontal cortex (Goldwater et al. 2009; Pavlides et al. 2002; Roozendaal et al. 2009). Neuroadaptation associated with ethanol exposure or stress plays a role in the onset of dysphoric symptoms that may manifest as stress-related psychiatric disorders or withdrawal-induced anxiety (Pandey et al. 2008*b*; Pittenger and Duman 2008; Roozendaal et al. 2009).

One molecule that has been implicated in synaptic plasticity and long-term memory formation is a protein, cAMP-responsive element binding (CREB) (Abel and Kandel 1998; Alberini 2009; Waltereit and Weller 2003). It also has been recognized as a critical modulator of neuroadaptation associated with alcoholism (Misra et al. 2001; Pandey 2004; Pandey et al. 2003, 2005) and the effects of stress (Barrot et al. 2002; Bilang-Bleuel et al. 2002; Carlezon et al. 2005). CREB is a transcription factor—that is, it helps regulate the first step in the conversion of the genetic information encoded in the DNA into finished protein products (i.e., transcription) by binding to specific DNA sequences in its target genes. To exert its effects, CREB must be activated by the addition of a phosphate group to (i.e., phosphorylation of) the amino acid serine at position 133 of the CREB protein. This phosphorylation is performed by enzymes, protein kinases, that are associated with various signaling cascades, including the mitogen-activated protein kinase (MAPK) pathway (Impey et al. 1999; Shaywitz and Greenberg 1999; Waltereit and Weller 2003). One target gene of CREB encodes a molecule, brain-derived neurotrophic factor (BDNF), which plays an important role in the regulation of synaptic plasticity and dendritic spine structure (Minichiello 2009; Poo 2001; Tao et al. 1998; Soule et al. 2006). (For more information on dendritic spines, see the textbox "Histone Acetylation and Dendritic Spines.") BDNF also may mediate changes in synaptic plasticity that accompany both alcohol exposure (Moonat et al. 2010, 2011; Pandey et al. 2008*b*) and stress (Briand and Blendy 2010; Duman and Monteggia 2006). Accordingly, researchers have begun to investigate how BDNF activity is controlled. These studies have determined that mechanisms contributing to the regulation of gene transcription that do not involve alterations of the DNA sequence (i.e., epigenetic mechanisms) seem to play a role in the regulation of BDNF activity as well as in synaptic plasticity (Guan et al. 2009; Lubin et al. 2008; Tsankova et al. 2006). Accordingly, this topic has become a focus of research in stress and alcoholism (Elliott et al. 2010; Hunter et al. 2009; Moonat et al. 2010; Pandey et al. 2008*a*; Qiang et al. 2010).

This article reviews research that attempts to describe the role of epigenetic mechanisms in the regulation of BDNF function in alcoholism and stress. After providing an overview of epigenetic mechanisms and their role in the control of gene transcription, the article will summarize research regarding the regulation of BDNF signaling, focusing on epigenetic mechanisms involved in the regulation of BDNF expression. Finally, the article will outline the potential role of the epigenetic control of BDNF signaling and synaptic plasticity in alcoholism and stress.

Epigenetic Regulation of Gene Transcription

The term epigenetics refers to chemical modifications occurring within a genome that may modulate gene expression without changing the DNA sequence (Holliday 2006; Murrell et al. 2005; Waddington 1942). Common epigenetic alterations include the chemical modification (e.g., addition or removal of acetyl groups) of the proteins around which the DNA is wrapped (i.e., histone proteins) to form the chromosomes and the direct addition of methyl groups (i.e., methylation) to DNA. These modifications are performed by enzymes,

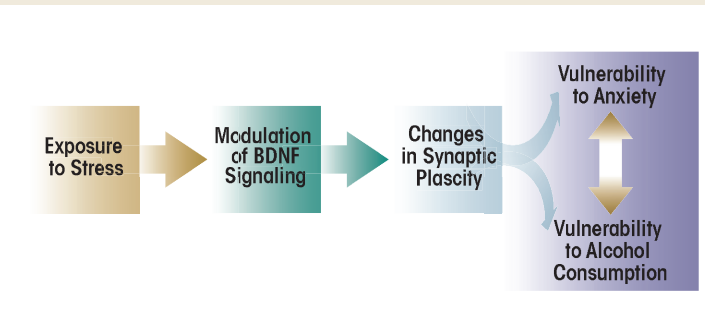


Figure 1 A psychiatric model for the relationship between stress, anxiety, and alcohol consumption and its modulation by brain-derived neurotrophic factor (BDNF) and synaptic plasticity. Exposure to stress is thought to result in the modulation of BDNF and synaptic plasticity in various brain regions. These changes may result in increased vulnerability to the development of stress-related disorders such as anxiety. Vulnerability to alcohol consumption also may be increased, either directly due to stress exposure or in response to the development of anxiety.

such as histone deacetylases (HDACs) and DNA methyltransferases (DNMTs). Both of these mechanisms work in concert to remodel the structure of the protein–DNA complex (i.e., the chromatin), thereby regulating the access of the transcriptional machinery to the DNA and, consequently, gene expression in the cell (Borrelli et al. 2008; Jenuwein and Allis 2001; Levenson and Sweatt 2005; for more information, also see Starkman et al. 2012).

Histone Acetylation

The basic unit of chromatin, a nucleosome, consists of four histone protein subtypes that form an octamer around which the DNA is wrapped (Jenuwein and Allis 2001; Smith 1991). Histone modification occurs at lysine amino acids near one end of the histone proteins and, as mentioned earlier, involves the addition and removal of acetyl groups. The level of acetylation of the histones determines how tightly the DNA is wound around the histones and how tightly the nucleosomes are stacked together. In the presence of many acetyl groups (i.e., hyperacetylation) at specific lysine residue of histones H3 and H4, the chromatin is relaxed and accessible to the transcriptional proteins, resulting in increased gene transcription; conversely, in the presence of only few acetyl groups (i.e., hypoacetylation), the chromatin is condensed, preventing access of transcriptional proteins and resulting in gene silencing (Smith 1991; Strahl and Allis 2000).

HDACs are enzymes that can remove acetyl groups from histone proteins; they seem to be key elements in the regulation of chromatin structure and function (figure 2) (Jenuwein and Allis 2001). Inhibition of HDAC enzymes by pharmacological intervention is effective in the treatment of some cancers, and numerous HDAC inhibitors have been approved or are in clinical trials for this purpose (Dokmanovic et al. 2007; Lane and Chabner 2009). Recently, HDAC inhibitors also have been explored as potential therapeutic agents in the treatment of psychiatric disorders, including stress-related disorders and addiction, and have become an important focus of research in the neuroscience field (Covington et al. 2009; Kumar et al. 2005; Pandey et al. 2008*a*; Renthal and Nestler 2008; Tsankova et al. 2007). Several HDAC isoforms have been identified and grouped into four classes based upon their regulation and cellular localization (de Ruijter et al. 2003; Dokmanovic et al. 2007). Specific HDAC variants (i.e., isoforms) recently have been identified as regulators of neuronal processes such as synaptic plasticity (Guan et al. 2009; Renthal and Nestler 2008). This suggests that use of isoform-specific HDAC inhibitors may increase the specificity and efficacy of these drugs in the treatment of psychiatric disorders.

HDAC-Induced Histone Deacetylation and Dendritic Spines

Dendritic spines are protuberances that make up the sites where incoming signals from other nerve cells are received (i.e., the postsynaptic terminals) along dendritic processes. The overall number of dendritic spines, their shape, and their distribution on the dendritic processes can change rapidly. This compartmentalization of dendritic spines may allow for the regulation of synaptic plasticity at an individual synapse (Yuste 2011; Higley and Sabatini 2008). For example, various intracellular signaling mechanisms, including brain-derived neurotrophic factor (BDNF) signaling via activity-regulated cytoskeleton-associated (Arc) protein, can regulate the structural and functional components of dendritic spines associated with long-term potentiation (LTP) and synaptic plasticity (Bramham et al. 2008; Minichiello 2009; Soule et al. 2006).

Epigenetic mechanisms also may play a role in the regulation of dendritic spines. Specifically, a recent study (Guan et al. 2009) noted that one histone deacetylase (HDAC) subtype, HDAC2, is involved in the regulation of dendritic spines. When studying mice that produced excessive levels of HDAC2, the investigators found that increased HDAC2 levels were associated with reduced memory formation in a fear-conditioning paradigm and that this impairment was associated with a reduction in dendritic spine density in the hippocampus. Treatment of HDAC2-overexpressing mice with HDAC inhibitors reversed these deficits. On the other hand, animals in which the gene encoding HDAC2 was inactivated (i.e., HDAC2 knockout animals) showed improved learning and increased dendritic spine density (Guan et al. 2009). These findings suggest that HDAC2 plays a role in the regulation of synaptic plasticity; however, future studies may be necessary to identify the specific genes that are regulated by HDAC2 in the control of neuronal function and structure. Given the involvement of brain-derived neurotrophic factor (BDNF) in synaptic plasticity, it may be useful to evaluate the potential regulation of BDNF signaling by HDAC2 in learning at the neuronal and behavioral levels.

DNA Methylation

The chromatin structure also can be modified by adding methyl groups to certain DNA building blocks (i.e., cytosine nucleotides) in a particular gene, resulting in transcriptional silencing (see figure 2). The level of DNA methylation is controlled by three DNMT subtypes that seem to be differentially regulated and preferentially methylate at specific DNA sequences (Antequera 2003; Bestor 2000; Okano et al. 1999). DNA methylation can inhibit transcription either directly, by blocking the binding of transcriptional machinery to DNA, or indirectly, via methyl-CpG binding domain proteins (MBDs) (Fan and Hutnick 2005; Wade 2001).

These proteins, including MeCP2, seem to directly regulate the condensation of chromatin structure and recruit HDACs and DNMTs, which may further enzymatically modify chromatin components (see figure 2) (Fuks et al. 2000; Kimura and Shiota 2003; Nan et al. 1998). Mutations in the *MeCP2* gene and, consequently, the resulting protein that alter transcription of the gene encoding BDNF and affect synaptic plasticity are thought to underlie a neurodevelopmental disorder, Rett syndrome (Chahrour and Zoghbi 2007; Chang et al. 2006; Monteggia and Kavalali 2009; Zhou et al. 2006). Thus, the coordinated actions of HDACs, DNMTs, and MBDs form a complex regulatory network that modulates neuronal function, and dysregulation of these proteins has been implicated in a variety of psychiatric disorders.

Researchers are beginning to identify the role of epigenetic mechanisms in the regulation of gene transcription related to alcohol exposure and the development of alcoholism (Kim and Shukla 2006; Moonat et al. 2010; Pandey et al. 2008*a*; Qiang et al. 2010). Moreover, histone modifications and DNA methylation are involved in the dysphoric states induced by acute and chronic stress (Elliott et al. 2010; Fuchikami et al. 2009; Hunter et al. 2009; Tsankova et al. 2006). Specifically, various studies have demonstrated that epigenetic mechanisms are involved in the regulation of *BDNF* gene transcription, which in turn plays a role in the modulation of synaptic structure and function (He et al. 2010; Lubin et al. 2008; Tsankova et al. 2006). This will be discussed in the following section.

The Regulation of BDNF Expression and Signaling

BDNF signaling seems to be an important factor in the intracellular processes which occur following neuronal activation (i.e., activity-dependent processes) that play a role in synaptic plasticity and the regulation of dendritic morphology (Messaoudi et al. 2007; Poo 2001; Soule et al. 2006; Ying et al. 2002). BDNF acts by binding to a receptor molecule,

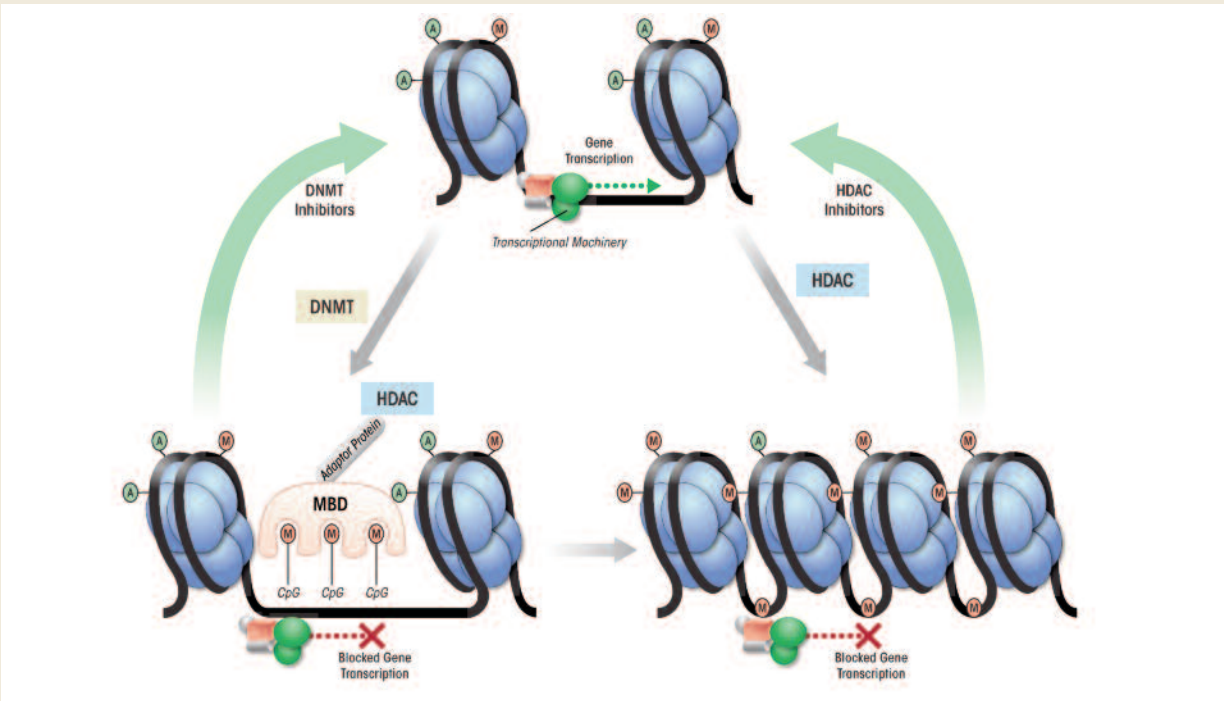


Figure 2 The coordinated actions of proteins involved in epigenetic modifications that regulate gene transcriptional processes. During the first step in the conversion of genetic information encoded in the DNA into gene products (i.e., during gene transcription), the DNA to be transcribed is associated with histone proteins (light blue) that are modified by the addition of acetyl groups (green). This modification results in a relaxed chromatin configuration that allows the transcriptional machinery access to the DNA. Enzymes, DNA methyltransferases (DNMTs), can add methyl groups (red) to the DNA at certain sequences of DNA building blocks (i.e., CpG islands). This methylation causes recruitment of methyl binding domain (MBD) protein complexes that also include repressor proteins, such as histone deacetylases (HDAC). The HDACs remove acetyl groups from histone proteins, resulting in a condensed chromatin that limits the binding of the transcriptional machinery, thereby decreasing gene transcription. Thus, activation of both DNMT and HDAC causes a reduction in gene transcription. Treatment with DNMT inhibitors and HDAC inhibitors may block these enzymatic processes and return the chromatin to a relaxed state, allowing gene transcription.

tyrosine kinase B (TrkB), which can phosphorylate other proteins as well as itself. The interaction of TrkB with BDNF results in dimerization and autophosphorylation of the receptor (Minichiello 2009; Reichardt 2006). When the TrkB receptor becomes phosphorylated, it can bind to "adaptor molecules" that then can initiate three primary intracellular signaling cascades (Impey et al. 1999; Minichiello 2009; Reichardt 2006):

- The MAPK pathway;
- The phospatidylinositol 3-kinase (PI3K) pathway; and
- The phospholipase Cγ (PLCγ) pathway.

The activation of these cascades, particularly the MAPK pathway, ultimately results in the recruitment and phosphorylation of two transcription factors, CREB and Elk-1, which in turn enhance the expression of a gene, *activity-regulated cytoskeleton-associated* (*Arc*) immediate-early gene,[1] (see figure 3) (Bramham et al. 2008; Pandey et al. 2008*b*; Ramanan et al. 2005; Ying et al. 2002). Arc protein plays a role in the induction of a process, long-term potentiation, and is believed to result in the proliferation of dendritic spines (Huang et al. 2007; Messaoudi et al. 2007; Pandey et al. 2008*b*; Ying et al. 2002). Thus, BDNF plays an important role in the regulation of synaptic plasticity by activating TrkB-coupled signaling and causing induction of *Arc* immediate-early gene.

BDNF is a member of the neurotrophin family whose activity is governed by complex regulatory mechanisms at the transcriptional, translational, and posttranslational levels of gene expression.[2] The gene encoding BDNF has a complex structure that allows for dynamic control over the expression of the gene region that encodes the actual BDNF protein by allowing for differential regulation of transcription via a wide variety of signaling and epigenetic mechanisms (Aid et al. 2007; Tao et al. 1998; Tsankova et al. 2004). For example, several regulatory elements (i.e., promoters) control *BDNF* transcription, with certain promoters active only in certain cells. As a result, several distinct *BDNF* transcripts (i.e., messenger RNAs [mRNAs]) can be generated that differ in the tissues and cells where they are produced; for example, certain *BDNF* mRNAs specifically are targeted to the neuronal dendrites (Aid et al. 2007; An et al. 2008; Greenberg et al. 2009; Timmusk et al. 1993). Specific *BDNF* transcripts also seem to be differentially regulated by activity-dependent processes. For example, some *BDNF* transcripts are regulated by the CREB transcription factor, and transcription of the same *BDNF* mRNAs is increased after consolidation of fear learning (Lubin et al. 2008; Ou and Gean 2007; Tao et al. 1998). In this manner, BDNF expression is regulated by CREB and, in turn, BDNF signaling also helps modulate CREB activity (Pandey et al. 2008*b*; Pizzorusso et al. 2000; Ying et al. 2002).

Role of Epigenetic Mechanisms

Epigenetic mechanisms, specifically histone modifications and DNA methylation, regulate BDNF expression via specific promoter regions for the *BDNF* gene. Huang and colleagues (2002) demonstrated that histone acetylation resulted in enhanced BDNF expression. Specifically, the level of histone acetylation associated with *BDNF* promoter II was increased in the hippocampus, suggesting a role for chromatin remodeling in the regulation of *BDNF*. Tsankova and colleagues (2004) also showed that histone acetylation influenced hippocampal BDNF expression in a model of electroconvulsive shock therapy, demonstrating that time- and promoter-dependent changes in histone acetylation levels were associated with similar changes in BDNF expression. Other investigators subsequently found that histone modifications were involved in the regulation of BDNF expression in the striatum during chronic cocaine exposure and in the hippocampus in a model of depression induced by chronic social-defeat stress (Kumar et al. 2005; Tsankova et al. 2006). Importantly, these studies determined that specific HDAC isoforms participated in the complex process of chromatin remodeling, suggesting a therapeutic role for isoform-specific HDAC inhibitors in alcohol and drugs of abuse as well as in depression (Kumar et al. 2005; Tsankova et al. 2006; Renthal and Nestler 2008). (Another role for HDAC activity—namely, in the regulation of dendritic spines—is discussed in the textbox "Histone Deacetylation and Dendritic Spines.")

As mentioned earlier, DNA methylation can inhibit transcription indirectly, via MBDs that seem to regulate the condensation of chromatin structure and recruit HDACs and DNMTs. One of these MBDs is MeCP2, which represses gene transcription via coordinated binding of methylated DNA, HDACs, and DNMT1 (Ballestar and Wolffe 2001). MeCP2 plays a role in the activity-dependent regulation of BDNF expression in neurons. Specifically, enhanced expression of one of the BDNF variants (i.e., BDNF exon IV) following arrival of a nerve impulse in the neurons (i.e., following depolarization) was associated with increased histone acetylation, reduced DNA methylation, and reduced MeCP2 binding at the promoter for that BDNF variant. This suggests that BDNF expression is regulated dynamically by chromatin remodeling (Martinowich et al. 2003). MeCP2-dependent regulation of this BDNF variant also is involved in regulating the formation of dendritic spines (Zhou et al. 2006).

The association between MeCP2 and BDNF exon IV levels is mediated at least in part by a protein, RACK1. This protein associates with histones H3 and H4 at the *BDNF* exon IV promoter and causes MeCP2 to dissociate from the *BDNF* gene (He et al. 2010). RACK1-mediated dissociation of MeCP2 from the *BDNF* gene leads to increased histone

[1] This gene also is known as activity-regulated gene 3.1 (Arg3.1).

[2] Transcription is the first step of gene expression, in which the genetic information encoded in the DNA is copied into an intermediate molecule, mRNA. In the second step of gene expression, translation, the mRNA then serves as a template for the synthesis of the proteins that are the gene products. After translation (i.e., posttranslationally) these proteins can be modified further by the addition of certain chemical groups.

acetylation at the *BDNF* exon IV promoter and, in turn, increases BDNF expression (He et al. 2010). Other studies found that reduction of DNA methylation levels in the *BDNF* exon IV promoter region increased BDNF expression during a fear conditioning experiment (Lubin et al. 2008). Of interest, in that study BDNF exon IV expression specifically was associated with the consolidation of fear memory, whereas increases in other BDNF variants (i.e., BDNF exons I and VI) occurred with the presentation of context alone (Lubin et al. 2008).

Taken together, these findings provide evidence for the overlap between histone modifications and DNA methylation in the regulation of *BDNF* gene expression, which may be associated with activity-dependent changes in synaptic plasticity.

BDNF and Epigenetic Mechanisms in Stress and Alcoholism

BDNF and Stress

Chronic stress has been linked with shrinkage of brain tissue (i.e., neuronal atrophy) and modulation of dendritic structure in the hippocampus (McEwen 2008; Watanabe et al. 1992) and was associated with reduced BDNF levels in that brain structure (Smith et al. 1995). In addition, both acute and chronic stress may modulate BDNF levels and structural plasticity in a variety of brain areas, including the hippocampus, prefrontal cortex, and amygdala (Calabrese et al. 2009;

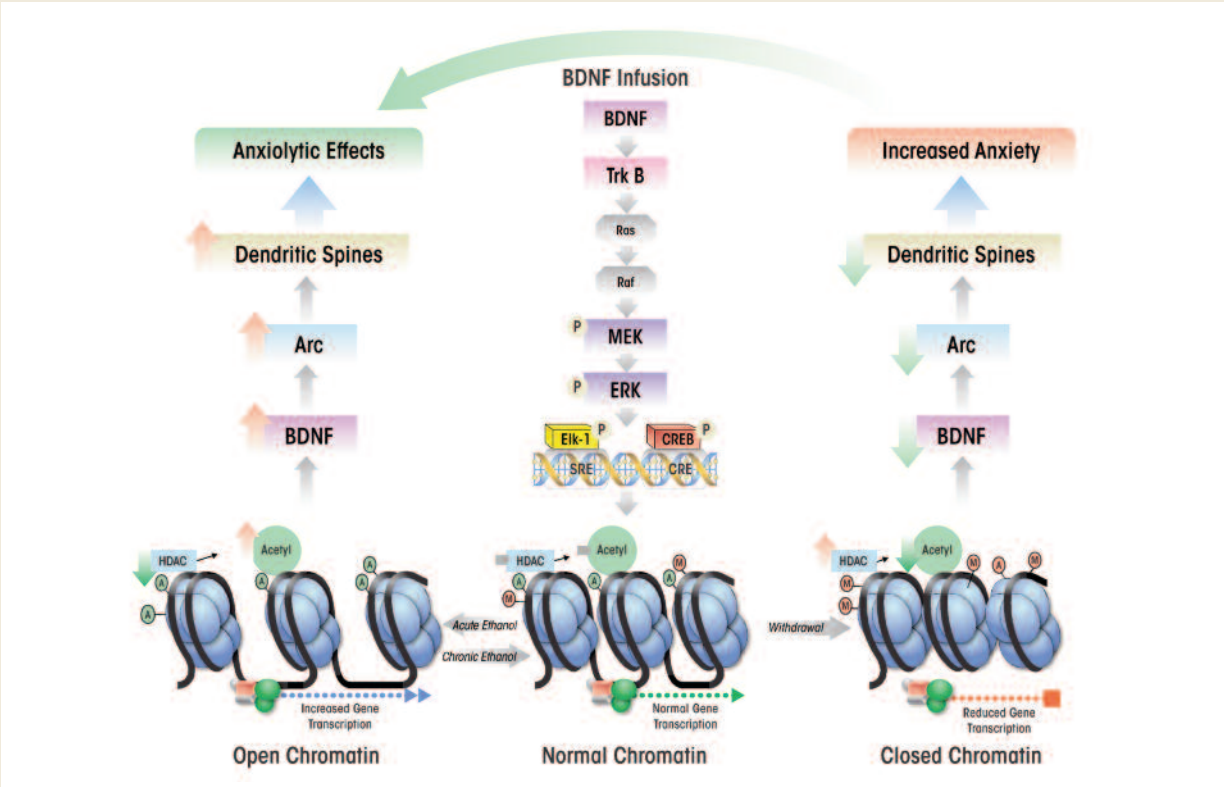


Figure 3 A hypothetical model for the role of brain-derived neurotrophic factor (BDNF) signaling and chromatin remodeling in central amygdaloid brain regions in the regulation of anxiety induced by acute ethanol and ethanol withdrawal. BDNF binding to tyrosine receptor kinase B (TrkB) triggers several signaling cascades that culminate in the activation of transcription factors, Elk-1 and cAMP-responsive element binding protein (CREB). Under normal conditions, histone deacetylase (HDAC) levels and histone acetylation are adequate to allow for normally regulated chromatin structure and gene transcription. Acute ethanol exposure inhibits HDAC, resulting in increased histone acetylation and an open chromatin conformation. This may lead to increased transcription of BDNF as well as higher levels of a protein, activity-regulated cytoskeleton associated protein (Arc), thereby increasing dendritic spine density. The modulation of these synaptic factors results in anxiety-reducing (i.e., anxiolytic) behavioral effects. In contrast during withdrawal from chronic ethanol exposure HDAC activity increases, resulting in a reduction of histone acetylation that in turn closes the chromatin conformation and reduces gene transcription. The resulting low BDNF levels decrease Arc and dendritic spine density, all of which are associated with anxiety-like behaviors. This model is further supported by the fact that exogenous infusion of BDNF into the CeA reduces anxiety-like behaviors in ethanol withdrawn rats and is associated with increased BDNF and Arc levels (Moonat et al. 2010; Pandey et al. 2008*a*, 2008*b*).

McEwen 2008; Pizarro et al. 2004). In the hippocampus, acute stress caused by immobilization as well as swim stress increased the levels of *BDNF* mRNA. This increase was associated with increased MeCP2 phosphorylation, suggesting that epigenetic mechanisms help mediate the effects of acute stress (Marmigere et al. 2003; Molteni et al. 2009). Increased BDNF expression may represent a protective mechanism in response to stress; conversely, reduced BDNF levels after exposure to repetitive and chronic stress appear to represent a dysregulation of this mechanism (Calabrese et al. 2009; McEwen 2008). This assumption is supported by findings that the antidepressant effects of medications used in chronic-stress models of depression are mediated by an increase in BDNF levels in the hippocampus (Nibuya et al. 1995; Shirayama et al. 2002; Tsankova et al. 2006). It also is interesting to note that low BDNF levels in the CeA and MeA mediate anxiety-like behaviors, and the anxiety-reducing (i.e., anxiolytic) effects of alcohol may be associated with an increase in BDNF signaling (Moonat et al. 2011; Pandey et al. 2006, 2008*b*). These observations clearly suggest that aberrations of BDNF signaling contribute to the development of stress-related dysphoric behaviors, and the BDNF signaling pathway therefore may be a promising potential therapeutic target for treatment of these disorders.

Role of Chromatin Remodeling. Researchers recently also have begun to investigate the role of chromatin remodeling in BDNF signaling associated with stress-related dysphoria. Using a model of depression induced by chronic stress, Tsankova and colleagues (2006) found that the levels of the *BDNF* exon IV and exon VI were reduced in the hippocampus and that this effect could be blocked by chronic antidepressant treatment (Tsankova et al. 2006). Further analyses found that this effect likely was associated with changes in histone methylation because chronic stress increased the levels of methylated histone H3 protein near the *BDNF* exons IV and VI promoters, which interferes with *BDNF* transcription. Conversely, treatment with antidepressants reduced the levels of histone methylation and increased the levels of acetylated H3 associated with these *BDNF* promoters, thereby increasing BDNF expression. Simultaneously, antidepressant treatment reduced the expression of HDAC5, but when the levels of HDAC5 were elevated through genetic engineering, the effects of antidepressant treatment were reduced (Tsankova et al. 2006).

The levels of several HDACs in the NAc also may influence the development of stress-related dysphoria. In contrast to the hippocampus, HDAC2 and HDAC5 levels in the NAc were reduced by chronic stress, suggesting opposing roles for histone modifications in the hippocampus and NAc in stress-related dysphoria (Renthal et al. 2007). Interestingly, systemic treatment with HDAC inhibitors or infusion of HDAC inhibitors into the NAc reduced stress-related dysphoria (Covington et al. 2009; Tsankova et al. 2006). Taken together, all these results suggest that histone modifications may be involved in the regulation of BDNF in stress-related dysphoria and the therapeutic effects of antidepressants.

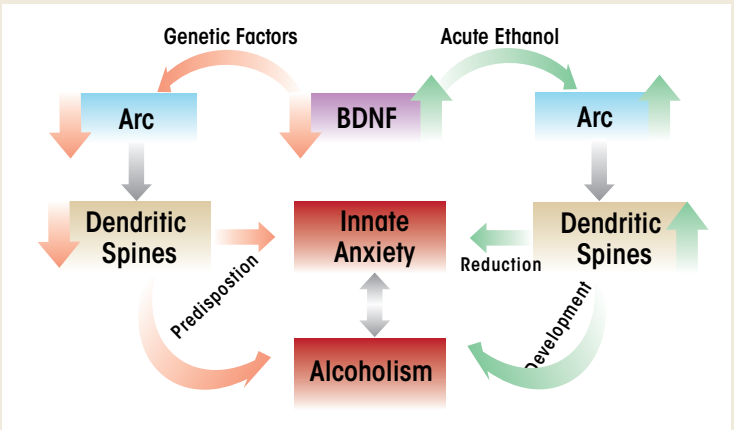


Figure 4 A hypothetical model for the role of amygdaloid brain-derived neurotrophic factor (BDNF) in the regulation of activity-regulated cytoskeleton-associated protein (Arc) and dendritic spine density in the comorbidity between innate anxiety and alcohol preference. Genetic factors may lead to innately low levels of amygdaloid BDNF that result in reduced Arc and dendritic spine density and which are associated with a predisposition to innate anxiety-like behaviors. Acute ethanol exposure increases BDNF signaling and associated synaptic factors, Arc, and dendritic spine density and results in a reduction of innate anxiety. Taken together, innate anxiety and a reduction of this anxiety by acute ethanol may be responsible for the development of alcoholism (Moonat et al. 2011).

Role of DNA Methylation. DNA methylation also plays a role in the development of stress-related dysphoria as well as synaptic plasticity in the NAc. Specifically, chronic stress increased expression of one DNA methyltransferase, DNMT3a, in the NAc, which was associated with an increase in depressive-like behavior (LaPlant et al. 2010). Infusion of a DNMT inhibitor into the NAc of chronically stressed animals reduced these observed behaviors. Conversely, overexpression of DNMT3a in the NAc precipitated a depression-like phenotype in animals that had not been exposed to stress. DNMT3a overexpression also resulted in the proliferation of dendritic spines (LaPlant et al. 2010). These results indicate that DNMT3a may contribute to stress-related dysphoria and control of dendritic spine structure. It would be interesting to expand upon these results and determine if a link exists between stress-associated changes in DNMT3a and methylation of the *BDNF* gene and alcoholism.

BDNF, Stress, and Alcoholism

Various researchers have explored the association of BDNF with ethanol preference, the effects of ethanol exposure, and dysphoric states associated with withdrawal from chronic ethanol exposure. BDNF deficits may lead to an increased preference for ethanol, because transgenic animals with reduced BDNF expression have a higher ethanol preference and conditioned place preference for ethanol compared with wild-type control animals (Hensler et al. 2003; McGough et al. 2004). Furthermore, ethanol exposure results in increased BDNF expression in the dorsal striatum. This increase involved a regulatory mechanism mediated by RACK1 because exogenous increases in RACK1 led to increased BDNF expression, resulting in reduced ethanol consumption (McGough et al. 2004). These findings suggest that BDNF in the dorsal striatum helps regulate neuronal homeostasis and prevent alcohol addiction (McGough et al. 2004). In addition, endogenous BDNF signaling in the dorsolateral striatum participates in the regulation of ethanol intake (Jeanblanc et al. 2009). Because, as mentioned earlier, MeCP2 is involved in the RACK1-mediated regulation of BDNF (He et al. 2010), future studies should determine whether chromatin remodeling affects BDNF expression in the dorsal striatum and, ultimately, ethanol's effects and ethanol preference.

Various studies have examined how BDNF impacts the interaction between alcohol preference and anxiety. For example, Pandey and colleagues (2006) reduced BDNF levels in the extended amygdala by introducing small molecules that can inhibit BDNF expression (i.e., antisense oligodeoxynucleotides) into the CeA or MeA. This caused increased voluntary ethanol intake and anxiety-like behaviors. The low BDNF levels resulted in reduced BDNF signaling, as evidenced by decreased levels of the phosphorylated forms of CREB and another regulatory molecule (Pandey et al. 2006). Both the effects on behavior and protein phosphorylation were reversed when BDNF was introduced together with the antisense oligonucleotides (Pandey et al. 2006). Additional studies identified a subsequent step in the signaling cascade induced by BDNF involving the Arc protein mentioned earlier. The findings suggested that the effects of reduced amygdaloid BDNF expression on ethanol preference and anxiety-like behaviors may be mediated by the downstream regulation of Arc (Pandey et al. 2008*b*). These behavioral changes were accompanied by a reduction in dendritic spine density in the CeA.

In an extension of these findings, investigators used an animal model of genetic predisposition to alcoholism and anxiety (i.e., selectively-bred alcohol-preferring [P] and nonpreferring [NP] rats) to study the role of BDNF in the extended amygdala. The studies found that compared with NP rats, P rats expressed lower levels of BDNF and Arc and had lower dendritic spine density in the CeA and MeA and that these characteristics were associated with high innate anxiety-like behaviors (Moonat et al. 2011; Prakash et al. 2008). Furthermore, acute ethanol exposure had anxiolytic effects that were associated with increased BDNF and Arc levels as well as increased dendritic spine density in the CeA and MeA in P rats, but not in NP rats (Moonat et al. 2011). These findings were consistent with earlier findings in Sprague-Dawley rats, which showed that increases in BDNF–Arc signaling and dendritic spine density in the extended amygdala were associated with the anxiolytic effects of acute ethanol (Pandey et al. 2008*b*). Withdrawal from chronic ethanol exposure provoked anxiety-like behaviors, which resulted in reduced BDNF signaling in the CeA and MeA, whereas BDNF infusion into the CeA normalized Arc levels and prevented anxiety-like behaviors (Pandey et al. 2008*b*). Taken together, these studies suggest that reduced BDNF–Arc signaling and synaptic plasticity contribute to both dysphoria associated with a genetic vulnerability for anxiety and to anxiety induced by environmental stressors, such as alcohol withdrawal (see figures 3 and 4).

Recent findings further suggest that the anxiolytic effects of acute ethanol exposure are associated with reduced HDAC activity and increased histone acetylation in the CeA and MeA (Pandey et al. 2008*a*). Conversely, withdrawal-induced anxiety following chronic ethanol treatment was linked with increased HDAC activity levels and reduced histone acetylation in these amygdaloid brain regions (see figure 3). Systemic administration of an agent that inhibits HDAC activity (i.e., trichostatin A) reduced the effects of withdrawal on histone acetylation and anxiety-like behaviors (Pandey et al. 2008*a*). Thus, treatment with HDAC inhibitors appears to have similar effects on withdrawal-induced anxiety as BDNF, and acute ethanol exposure may have similar effects on histone acetylation and BDNF levels (Pandey et al. 2008*a*, 2008*b*). Therefore, it may be important to study the potential regulation of amygdaloid BDNF by chromatin remodeling and its role in dysphoria associated with the development of alcoholism. Similarly, it may be interesting to explore the possibility that innate abnormalities in chromatin structure may affect BDNF levels, resulting in innate anxiety-like behaviors, such as those demonstrated by P rats, that may be critical to the development of alcoholism.

Conclusions

The studies reviewed here suggest that the reduction of BDNF levels may play a role in the neuroadaptation to repetitive or chronic exposure to alcohol or stress and the development of dysphoric states. Moreover, it appears that abnormalities in BDNF signaling serve as predisposing factors to innate dysphoric states that may associated with alcohol-drinking behaviors, such as anxiety (see figure 4). It also is possible that the environmental effects and genetic factors involved in an increased vulnerability to stress and alcoholism may be related to a common epigenetic mechanism that results in the dysregulation of BDNF signaling in various brain regions. Future studies are necessary to further evaluate the role of specific HDAC and DNMT variants that are involved in the epigenetic regulation of BDNF or other genes associated with synaptic plasticity during the development of pathological

behaviors associated with stress and alcohol addiction. Finally, the development and assessment of specific pharmacological agents that act via epigenetic mechanisms, such as HDAC and DNMT inhibitors, could have a significant psychotherapeutic impact on the development of stress-related disorders and the comorbidity with alcoholism. ■

Acknowledgements

The work described here from the laboratory of Dr. Subhash Pandey was supported by National Institute on Alcohol Abuse and Alcoholism grants AA-016690, AA-019971, AA-010005, and AA-013341 and by the Department of Veterans Affairs (Merit Review Grant; Research Career Scientist award).

Financial Disclosure

The authors declare that they have no competing financial interests.

References

Abel, T., and Kandel, E. Positive and negative regulatory mechanisms that mediate long-term memory storage. *Brain Research. Brain Research Reviews* 26(2–3):360–378, 1998. PMID: 9651552

Aid, T.; Kazantseva, A.; Piirsoo, M.; et al. Mouse and rat BDNF gene structure and expression revisited. *Journal of Neuroscience Research* 85(3):525–535, 2007. PMID: 17149751

Alberini, C.M. Transcription factors in long-term memory and synaptic plasticity. *Physiological Reviews* 89(1):121–145, 2009. PMID: 19126756

Alheid, G.F. Extended amygdala and basal forebrain. *Annals of the New York Academy of Sciences* 985:185–205, 2003. PMID: 12724159

An, J.J.; Gharami, K.; Liao, G.Y.; et al. Distinct role of long 3′ UTE BDNF mRNA in spine morphology and synaptic plasticity in hippocampal neurons. *Cell* 134(1):175–187, 2008. PMID: 18614020

Antequera, F. Structure, function and evolution of CpG island promoters. *Cellular and Molecular Life Sciences* 60(8):1647–1658, 2003. PMID: 14504655

Ballestar, E., and Wolffe, A.P. Methyl-CpG-binding proteins. Targeting specific gene repression. *European Journal of Biochemistry* 268(1):1–6, 2001. PIA: 11121095

Barrot, M.; Olivier, J.D.; Perrotti, L.I.; etc al. CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. *Proceedings of the National Academy of Sciences of the United States of America* 99(17):11435–11440, 2002. PMID: 12165570

Bestor, T.H. The DNA methyltransferases of mammals. *Human Molecular Genetics* 9(16):2395–2402, 2000. PMID: 11005794

Bilang-Bleuel, A.; Rech, J.; De Carli, S.; et al. Forced swimming evokes a biphasic response in CREB phosphorylation in extrahypothalamic limbic and neocortical brain structures in the rat. *European Journal of Neuroscience* 15(6):1048–1060, 2002. PMID: 11918664

Bolton, J.M.; Robinson, J.; and Sareen, J. Self-medication of mood disorders with alcohol and drugs in the National Epidemiologic Survey on Alcohol and Related Conditions. *Journal of Affective Disorders* 115(3):367–375, 2009. PMID: 19004504

Borrelli, E.; Nestler, E.J.; Allis, C.D.; and Sassone-Corsi, P. Decoding the epigenetic language of neuronal plasticity. *Neuron* 60(6):961–974, 2008. PMID: 19109904

Bramham, C.R.; Worley, P.F.; Moore, M.J.; and Guzowski, J.F. The immediate early gene arc/arg3.1: Regulation, mechanisms, and function. *Journal of Neuroscience* 28(46):11760–11767, 2008. PMID: 19005037

Breese, G.R.; Sinha, R.; and Heilig, M. Chronic alcohol neuroadaptation and stress contribute to susceptibility for alcohol craving and relapse. *Pharmacology & Therapeutics* 129(2):149–171, 2011. PMID: 20951730

Briand, L.A., and Blendy, J.A. Molecular and genetic substrates linking stress and addiction. *Brain Research* 1314:219–234, 2010. PMID: 19900417

Calabrese, F.; Molteni, R.; Racagni, G.; and Riva, M.A. Neuronal plasticity: A link between stress and mood disorders. *Psychoneuroendocrinology* 34 (Suppl. 1):S208–S216, 2009. PMID: 19541429

Carlezon, W.A., Jr.; Duman, R.S.; and Nestler, E.J. The many faces of CREB. *Trends in Neurosciences* 28(8):436–445, 2005. PMID: 15982754

Carpenter-Hyland, E.P., and Chandler, L.J. Homeostatic plasticity during alcohol exposure promotes enlargement of dendritic spines. *European Journal of Neuroscience* 24(12):3496–3506, 2006. PMID: 17229098

Chahrour, M., and Zoghbi, H.Y. The story of Rett syndrome: From clinic to neurobiology. *Neuron* 56(3):422–437, 2007. PMID: 17988628

Chang, Q.; Khare, G.; Dani, V.; et al. The disease progression of Mecp2 mutant mice is affected by the level of BDNF expression. *Neuron* 49(3):341–348, 2006. PMID: 16446138

Cloninger, C.R. Neurogenetic adaptive mechanisms in alcoholism. *Science* 236(4800): 410–416, 1987. PMID: 2882604

Covington, H.E., 3rd; Maze, I.; LaPlant, Q.C.; et al. Antidepressant actions of histone deacetylase inhibitors. *Journal of Neuroscience* 29(37):11451–11460, 2009. PMID: 19759294

Crabbe, J.C. Alcohol and genetics: New models. *American Journal of Medical Genetics* 114(8):969–974, 2002. PMID: 12457395

De Ruijter, A.J.; van Gennip, A.H.; Caron, H.N.; et al. Histone deacetylases (HDACs): Characterization of the classical HDAC family. *Biochemical Journal* 370(Pt 3):737–749, 2003. PMID: 12429021

Dokmanovic, M.; Clarke, C.; and Marks, P.A. Histone deacetylase inhibitors: Overview and perspectives. *Molecular Cancer Research* 5(10):981–989, 2007. PMID: 17951399

Duman, R.S., and Monteggia, L.M. A neurotrophic model for stress-related mood disorders. *Biological Psychiatry* 59(12):1116–1127, 2006. PMID: 16631126

Elliott, E.; Ezra-Nevo, G.; Regev, L.; et al. Resilience to social stress coincides with functional DNA methylation of the Crf gene in adult mice. *Nature Neuroscience* 13(11): 1351–1353, 2010. PMID: 20890295

Fan, G., and Hutnick, L. Methyl-CpG binding proteins in the nervous system. *Cell Research* 15(4):255–261, 2005. PMID: 15857580

Fuchikami, M.; Morinobu, S.; Kurata, A.; et al. Single immobilization stress differentially alters the expression profile of transcripts of the brain-derived neurotrophic factor (BDNF) gene and histone acetylation at its promoters in the rat hippocampus. *International Journal of Neuropsychopharmacology* 12(1):73–82, 2009. PMID: 18544182

Fuks, F.; Burgers, W.A.; Brehm, A.; et al. DNA methyltransferase Dnmt1 associates with histone deacetylase activity. *Nature Genetics* 24(1):88–91, 2000. PMID: 10615135

Goldwater, D.S.; Pavlides, C.; Hunter, R.G.; et al. Structural and functional alterations to rat medial prefrontal cortex following chronic restraint stress and recovery. *Neuroscience* 164(2):798–808, 2009. PMID: 19723561

Gordon, H.W. Early environmental stress and biological vulnerability to drug abuse. *Psychoneuroendocrinology* 27(1–2):115–126, 2002. PMID: 11750773

Grant, B.F.; Stinson, F.S.; Dawson, D.A.; et al. Prevalence and co-occurrence of substance use disorders and independent mood and anxiety disorders: Results from the National Epidemiologic Survey on Alcohol and Related Conditions. *Archives of General Psychiatry* 61(8):807-816, 2004. PMID: 15289279

Greenberg, M.E.; Xu, B.; Lu, B.; and Hempstead, B.L. New insights in the biology of BDNF synthesis and release: Implications in CNS function. *Journal of Neuroscience* 29(41):12764–12767, 2009. PMID: 19828787

Guan, J.S.; Haggarty, S.J.; Giacometti, E.; et al. HDAC2 negatively regulates memory formation and synaptic plasticity. *Nature* 459(7243):55–60, 2009. PMID: 19424149

Guerri, C., and Pascual, M. Mechanisms involved in the neurotoxic, cognitive, and neurobehavioral effects of alcohol consumption during adolescence. *Alcohol* 44(1):15–26, 2010. PMID: 20113871

He, D.Y.; Neasta, J.; and Ron, D. Epigenetic regulation of BDNF expression via the scaffolding protein RACK1. *Journal of Biological Chemistry* 285(25):19043–19050, 2010. PMID: 20410295

Hellemans, K.G.; Sliwowska, J.H.; Verma, P.; and Weinberg, J. Prenatal alcohol exposure: Fetal programming and later life vulnerability to stress, depression and anxiety disorders. *Neuroscience and Biobehavioral Reviews* 34(6):791–807, 2010. PMID: 19545588

Hensler, J.G.; Ladenheim, E.E.; and Lyons, W.E. Ethanol consumption and serotonin-1A (5-HT1A) receptor function in heterozygous BDNF (+/-) mice. *Journal of Neurochemistry* 85(5):1139–1147, 2003. PMID: 12753073

Higley, M.J., and Sabatini, B.L. Calcium signaling in dendrites and spines: Practical and functional considerations. *Neuron* 59(6):902–913, 2008. PMID: 18817730

Huang, F.; Chotiner, J.K.; and Steward, O. Actin polymerization and ERK phosphorylation are required for Arc/Arg3.1 mRNA targeting to activated synaptic sites on dendrites. *Journal of Neuroscience* 27(34):9054–9067, 2007. PMID: 17715342

Huang, Y.; Doherty, J.J.; and Dingledine, R. Altered histone acetylation at glutamate receptor 2 and brain-derived neurotrophic factor genes is an early event triggered by status epilepticus. *Journal of Neuroscience* 22(19):8422–8428, 2002. PMID: 12351716

Hunter, R.G.; McCarthy, K.J.; Milne, T.A.; et al. Regulation of hippocampal H3 histone methylation by acute and chronic stress. *Proceedings of the National Academy of Sciences of the United States of America* 106(49):20912–20917, 2009. PMID: 19934035

Impey, S.; Obrietan, K.; and Storm, D.R. Making new connections: Role of ERK/MAP kinase signaling in neuronal plasticity. *Neuron* 23(1):11–14, 1999. PMID: 10402188

Jeanblanc, J.; He, D.Y.; Carnicella, S.; et al. Endogenous BDNF in the dorsolateral striatum gates alcohol drinking. *Journal of Neuroscience* 29(43):13494–13502, 2009. PMID: 19864562

Jenuwein, T., and Allis, C.D. Translating the histone code. *Science* 293(5532):1074–1080, 2001. PMID: 11498575

Kim J.S., and Shukla, S.D. Acute in vivo effect of ethanol (binge drinking) on histone H3 modifications in rat tissues. *Alcohol and Alcoholism* 41(2):126–132, 2006. PMID: 16314425

Kimura, H., and Shiota, K. Methyl-CpG-binding protein, MeCP2, is a target molecule for maintenance DNA methyltransferase, Dnmt1. *Journal of Biological Chemistry* 278(7):4806–4812, 2003. PMID: 12473678

Koob, G.F. Alcoholism: Allostasis and beyond. *Alcoholism: Clinical and Experimental Research* 27(2):232–243, 2003. PMID: 12605072

Koob, G.F. A role for brain stress systems in addiction. *Neuron* 59(1):11–34, 2008. PMID: 18614026

Koob, G.F. Brain stress systems in the amygdala and addiction. *Brain Research* 1293:61–75, 2009. PMID: 19332030

Koob, G.F., and Volkow, N.D. Neurocircuitry of addiction. *Neuropsychopharmacology* 35(1):217–238, 2010. PMID: 19710631

Kumar, A.; Choi, K.H.; Renthal, W.; et al. Chromatin remodeling is a key mechanism underlying cocaine-induced plasticity in striatum. *Neuron* 48(2):303–314, 2005. PMID: 16242410

Lane, A.A., and Chabner, B.A. Histone deacetylase inhibitors in cancer therapy. *Journal of Clinical Oncology* 27(32):5459–5468, 2009. PMID: 19826124

Laplant, Q.; Vialou, V.; Covington, H.E., 3rd; et al. Dnmt3a regulates emotional behavior and spine plasticity in the nucleus accumbens. *Nature Neuroscience* 13(9):1137–1143, 2010. PMID: 20729844

Levenson, J.M., and Sweatt, J.D. Epigenetic mechanisms in memory formation. *Nature Reviews. Neuroscience* 6(2):108–118, 2005. PMID: 15654323

Lubin, F.D.; Roth, T.L.; and Sweatt, J.D. Epigenetic regulation of BDNF gene transcription in the consolidation of fear memory. *Journal of Neuroscience* 28(42):10576–10586, 2008. PMID: 18923034

Marmigere, F.; Givalois, L.; Rage, F.; et al. Rapid induction of BDNF expression in the hippocampus during immobilization stress challenge in adult rats. *Hippocampus* 13(5):646–655, 2003. PMID: 12921353

Martinowich, K.; Hattori, D.; Wu, H.; et al. DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation. *Science* 302(5646):890–893, 2003. PMID: 14593184

McEwen, B.S. Central effects of stress hormones in health and disease: Understanding the protective and damaging effects of stress and stress mediators. *European Journal of Pharmacology* 583(2-3):174–185, 2008. PMID: 18282566

McGough, N.N.; He, D.Y.; Logrip, M.L.; et al. RACK1 and brain-derived neurotrophic factor: A homeostatic pathway that regulates alcohol addiction. *Journal of Neuroscience* 24(46):10542–10552, 2004. PMID: 15548669

Messaoudi, E.; Kanhema, T.; Soule, J.; et al. Sustained Arc/Arg3.1 synthesis controls long-term potentiation consolidation through regulation of local actin polymerization in the dentate gyrus in vivo. *Journal of Neuroscience* 27(39):10445–10455, 2007. PMID: 17898216

Minichiello, L. TrkB signalling pathways in LTP and learning. *Nature Reviews. Neuroscience* 10(12):850–860, 2009. PMID: 19927149

Misra, K.; Roy, A.; and Pandey, S.C. Effects of voluntary ethanol intake on the expression of Ca(2+) /calmodulin-dependent protein kinase IV and on CREB expression and phosphorylation in the rat nucleus accumbens. *Neuroreport* 12(18):4133–4137, 2001. PMID: 11742252

Molteni, R.; Calabrese, F.; Cattaneo, A.; et al. Acute stress responsiveness of the neurotrophin BDNF in the rat hippocampus is modulated by chronic treatment with the antidepressant duloxetine. *Neuropsychopharmacology* 34(6):1523–1532, 2009. PMID: 19020498

Monteggia, L.M., and Kavalali, E.T. Rett syndrome and the impact of MeCP2 associated transcriptional mechanisms on neurotransmission. *Biological Psychiatry* 65(3):204–210, 2009. PMID: 19058783

Moonat, S.; Sakharkar, A.J.; Zhang, H.; and Pandey, S.C. The role of amygdaloid brain-derived neurotrophic factor, activity-regulated cytoskeleton-associated protein and dendritic spines in anxiety and alcoholism. *Addiction Biology* 16(2):238–250, 2011. PMID: 21182574

Moonat, S.; Starkman, B.G.; Sakhakar, A.; and Pandey, S.C. Neuroscience of alcoholism: Molecular and cellular mechanisms. *Cellular and Molecular Life Sciences* 67(1):73–88, 2010. PMID: 19756388

Nan, X.; Ng, H.H.; Johnson, C.A.; et al. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* 393(6683):386–389, 1998. PMID: 9620804

Nibuya, M.; Morinobu, S.; and Duman, R.S. Regulation of BDNF and trkB mRNA in rat brain by chronic electroconvulsive seizure and antidepressant drug treatments. *Journal of Neuroscience* 15(11):7539–7547, 1995. PMID: 7472505

Okano, M.; Bell, D.W.; Haber, D.A.; and Li, E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 99(3):247–257, 1999. PMID: 10555141

Ou, L.C., and Gean, P.W. Transcriptional regulation of brain-derived neurotrophic factor in the amygdala during consolidation of fear memory. *Molecular Pharmacology* 72(2):350–358, 2007. PMID: 17456785

Pandey, S.C. Anxiety and alcohol abuse disorders: A common role for CREB and its target, the neuropeptide Y gene. *Trends in Pharmacological Sciences* 24(9):456–460, 2003. PMID: 12967770

Pandey, S.C. The gene transcription factor cyclic AMP-responsive element binding protein: Role in positive and negative affective states of alcohol addiction. *Pharmacology & Therapeutics* 104(1):47–58, 2004. PMID: 15500908

Pandey, S.C.; Roy, A.; and Zhang, H. The decreased phosphorylation of cyclic adenosine monophosphate (cAMP) response element binding (CREB) protein in the central amygdala acts as a molecular substrate for anxiety related to ethanol withdrawal in rats. *Alcoholism: Clinical and Experimental Research* 27(3):396–409, 2003. PMID: 12658105

Pandey, S.C.; Ugale, R.; Zhang, H.; et al. Brain chromatin remodeling: A novel mechanism of alcoholism. *Journal of Neuroscience* 28(14):3729–3737, 2008*a*. PMID: 18385331

Pandey, S.C.; Zhang, H.; Roy, A.; and Misra, K. Central and medial amygdaloid brain-derived neurotrophic factor signaling plays a critical role in alcohol-drinking and anxiety-like behaviors. *Journal of Neuroscience* 26(32):8320–8331, 2006. PMID: 16899727

Pandey, S.C.; Zhang, H.; Roy, A.; and Xu, T. Deficits in amygdaloid cAMP-responsive element-binding protein signaling play a role in genetic predisposition to anxiety and alcoholism. *Journal of Clinical Investigation* 115(10):2762–2773, 2005. PMID: 16200210

Pandey, S.C.; Zhang, H.; Ugale, R.; et al. Effector immediate-early gene Arc in the amygdala plays a critical role in alcoholism. *Journal of Neuroscience* 28(10):2589–2600, 2008*b*. PMID: 18322102

Pavlides, C.; Nivon, L.G.; and McEwen, B.S. Effects of chronic stress on hippocampal long-term potentiation. *Hippocampus* 12(2):245–257, 2002. PMID: 12000121

Pittenger, C., and Duman, R.S. Stress, depression, and neuroplasticity: A convergence of mechanisms. *Neuropsychopharmacology* 33(1):88–109, 2008. PMID: 17851537

Pizarro, J.M.; Lumley, L.A.; Medina, W.; et al. Acute social defeat reduces neurotrophin expression in brain cortical and subcortical areas in mice. *Brain Research* 1025(1–2):10–20, 2004. PMID: 15464739

Pizzorusso, T.; Ratto, G.M.; Putignano, E.; and Maffei, L. Brain-derived neurotrophic factor causes cAMP response element-binding protein phosphorylation in absence of calcium increases in slices and cultured neurons from rat visual cortex. *Journal of Neuroscience* 20(8):2809–2816, 2000. PMID: 10751432

Poo, M.M. Neurotrophins as synaptic modulators. *Nature Reviews. Neuroscience* 2(1):24–32, 2001. PMID: 11253356

Prakash, A.; Zhang, H.; and Pandey, S.C. Innate differences in the expression of brain-derived neurotrophic factor in the regions within the extended amygdala between alcohol preferring and nonpreferring rats. *Alcoholism: Clinical and Experimental Research* 32(6):909–920, 2008. PMID: 18445109

Qiang, M.; Denny, A.; Chen, J.; et al. The site specific demethylation in the 5′-regulatory area of NMDA receptor 2B subunit gene associated with CIE-induced up-regulation of transcription. *PLoS One* 5(1):e8798, 2010. PMID: 20098704

Ramanan, N.; Shen, Y.; Sarsfield, S.; et al. SRF mediates activity-induced gene expression and synaptic plasticity but not neuronal viability. *Nature Neuroscience* 8(6):759–767, 2005. PMID: 15880109

Reichardt, L.F. Neurotrophin-regulated signalling pathways. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 361(1473):1545–1564, 2006. PMID: 16939974

Renthal, W.; Maze, I.; Krishnan, V.; et al. Histone deacetylase 5 epigenetically controls behavioral adaptations to chronic emotional stimuli. *Neuron* 56(3):517–529, 2007. PMID: 17988634

Renthal, W., and Nestler, E.J. Epigenetic mechanisms in drug addiction. *Trends in Molecular Medicine* 14(8):341–350, 2008. PMID: 18635399

Roberto, M.; Nelson, T.E.; Ur, C.L.; and Gruol, D.L. Long-term potentiation in the rat hippocampus is reversibly depressed by chronic intermittent ethanol exposure. *Journal of Neurophysiology* 87(5):2385–2397, 2002. PMID: 11976376

Robinson, J.; Sareen, J.; Cox, B.J.; and Bolton, J. Self-medication of anxiety disorders with alcohol and drugs: Results from a nationally representative sample. *Journal of Anxiety Disorders* 23(1):38–45, 2009. PMID: 18571370

Roozendaal, B.; McEwen, B.S.; and Chatterji, S. Stress, memory and the amygdala. *Nature Reviews. Neuroscience* 10(6):423–433, 2009. PMID: 19469026

Schuckit, M.A., and Hesselbrock, V. Alcohol dependence and anxiety disorders: What is the relationship? *American Journal of Psychiatry* 151(12):1723–1734, 1994. PMID: 7977877

Self, D.W., and Nestler, E.J. Relapse to drug-seeking: Neural and molecular mechanisms. *Drug and Alcohol Dependence* 51(1–2):49–60, 1998. PMID: 9716929

Shaywitz, A.J., and Greenberg, M.E. CREB: A stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annual Review of Biochemistry* 68:821–861, 1999. PMID: 10872467

Shirayama, Y.; Chen, A.C.; Nakagawa, S.; et al. Brain-derived neurotrophic factor produces antidepressant effects in behavioral models of depression. *Journal of Neuroscience* 22(8):3251–3261, 2002. PMID: 11943826

Sinha, R. The role of stress in addiction relapse. *Current Psychiatry Reports* 9(5):388–395, 2007. PMID: 17915078

Sinha, R. Chronic stress, drug use, and vulnerability to addiction. *Annals of the New York Academy of Sciences* 1141:105–130, 2008. PMID: 18991954

Smith, M.A.; Makino, S.; Kvetnansky, R.; and Post, R.M. Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. *Journal of Neuroscience* 15(3 Pt 1):1768–1777, 1995. PMID: 7891134

Smith, M.M. Histone structure and function. *Current Opinion in Cell Biology* 3(3):429–437, 1991. PMID: 1892654

Smith, S.M., and Vale, W.W. The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. *Dialogues in Clinical Neuroscience* 8(4):383–395, 2006. PMID: 17290797

Soule, J.; Messaoudi, E.; and Bramham, C.R. Brain-derived neurotrophic factor and control of synaptic consolidation in the adult brain. *Biochemical Society Transactions* 34(Pt 4):600–604, 2006. PMID: 16856871

Starkman, B.G.; Sakharkar, A.J.; and Pandey, S.C. Epigenetics—Beyond the genome in alcoholism. *Alcohol Research: Current Reviews* 34(3):325–337, 2012.

Strahl, B.D., and Allis, C.D. The language of covalent histone modifications. *Nature* 403(6765):41–45, 2000. PMID: 10638745

Tao, X.; Finkbeiner, S.; Arnold, D.B.; et al. Ca^{2+} influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. *Neuron* 20(4):709–726, 1998. PMID: 9581763

Timmusk, T.; Palm, K.; Metsis, M.; et al. Multiple promoters direct tissue-specific expression of the rat BDNF gene. *Neuron* 10(3):475–489, 1993. PMID: 8461137

Tsankova, N.; Renthal, W.; Kumar, A.; and Nestler, E.J. Epigenetic regulation in psychiatric disorders. *Nature Reviews. Neuroscience* 8(5):355–367, 2007. PMID: 17453016

Tsankova, N.M.; Berton, O.; Renthal, W.; et al. Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. *Nature Neuroscience* 9(4):519–525, 2006. PMID: 16501568

Tsankova, N.M.; Kumar, A.; and Nestler, E.J. Histone modifications at gene promoter regions in rat hippocampus after acute and chronic electroconvulsive seizures. *Journal of Neuroscience* 24(24):5603–5610, 2004. PMID: 15201333

Uhart, M., and Wand, G.S. Stress, alcohol and drug interaction: An update of human research. *Addiction Biology* 14(1):43–64, 2009. PMID: 18855803

Wade, P.A. Methyl CpG binding proteins: Coupling chromatin architecture to gene regulation. *Oncogene* 20(24):3166–3173, 2001. PMID: 11420733

Waltereit, R., and Weller, M. Signaling from cAMP/PKA to MAPK and synaptic plasticity. *Molecular Neurobiology* 27(1):99–106, 2003. PMID: 12668903

Watanabe, Y.; Gould, E.; and McEwen, B.S. Stress induces atrophy of apical dendrites of hippocampal CA3 pyramidal neurons. *Brain Research* 588(2):341–345, 1992. PMID: 1393587

Ying, S.W.; Futter, M.; Rosenblum, K.; et al. Brain-derived neurotrophic factor induces long-term potentiation in intact adult hippocampus: Requirement for ERK activation coupled to CREB and upregulation of Arc synthesis. *Journal of Neuroscience* 22(5):1532–1540, 2002. PMID: 11880483

Yuste, R. Dendritic spines and distributed circuits. *Neuron* 71(5):772–781, 2011. PMID: 21903072

Zhou, F.C.; Anthony, B.; Dunn, K.W.; et al. Chronic alcohol drinking alters neuronal dendritic spines in the brain reward center nucleus accumbens. *Brain Research* 1134(1):148–161, 2007. PMID: 17198693

Zhou, Z.; Hong, E.J.; Cohen, S.; et al. Brain-specific phosphorylation of MeCP2 regulates activity-dependent BDNF transcription, dendritic growth, and spine maturation. *Neuron* 52(2):255–269, 2006. PMID: 17046689

Genetic and Environmental Determinants of Stress Responding

Toni-Kim Clarke, Ph.D.; Charlotte Nymberg, M.Sc.; and Gunter Schumann, M.D.

Toni-Kim Clarke, Ph.D., *is a postdoctoral fellow at the Center for Neurobiology and Behavior, Department of Psychiatry, University of Pennsylvania, Perelman School of Medicine, Philadelphia, Pennsylvania.*

Charlotte Nymberg, M.Sc., *is a doctural student, and* **Gunter Schumann, M.D.,** *is a professor and chair of biological psychiatry at the Medical Research Council (MRC) Social, Genetic, and Developmental Psychiatry Centre (SGDP), Institute of Psychiatry, King's College, London, United Kingdom.*

The risk for alcohol dependence throughout development is determined by both genetic and environmental factors. Genetic factors that are thought to modulate this risk act on neurobiological pathways regulating reward, impulsivity, and stress responses. For example, genetic variations in pathways using the brain signaling molecule (i.e., neurotransmitter) dopamine, which likely mediate alcohol's rewarding effects, and in two hormonal systems involved in the stress response (i.e., the hypothalamic–pituitary–adrenal axis and the corticotropin-releasing factor system) affect alcoholism risk. This liability is modified further by exposure to environmental risk factors, such as environmental stress and alcohol use itself, and the effects of these factors may be enhanced in genetically vulnerable individuals. The transition from alcohol use to dependence is the result of complex interactions of genes, environment, and neurobiology, which fluctuate throughout development. Therefore, the relevant genetic and environmental risk factors may differ during the different stages of alcohol initiation, abuse, and dependence. The complex interaction of these factors is yet to be fully elucidated, and translational studies, ranging from animal studies to research in humans, and well-characterized longitudinal studies are necessary to further understand the development of alcohol dependence. **KEY WORDS: Alcohol dependence; alcoholism; alcohol use and abuse; alcohol and other drug use initiation; risk factors; genetic factors; environmental factors; stress; stress response; neurobiology; biological development; brain; hypothalamic–pituitary–adrenal axis; corticotropin-releasing factor system; animal studies; human studies; literature review**

The development of alcohol dependence is a complex process influenced by both genetic and environmental risk factors (Prescott and Kendler 1999). The relative contributions of genetic and environmental influences fluctuate across development. During adolescence the initiation of alcohol use is strongly influenced by environmental factors (Dick et al. 2007; Heath et al. 1997; Karvonen 1995; Latendresse et al. 2008; McGue et al. 2000), whereas the genetic contribution to alcohol use at this stage is nonspecific and increases the risk for general externalizing behavior (Moffitt 1993; Moffitt et al. 2002). Specific genetic factors increasingly become relevant, however, as patterns of alcohol use are established (Hopfer et al. 2003; Pagan et al. 2006), particularly in mid-adulthood when dependence tends to emerge (Kendler et al. 2010; Schuckit et al. 1995). Gene–environment interactions also play a role because the influence of certain genetic factors seems to increase when a person is exposed to relevant environmental risk factors (Uhart and Wand 2009). Therefore, the development of dependence can be conceptualized within a temporal framework of genes, environment, and behavior.

The purpose of this review is to explore, within this framework, the contribution of some of the neurobiological systems that are important for the development of alcohol dependence. One of these is the mesolimbic dopaminergic system, which is involved in inducing the rewarding effects of alcohol and plays a central role in early alcohol use. Another pathway that also has been implicated in alcohol abuse, and particularly in the transition to alcohol dependence, involves two stress-response systems, the hypothalamic–pituitary–adrenal (HPA) axis and the extrahypothalamic corticotropin-releasing factor (CRF) stress response system, which mediate the interaction of psychosocial stress and early alcohol use. Both of these systems exemplify how the effects of genes and environment may be augmented during critical periods of alcohol use and dependence across the lifespan. For example, the dopaminergic system undergoes developmental transformations during adolescence that are associated with increased reward sensitivity and risk taking (Spear 2000), which presents a window of vulnerability for exposure to alcohol and stress. Then, as alcohol use continues through life, chronic exposure to alcohol can enhance the activity of (i.e., upregulate) the HPA and CRF systems. This dysregulation of the stress response systems

becomes a pathological feature of alcohol dependence, perpetuating chronic alcohol drinking based on an allostatic shift[1] of the CRF system (Koob 2010). Moreover, the HPA, CRF, and dopaminergic systems can influence early alcohol drinking as a result of gene–environment interactions. This article will summarize the literature that has explored how genetic variation within the dopaminergic and stress response systems can influence the risk of alcohol dependence and how the exposure to relevant environmental risk factors and their interaction with genetic variants may influence alcoholism pathology. The effects of genes and environment on alcohol dependence will be discussed in a developmental framework from early childhood to adolescence as well as in the context of the development of dependence, when drinking behavior shifts from recreational use to dependence.

Role of Dopaminergic and Stress Response Systems in Alcohol Initiation and Early Alcohol Use

Environmental Factors and the Dopaminergic System

Several environmental factors have been shown to influence the initiation of alcohol consumption and its use during adolescence, including the level and quality of parental monitoring, peer-group influences, alcohol availability, and socioregional effects (Dick et al. 2007; Heath et al. 1997; Karvonen 1995; Latendresse et al. 2008; McGue et al. 2000). Thus, maternal and paternal alcohol use has been positively correlated with adolescent alcohol use at ages 14 and 17 (Latendresse et al. 2008). Moreover, the level of urbanization was found to correlate with alcohol use in Finnish adolescents at ages 16 and 18 (Karvonen 1995), and peer-group drinking behavior was one of the strongest predictors of problematic drinking in a cohort of Spanish adolescents (Ariza Cardenal and Nebot Adell 2000).

Once alcohol use has been initiated, neuronal networks are activated that engage the brain circuits mediating the rewarding effects of alcohol use (i.e., the reward neurocircuitry). This activation attributes salience to alcohol and serves as an incentive for alcohol use to continue (Robinson and Berridge 1993). Neuronal networks that are known to mediate these effects include those using the signaling molecules (i.e., neurotransmitters) glutamate and γ-aminobutyric acid (GABA) as well as the endogenous opioids (Gass and Olive 2008; Malcolm 2003; Oswald and Wand 2004). In addition, signal transmission involving the neurotransmitter dopamine in the mesolimbic system (Di Chiara and Imperato 1988) is particularly important for the establishment of regular alcohol consumption because alcohol-induced dopamine release is believed to contribute to the rewarding effects of alcohol (for reviews see, Soderpalm et al. 2009; Tupala and Tiihonen 2004). The mesolimbic system is a set of interconnected brain structures including the ventral tegmental area (VTA), nucleus accumbens (NAc), and components of the limbic system (e.g., the amygdala). Studies in rats found that alcohol consumption can increase dopamine signaling in the NAc (Weiss et al. 1996). Conversely, dopaminergic neurotransmission is decreased during withdrawal in the NAc and VTA of rats treated chronically with ethanol (Diana et al. 1993).

Environmental risk factors during early life and adolescence may interact with the dopaminergic system to influence alcohol intake. Two such factors are exposure to environmental stress and alcohol consumption itself. The developing adolescent brain undergoes substantial changes in the strength with which signals are transmitted between neurons (i.e., in synaptic plasticity) (Bava and Tapert 2010; Giedd 2003). These changes include increased dopaminergic inputs to the prefrontal cortex that peak during adolescence and decrease later in life (Kalsbeek et al. 1988; Rosenberg and Lewis 1994). Furthermore, dopamine levels in the NAc also peak during adolescence, before decreasing during subsequent brain maturation (Philpot and Kirstein 2004). These neuronal alterations are believed to promote sensation-seeking and risk-taking behavior during adolescence, which in turn increase the propensity for alcohol initiation and alcohol use (Spear 2000). Exposure to alcohol and/or stress during early life (i.e., from the prenatal period through adolescence) has been shown to have lasting consequences on the dopamine system that have a significant impact on the risk for alcohol abuse.

The Effects of Early Alcohol Use on the Dopaminergic System

Studies in rats found that exposure to alcohol during the prenatal period decreases the levels of two important enzymes involved in regulating dopamine activity—the dopamine transporter and the dopamine hydroxylase enzyme—in the VTA (Szot et al. 1999). Moreover, rats chronically treated with ethanol during adolescence displayed persistently elevated baseline dopamine levels in the NAc during adulthood, even after a period of 15 days abstinence (Badanich et al. 2007). Finally, repeated ethanol injections in preadolescent and adolescent rats increased subsequent dopamine activity in the NAc, with the largest increases observed in preadolescence. Early ethanol exposure in these rats decreased the ability of subsequent ethanol injections to elicit dopamine release from the NAc (Philpot and Kirstein 2004). These findings suggest that ethanol exposure in early life may influence the response to alcohol in later life. Indeed, additional studies have confirmed that both pre- and postnatal exposure to alcohol increase the sensitivity of rats to the locomotor effects of alcohol and to an agent that mimics dopamine's effects (i.e., a dopamine agonist), apomorphine

[1] The term allostasis refers to the process through which various biological processes attempt to restore the body's internal balance (i.e., homeostasis) when an organism is threatened by various types of stress in the internal or external environment. Allostatic responses can involve alterations in HPA axis function, the nervous system, various signaling molecules in the body, or other systems.

(Barbier et al. 2009). Therefore, at least in rodents, early alcohol exposure seems to confer lasting effects on neuronal dopamine activity that can alter behavioral responses to subsequent alcohol exposure. Indeed, rats chronically treated with ethanol both prenatally and during adolescence also show an increased preference for alcohol and increased alcohol intake as adults (Barbier et al. 2009; Pascual et al. 2009). Furthermore, stress-induced alcohol consumption was associated with an earlier age of drinking onset in Wistar rats (Fullgrabe et al. 2007; Siegmund et al. 2005).

Studies in humans have confirmed the potential long-lasting impact of early alcohol exposure, demonstrating that an early initiation of alcohol use is associated with an increased risk of later problems with alcohol. For example, Hawkins and colleagues (1997) noted that the earlier drinking is initi-

The Extrahypothalamic Corticotropin-Releasing Factor System and the Transition to Alcohol Dependence

As described in the main article, corticotropin-releasing factor (CRF) is a key component of one of the body's main stress response systems, the hypothalamic–pituitary–adrenal (HPA) axis. Moreover, activation of the HPA axis in response to stressful situations as well as alcohol ingestion plays an important role in the development of alcohol dependence. However, studies in rodents and macaques have shown that enhanced activity (i.e., upregulation) of the CRF system in response to chronic alcohol exposure in several brain regions not immediately related to the HPA system (e.g., the amygdala) also is a key characteristic of alcohol dependence. CRF is an anxiety-inducing peptide, and rodent models of motivation have demonstrated that CRF, administered either directly into the brain or under the skin, induces conditioned place aversion (Cador et al. 1992). In addition, studies in mice found that transient elevation of CRF levels in the forebrain during early development increased anxiety in later life compared with control animals (Kolber et al. 2010).

Studies of a rat strain bred for high alcohol preference (i.e., the mSP rats) found that the animals display an increased behavioral sensitivity to stress and a lowered threshold for stress-induced reinstatement of alcohol-seeking behavior (Hansson et al. 2006). Gene expression analyses across different brain regions of the mSP strain revealed a significantly enhanced expression of a gene, *CRF1,* which encodes one of the CRF receptors. Additional gene sequence analyses of the mSP rats identified a DNA variation (i.e., polymorphism) in a regulatory region (i.e., the promoter) of the *CRF1* gene that is unique to the mSP rats, suggesting that segregation of this polymorphism may have occurred during selection for the alcohol preference trait. However, alcohol consumption reduced CRF1 levels in the amygdala and the nucleus accumbens (NAc) in mSP rats, indicating that the animals may consume alcohol to reduce CRF activity in these regions (Hansson et al. 2007).

Studies in *Rhesus* macaques also have confirmed the link between the CRF system, stress, and alcohol because a polymorphism (–248C/T) in the promoter of the CRF gene was associated with differential behavioral and hormonal responses to stress. Animals that carried the T allele DNA variant at this site displayed greater HPA axis responses to separation stress and increased alcohol intake if they were exposed to early-life adversity in the form of peer rearing (Barr et al. 2009). These findings demonstrate that genetic variation in the CRF system associated with increased sensitivity to stressors also is correlated with increased alcohol consumption in both rats and primates. Because alcohol consumption is known to reduce the activity of the HPA axis, hyperactivity of this system in animals carrying risk variants of the CRF gene likely is a motivating factor for alcohol consumption in these animals, and this effect is enhanced when the animals are exposed to stressors.

Animal studies also have demonstrated that agents that block the activity of the CRF1 receptor (i.e., CRF1 antagonists) may be suitable for treatment of alcohol dependence (Gehlert et al. 2007). Although animals do not exhibit all aspects of alcohol dependence found in humans, certain components of the disorder can be modeled in rodents. Thus, researchers induced a "postdependent state" in rats by first subjecting the animals to involuntary intermittent exposure to alcohol vapor and then allowing them 3 weeks of recovery from the exposure (Sommer et al. 2008). After this recovery period, the animals displayed increased CRF1 levels in the amygdala, comparable to those observed in mSP rats at baseline. In addition, the postdependent animals exhibited increased fear suppression of behavior that persisted for 3 months after cessation of alcohol exposure, as well as increased voluntary alcohol consumption. This postdependent phenotype could be reversed by a CRF1 antagonist, 3-(4-chloro-2-morpholin-4-yl-thiazol-5-yl)-8-(1-ethylpropyl)-2,6-dimethyl-

ated in adolescence, the greater the levels of alcohol misuse at ages 17 to 18. Furthermore, people who begin drinking at age 14 or younger are more likely to become alcohol dependent later in life (Grant and Dawson 1997). Few studies have been conducted to determine the precise mechanism by which early alcohol exposure affects the risk for subsequent alcohol abuse and dependence. However, Pascual and colleagues (2009) demonstrated that in adolescent rats chronically treated with ethanol, two neurotransmitter receptors—dopamine receptor 2 (DRD2) and glutamate receptor (NMDAR2B)—show lower levels of a chemical modification (i.e., phosphorylation) in the prefrontal cortex compared with adults chronically treated with ethanol. This finding suggests that alcohol use during adolescence causes neurobiological changes to the dopamine system that are not observed in adult animals.

imidazo[1,2-b]pyridazine (MTIP) (Funk et al. 2006; Sommer et al. 2008), confirming the role of increased CRF activity during alcohol dependence. Other studies also demonstrated that selective CRF1 antagonists reduced alcohol self-administration in alcohol-dependent animals but had no effect in alcohol-naïve animals (Funk et al. 2006, 2007). The exposure to stress, which often triggers relapse in abstaining alcoholics, also reinstates alcohol-seeking behavior in postdependent animals. CRF1 antagonists can suppress this behavior in animals (Le et al. 2000; Liu and Weiss 2002; Marinelli et al. 2007), further confirming their relevance as a potential pharmacotherapy for alcohol dependence. Finally, CRF1 antagonists can block the anxiety-like responses exhibited during withdrawal from alcohol in animals (Breese et al. 2005).

The potential of CRF1 antagonists in the treatment of alcohol dependence now also is being considered in humans. CRF1 antagonists previously have been assessed in the treatment of depression and anxiety (Zobel et al. 2000) and Phase II/Phase III clinical trials with these agents currently are underway for the treatment of alcohol use disorders (www.clinicaltrials.gov; Zorrilla and Koob 2010). The results of these trials may pave the way for the clinical consideration of CRF1 antagonists for addictive disorders. If such compounds are efficacious in humans, pharmacogenetic studies may identify those patients who are most amenable to CRF1 antagonist treatment, especially among those who are exposed to high levels of lifetime stress.

References

Barr, C.S.; Dvoskin, R.L.; Gupte, M.; et al. Functional CRH variation increases stress-induced alcohol consumption in primates. *Proceedings of the National Academy of Sciences of the United States of America* 106:14593–14598, 2009. PMID: 19706546

Breese, G.R.; Chu, K.; Dayas, C.V.; et al. Stress enhancement of craving during sobriety: A risk for relapse. *Alcoholism: Clinical and Experimental Research* 29:185–195, 2005. PMID: 15714042

Cador, M.; Ahmed, S.H.; Koob, G.F.; et al. Corticotropin-releasing factor induces a place aversion independent of its neuroendocrine role. *Brain Research* 597:304–309, 1992. PMID: 1473001

Funk, C.K.; O'Dell, L.E.; Crawford, E.F.; and Koob, G.F. Corticotropin-releasing factor within the central nucleus of the amygdala mediates enhanced ethanol self-administration in withdrawn, ethanol-dependent rats. *Journal of Neuroscience* 26:11324–11332, 2006. PMID: 17079660

Funk, C.K.; Zorrilla, E.P.; Lee, M.J.; et al. Corticotropin-releasing factor 1 antagonists selectively reduce ethanol self-administration in ethanol-dependent rats. *Biological Psychiatry* 61:78–86, 2007. PMID: 16876134

Gehlert, D.R.; Cippitelli, A.; Thorsell, A.; et al. 3-(4-Chloro-2-morpholin-4-yl-thiazol-5-yl)-8-(1-ethylpropyl)-2,6-dimethyl- imidazo[1,2-b]pyridazine: A novel brain-penetrant, orally available corticotropin-releasing factor receptor 1 antagonist with efficacy in animal models of alcoholism. *Journal of Neuroscience* 27:2718–2726, 2007. PMID: 17344409

Hansson, A.C.; Cippitelli, A.; Sommer, W.H.; et al. Region-specific down-regulation of Crhr1 gene expression in alcohol-preferring msP rats following ad lib access to alcohol. *Addiction Biology* 12:30–34, 2007. PMID: 17407495

Hansson, A.C.; Cippitelli, A.; Sommer, W.H.; et al. Variation at the rat Crhr1 locus and sensitivity to relapse into alcohol seeking induced by environmental stress. *Proceedings of the National Academy of Sciences of the United States of America* 103:15236–15241, 2006. PMID: 17015825

Kolber, B.J.; Boyle, M.P.; Wieczorek, L.; et al. Transient early-life forebrain corticotropin-releasing hormone elevation causes long-lasting anxiogenic and despair-like changes in mice. *Journal of Neuroscience* 30:2571–2581, 2010. PMID: 20164342

Le, A.D.; Harding, S.; Juzytsch, W.; et al. The role of corticotrophin-releasing factor in stress-induced relapse to alcohol-seeking behavior in rats. *Psychopharmacology* (Berlin) 150:317–324, 2000. PMID: 10923760

Liu, X., and Weiss, F. Additive effect of stress and drug cues on reinstatement of ethanol seeking: Exacerbation by history of dependence and role of concurrent activation of corticotropin-releasing factor and opioid mechanisms. *Journal of Neuroscience* 22:7856–7861, 2002. PMID: 12223538

Marinelli, P.W.; Funk, D.; Juzytsch, W.; et al. The CRF1 receptor antagonist antalarmin attenuates yohimbine-induced increases in operant alcohol self-administration and reinstatement of alcohol seeking in rats. *Psychopharmacology* (Berlin) 195:345–355, 2007. PMID : 17705061

Sommer, W.H.; Rimondini, R.; Hansson, A.C.; et al. Upregulation of voluntary alcohol intake, behavioral sensitivity to stress, and amygdala Crhr1 expression following a history of dependence. *Biological Psychiatry* 63:139–145, 2008. PMID: 17585886

Zobel, A.W.; Nickel, T.; Kunzel, H.E.; et al. Effects of the high-affinity corticotropin-releasing hormone receptor 1 antagonist R121919 in major depression: The first 20 patients treated. *Journal of Psychiatric Research* 34:171–181, 2000. PMID: 10867111

Zorrilla, E.P., and Koob, G.F. Progress in corticotropin-releasing factor-1 antagonist development. *Drug Discovery Today* 15:371–383, 2010. PMID: 20206287

The Effects of Environmental Stress on the Dopaminergic System

Environmental stress is one of the most pertinent risk factors for alcohol dependence. The exposure to early-life stress sensitizes animals to drugs of abuse (Fahlke et al. 1994; Piazza et al. 1991; Shaham and Stewart 1994) and also increases alcohol consumption in later life (Fahlke et al. 2000). Alterations in the dopaminergic mesolimbic system that persist into adulthood are believed to explain, at least in part, these behavioral adaptations (for review, see Rodrigues et al. 2011). For example, studies in rats found that chronic exposure to cold stress in adolescence altered both basal and stress-evoked release of dopamine and another neurotransmitter, norepinephrine,[2] in the medial prefrontal cortex, NAc, and striatum compared with stress-naïve rats (Gresch et al. 1994). Other studies in Sprague-Dawley rats demonstrated that stress caused by separation from the mother during the first 2 weeks of life blunted the animals' dopamine response to restraint stress in adulthood (Jahng et al. 2010). Although no human studies analyzing the effect of early-life stress and alcohol sensitization exist, imaging studies using functional magnetic resonance imaging (fMRI) to analyze reward anticipation have found that childhood adversity is associated with blunted subjective responses to reward-predicting cues as well as with impaired reward-related learning and motivation (Dillon et al. 2009). Such findings demonstrate that early environmental experiences can alter the impact of a reward and that similar effects can be observed across species.

Other studies have evaluated the effects of early-life stress on alcohol consumption or alcohol dependence. Such studies found that even exposure to prenatal stress can have an impact on later alcohol-related behaviors because the offspring of mice that repeatedly were restrained during the last 7 days of gestation subsequently demonstrated enhanced alcohol consumption—an effect that has been linked to persistently elevated dopaminergic and glutamatergic neurotransmission in the forebrain (Campbell et al. 2009). In humans, retrospective studies examining early-life experiences and alcohol consumption found that childhood stressors were associated with alcohol dependence during adulthood (Ducci et al. 2009; Pilowsky et al. 2009). In a study of the adult American population (i.e., the National Epidemiologic Survey on Alcohol and Related Conditions [NESARC]), two or more stressful life events in childhood significantly increased the risk for alcohol dependence in adulthood (Pilowsky et al. 2009). Furthermore, early initiation of alcohol use in human adolescents is associated with exposure to traumatic life events and symptoms of posttraumatic stress disorder (Wu et al. 2010).

Thus, exposure to stress and/or alcohol consumption during early life may influence dopaminergic neurotransmission, with lasting adaptations into adulthood and notable consequences for subsequent alcohol use. However, the impact on different individuals varies, and a portion of this variability can be attributed to genetic factors. Indeed, studies of rats have shown that exposure to chronic unpredictable stress increases the levels of a dopamine-metabolizing enzyme, tyrosine hydroxylase (TH), in the VTA but that the extent of this increase differs drastically between different rat strains (Ortiz et al. 1996). Additional research in *Rhesus macaques* identified a variation (i.e., polymorphism) in the gene encoding dopamine receptor 1 (DRD1)[3] that was associated with increased alcohol consumption in animals exposed to peer-rearing conditions compared with maternally reared animals that carried the same polymorphism (Newman et al. 2009).

Studies in humans also have shown that genetic factors mediate the effects of stress and alcohol on the risk for alcohol dependence. Schmid and colleagues (2009) analyzed 291 young adults in the Mannheim Study of Children at Risk for two polymorphisms in the gene encoding the dopamine transporter. The investigators found that the age of first alcohol use and of intensive alcohol consumption mediated the association between these polymorphisms and early alcohol abuse and dependence. Genetic variation in another gene, *KCNJ6*, which is expressed in the brain, mediates the effects of early-life stress on alcohol abuse in adolescence. It induces inhibition of neuronal signaling at the level of the signal-receiving (i.e., postsynaptic) dopaminergic neurons (Kuzhikandathil et al. 1998). Furthermore, the protein encoded by the *KCNJ6* gene, the membrane potasium channel GIRK2, is co-expressed in TH-positive cells of mice (Schein et al. 1998). Individuals who carry a certain *KCNJ6* variant and are exposed to high levels of psychosocial stress in early life display increased risky drinking behavior in adolescence; moreover, the same polymorphism is associated with alcohol dependence in adults (Clarke et al. 2011).

Genes in other neurobiological systems also mediate the effects of early-life stress on alcohol consumption, including genes encoding the serotonin receptor (Laucht et al. 2009) and the GABA receptor subunit α-2 (*GABRA2*) (Enoch et al. 2010). Another important gene is that encoding the μ-opioid receptor (*OPRM1)*. It also moderates the effects of stress and alcohol with implications not only for alcohol use but also for recovery from alcohol dependence. Alcohol activates the μ-opioid receptor in the VTA, which causes inhibition of GABAergic neurons; this in turn results in disinhibition of dopaminergic neurons and, thus, increased dopamine release in the ventral striatum (Spanagel 2009). In macaques, a certain polymorphism in the *OPRM1* gene (i.e., the C77G polymorphism) predicts the degree of distress upon exposure to maternal separation (Barr et al. 2008). In humans, the equivalent polymorphism (i.e., the A118G polymorphism) is associated

[2] Norepinephrine also is known as noradrenaline.

[3] The variation was located at the beginning of the gene, in a DNA region that did not encode a part of the final protein (i.e., in the 5′ untranslated region of the gene).

with the quality of parent–child interactions under conditions of poor parenting (Copeland et al. 2011). Finally, in both macaques and humans the same polymorphisms are associated with subjective/behavioral responses to alcohol (Barr et al. 2007, 2008; Ramchandani et al. 2010). The role of this polymorphism further has been demonstrated in studies using a μ-opioid receptor antagonist, naltrexone, that commonly is used to treat alcohol dependence. In heavy drinkers, the A118G polymorphism mediates the effects of naltrexone on positive mood, craving, and enjoyment from alcohol (Ray and Hutchison 2004). Furthermore, the presence or absence of the A118G polymorphism can help predict which individuals will benefit from naltrexone treatment for alcohol dependence (Oslin et al. 2003).

Taken together, the findings described here indicate that early exposure to alcohol and stress can increase the subsequent risk for alcohol dependence, at least in part because they induce changes in the dopamine system. However, these effects are moderated by genetic factors in the dopamine pathways and other neurobiological systems.

Brain Stress Response Systems and the Development of Alcohol Dependence

As indicated by the observations discussed in the preceding section, the dopamine system is an important neurobiological system mediating early alcohol use. In addition, stress response systems in the brain have been implicated in alcohol initiation and in the escalation of alcohol use from episodic use to abuse and, ultimately, dependence. Stress responses are crucial for survival by allowing the organism to coordinate appropriate behavioral adaptations to adverse stimuli and are essential homeostatic processes. Central components of the stress response include activation of the HPA axis, increases in norepinephrine turnover in a brain region, the locus coeruleus, and activation of CRF systems (Habib et al. 2001). CRF acts through two pathways. First, it acts as a signaling hormone inside the HPA axis, where it is released from the paraventricular nucleus of the

The IMAGEN Study

The IMAGEN study (www.imagen-europe.com) is the first study aimed at identifying the genetic and neurobiological basis of individual variability in impulsivity, reinforcer sensitivity, and emotional reactivity, as well as determining their predictive value for the development of common psychiatric disorders. The data collection of IMAGEN began in 2007. Since then, the study has collected comprehensive behavioral and neuropsychological data, as well as functional/structural neuroimaging data for 2,000 14-year-old adolescents. These data are complemented by genome-wide association (GWA) data on the study participants. These genetic analyses target approximately 600,000 DNA markers distributed across the genome, using the Illumina Quad 660 chip.

Data from the first wave of IMAGEN became available in 2010 in an extensive database (Schumann et al. 2010), and since then several articles have been published on the dataset, contributing toward a greater understanding of the adolescent brain. For example, Peters and colleagues (2010) showed that adolescent smokers display lower activation of the ventral striatum during reward anticipation compared to their nonsmoking peers. Other studies identified gender-dependent amygdala lateralization during face processing and created probabilistic maps of the face network in the adolescent brain (Schneider et al. 2010; Tahmasebi et al. 2010).

The sample will be followed up at age 16 to investigate the predictive value of genetic factors and intermediate phenotypes for the development of mental disorders, such as alcohol dependence. The full dataset from the follow-up will be completed in 2012. A second follow-up is planned to be completed when the participants reach age 18.

In conclusion, IMAGEN integrates technological and methodological advances in the field of cognitive neuroscience as well as in the fields of human and molecular genetics. This comprehensive approach, together with the large sample sizes, will provide new insights into the interplay between genes and environments that results in individual variability in brain structure, function, and psychological traits. The complex phenotypic and genotypic profiling provided by IMAGEN will be vital in identifying biomarkers that aid in earlier diagnosis and in the developments of treatments for psychiatric disorders, including alcohol dependence.

References

Schneider, S.; Peters, J.; Bromberg, U.; et al. Boys do it the right way: Sex-dependent amygdala lateralization during face processing in adolescents. *NeuroImage* 56:1847–1853, 2011. PMID: 21316467

Schumann, G.; Loth, E.; Banaschewski, T.; et al. The IMAGEN study: Reinforcement-related behaviour in normal brain function and psychopathology. *Molecular Psychiatry* 15:1128–1139, 2010. PMID: 21102431

Tahmasebi, A.M.; Artiges, E.; Banaschewski, T.; et al. Creating probabilistic maps of the face network in the adolescent brain: A multicentre functional MRI study. *Human Brain Mapping,* 2010 [Epub ahead of print]. PMID: 21416563

hypothalamus. It then is transported to the anterior pituitary, where it binds to CRF receptors (CRF1 and CRF2), thereby eliciting the release of adrenocorticotrophic hormone (ACTH). ACTH production ultimately results in the release of stress hormones (i.e., glucocorticoids) from the adrenal glands. The main glucocorticoid in humans is cortisol. Second, CRF acts outside of the hypothalamus (i.e., extrahypothalamically) because immunological tests have detected its presence in the extended amygdala and the brainstem (Swanson et al. 1983).

Studies have demonstrated that exaggerated HPA axis responses to stress can precede the onset of alcoholism. Nondependent sons of alcoholic fathers (who are at increased risk of alcoholism) displayed increased cortisol and ACTH responses to psychosocial stress compared with people with no family history of alcoholism (Uhart et al. 2006; Zimmermann et al. 2004*a*, *b*). Furthermore, alcohol had a greater attenuating effect on ACTH and a related hormone (i.e., arginine vasopressin [AVP]) in people with alcoholic fathers, suggesting that alcohol may be more rewarding for such individuals (Zimmermann et al. 2004*b*). These findings also indicate that interindividual differences in HPA axis activity may underlie some of the variation observed in the vulnerability to alcohol dependence.

As alcohol dependence develops, the stress response systems are upregulated, and this hyperactivity may in fact be a pathological component of dependence (Koob 2008). It has been hypothesized that as dependence develops, the motivation for alcohol use shifts from positive reinforcement, whereby alcohol is consumed for its pleasurable effects, to negative reinforcement—that is, the drinker consumes alcohol to alleviate the negative emotional effects encountered during withdrawal and into protracted abstinence (Koob and Le Moal 2008). The development of negative emotional states has been proposed to include the recruitment and subsequent deregulation of various brain stress system, including the HPA axis, extrahypothalamic CRF, and various others[4] (George et al. 2008; Koob 2008). Genetic variation in genes encoding components of these stress response systems therefore may be relevant for the risk for alcohol dependence.

Genetic Influences on Stress Responding and Their Role in Alcohol Dependence

The variability between individuals in stress responding results at least partially from inherited factors (Armbruster et al. 2009; Linkowski et al. 1993; Meikle et al. 1988) that also may influence the risk of alcohol dependence. For example, polymorphisms that affect only a single DNA building block (i.e., single nucleotide polymorphisms [SNPs]) in the gene encoding CRF1 were associated with alcohol consumption and a lifetime prevalence of drunkenness in two independent samples (Treutlein et al. 2006). One of those polymorphisms, known as rs1876831, was found to moderate the effects of stress on drinking. Thus, adolescents at age 15 who had experienced negative life events in

[4] Additional brain stress response systems involve the signaling molecules norepinephrine, neuropeptide Y, tachykinins, and dynorphins.

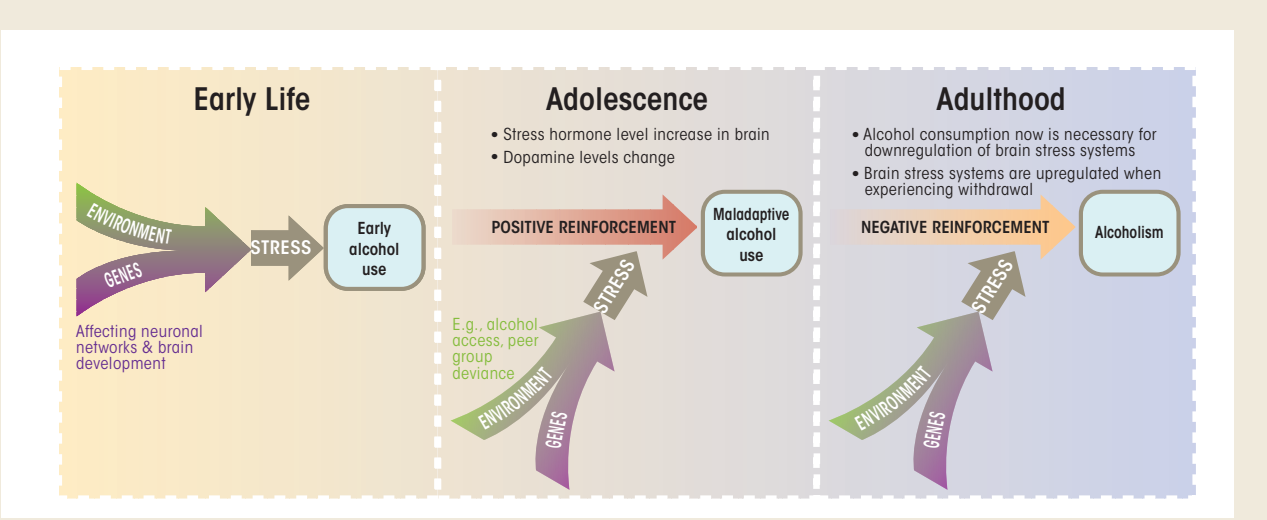


Figure Schematic depiction of the typical progression from alcohol use to alcohol dependence. Both genetic and environmental factors influence each stage of disease progression. Early-life experiences, including prenatal environments and early-life stressors, may affect the onset of alcohol use. In adolescence, heightened sensation seeking, resulting from an increase in cortical dopamine neurons, often results in experimentation with alcohol. In adulthood, alcohol use may occur to downregulate brain stress systems in individuals suffering from alcohol dependence. Thus, early alcohol use is motivated by positive reinforcement, whereas later stages are driven by negative reinforcement, when alcohol is consumed to alleviate negative emotional states.

the past 3 years and who carried the variant (i.e., allele) of rs1876831 that was associated with increased risk of drinking displayed increased alcohol consumption per drinking occasion and greater lifetime rates of heavy drinking (Blomeyer et al. 2008). A similar effect also was observed at age 19, when the risk allele was associated with earlier age of onset of alcohol use and higher alcohol consumption in individuals exposed to stressful life events (Schmid et al. 2010). Furthermore, a gene–environment interaction was detected with a combination of several gene variants (i.e., a haplotype) in the *CRF1* gene (which also contains rs1876831) and childhood sexual abuse in a large cohort of Australians recruited for the Nicotine Genetics Project (Saccone et al. 2007). Individuals who had experienced childhood abuse but carried a protective polymorphism of the *CRF1* gene had lower lifetime alcohol consumption scores and rates of alcohol dependence (Nelson et al. 2009).

Further genetic factors mediating the association between the stress response and alcohol consumption are found in genes encoding the receptors to which cortisol binds after it is released from the adrenal gland when the HPA becomes activated (Bjorntorp 2001). Cortisol binds to glucocorticoid receptors (GRs) that are made up of two identical subunits (i.e., form homodimers). These receptors interact with certain DNA sequences, glucocorticoid response elements (GREs), in the target genes, thereby activating those genes as part of the stress response (Gower 1993; Simons et al. 1992). The GRs are encoded by a family of genes known as nuclear member subfamily 3 (*NR3C*) genes.

Researchers have identified functional polymorphisms in the genes encoding two receptors, NR3C1 and NR3C2, which are associated with differential responses to stress (Wust et al. 2004). For example, a SNP, N363S that results in an altered receptor, protein (i.e., a non-synonymous SNP) in *NR3C1* is associated with increased glucocorticoid sensitivity (Huizenga et al. 1998) as well as elevated levels of cortisol in the saliva of healthy people in response to psychosocial stress (Wust et al. 2004). Moreover, a haplotype that includes three SNPs and is located in a noncoding region of the *NR3C1* gene also is associated with enhanced sensitivity to glucocorticoids (Stevens et al. 2004). Because chronic alcohol consumption can increase HPA axis activity in animals and humans (Rivier 1996; Rivier and Lee 1996; Waltman et al. 1994), polymorphisms in genes encoding components of the HPA axis may increase the risk for alcohol abuse. Indeed, a recent study of 26 SNPs across the *NR3C1* gene in 4,534 adolescents identified several variants that were associated with onset of drinking and drunkenness by age 14, suggesting that genetic variation in *NR3C1* can influence the risk of alcohol abuse in adolescence (Desrivieres 2010). Likewise, variants in the gene encoding the ACTH precursor, promelanocortin *(POMC)*, have been associated with substance abuse, including alcohol abuse (Zhang et al. 2009).

Genes encoding components of the norepinephrine stress response system also have been linked to variability in the response to stress. Thus, polymorphisms in the *ADRA2A* gene, which encodes adrenergic receptors that inhibit norepinephrine release from the neuron, are associated with certain aspects of the stress response as determined by measuring blood pressure and heart rate (Finley et al. 2004). In addition, variants in the *ADRA2A* gene are associated with alcohol abuse phenotypes in humans. For example, in a study analyzing 23 SNPs in *ADRA2A* as well as in a gene *SLC6A2* (which encodes the norepinephrine transporter, NET1) in association with adult alcohol dependence identified two SNPs in *ADRA2A* associated with a positive family history of alcoholism and four SNPs in *SLC6A2* associated with adult alcohol dependence (Clarke et al. 2010).

All of these studies demonstrate that genes that regulate stress responding also influence the risk for alcohol dependence. Thus, people who display increased sensitivity to stress may consume alcohol to dampen the exaggerated stress responses and therefore may find alcohol more rewarding. These people also may more readily experience the negative emotional states associated with withdrawal after chronic alcohol exposure, which may accelerate the transition to dependence. However, the precise relationship between genes, stress, and alcohol use is complex, and gene–environment interactions are notoriously difficult to elucidate (Flint and Munafo 2008). Therefore, translational studies analyzing the effects of genetic factors and stress and their interactions under tightly controlled experimental conditions using animal models are warranted (Barr and Goldman 2006). Indeed, the study of the extrahypothalamic CRF system in animals has helped to clearly delineate the role of brain stress systems in the pathology of alcoholism, and this system is now a plausible target for future alcoholism pharmacotherapies. (For more information on these studies, see the sidebar "The Extrahypothalamic CRF System and the Transition to Alcohol Dependence.")

Another confounding issue for the study of gene–environment interactions is that many studies are conducted retrospectively, and the participants' recall of environmental risk factors may not be accurate. Therefore, prospective longitudinal studies are of great importance to advance the field of gene–environment interactions in alcohol dependence. One study that illustrates how such methodological issues can be addressed is the IMAGEN study, a longitudinal initiative funded by the Framework 6 program of the European Commission and the Medical Research Council that tracks the interplay between genetic polymorphisms and environmental stressors from early adolescence onward. The study collects neuropsychological, behavioral, and functional/structural neuroimaging data and also conducts genetic analyses on a sample of 2,000 adolescents from age 14 onward. (For more information

on this study, see the sidebar "The IMAGEN Study.")

Conclusion and Future Perspectives

Dopaminergic and stress response pathways jointly are engaged upon the commencement of alcohol consumption. Genetic polymorphisms within these pathways may affect the risk of developing alcohol dependence. The effects of exposure to environmental stressors that increase the risk of developing alcohol dependence may be augmented in genetically vulnerable individuals. In some cases, these genetic variants may vary the impact that a particular stressor has within a specific time window (see the figure). To elucidate the role of alcohol usage as a consequence of environmental stressors, and as an environmental stressor in itself, longitudinal studies of the interplay between genes and environments are needed.

The IMAGEN study is an ongoing longitudinal study that attempts to address the role of genes and the environment in alcohol use. The extensive phenotypic database available from this study will allow researchers to test the hypothesis that overactivity of the brain's stress systems, resulting from childhood maltreatment and neglect, may affect brain development and ultimately behaviors such as alcohol use. Alcohol use patterns of the IMAGEN participants are recorded to investigate the long-term effects of early intoxication on cognitive development and behavior. Finally, genetic analyses investigating the association of genetic markers distributed across the genome with specific traits or behaviors (i.e., genomewide association data) are available for each participant and may demonstrate the relationship between genes of the stress response system and intermediate phenotypes (Schumann et al. 2010).

Longitudinal gene–neuroimaging studies, such as the IMAGEN study, aim to clarify the role of the HPA axis and supplementary stress systems in the development and maintenance of alcohol dependence. Such studies will elucidate how alcohol use fluctuates throughout development under the influence of genetic and environmental factors. A better understanding of these factors will promote novel therapies for alcohol dependence as well as approaches to prevent the disorder. ■

Financial Disclosure

The authors declare that they have no competing financial interests.

References

Ariza Cardenal, C., and Nebot Adell, M. Factors associated with problematic alcohol consumption in schoolchildren. *Journal of Adolescent Health* 27:425–433, 2000. PMID: 11090745

Armbruster, D.; Mueller, A.; Moser, D.A.; et al. Interaction effect of D4 dopamine receptor gene and serotonin transporter promoter polymorphism on the cortisol stress response. *Behavioral Neuroscience* 123:1288–1295, 2009. PMID: 20001112

Badanich, K.A.; Maldonado, A.M.; and Kirstein, C. L. Chronic ethanol exposure during adolescence increases basal dopamine in the nucleus accumbens septi during adulthood. *Alcoholism: Clinical and Experimental Research* 31:895–900, 2007. PMID: 17391340

Barbier, E.; Houchi, H.; Warnault, V.; et al. Effects of prenatal and postnatal maternal ethanol on offspring response to alcohol and psychostimulants in Long Evans rats. *Neuroscience* 161:427–440, 2009. PMID: 19348874

Barr, C.S., and Goldman, D. Non-human primate models of inheritance vulnerability to alcohol use disorders. *Addiction Biology* 11:374–385, 2006. PMID: 16961765

Barr, C.S.; Schwandt, M.; Lindell, S.G.; et al. Association of a functional polymorphism in the mu-opioid receptor gene with alcohol response and consumption in male rhesus macaques. *Archives of General Psychiatry* 64:369–376, 2007. PMID: 17339526

Barr, C.S.; Schwandt, M.L.; Lindell, S.G.; et al. Variation at the mu-opioid receptor gene (OPRM1) influences attachment behavior in infant primates. *Proceedings of the National Academy of Sciences of the United States of America* 105:5277–5281, 2008. PMID: 18378897

Bava, S., and Tapert, S.F. Adolescent brain development and the risk for alcohol and other drug problems. *Neuropsychology Review* 20:398–413, 2010. PMID: 20953990

Bjorntorp, P. Do stress reactions cause abdominal obesity and comorbidities? *Obesity Reviews* 2:73–86, 2001. PMID: 12119665

Blomeyer, D.; Treutlein, J.; Esser, G.; et al. Interaction between CRHR1 gene and stressful life events predicts adolescent heavy alcohol use. *Biological Psychiatry* 63:146–151, 2008. PMID: 17597588

Campbell, J.C.; Szumlinski, K.K.; and Kippin, T.E. Contribution of early environmental stress to alcoholism vulnerability. *Alcohol* 43:547–554, 2009. PMID: 19913199

Clarke, T.K.; Dempster, E.; Docherty, S.J.; et al. Multiple polymorphisms in genes of the adrenergic stress system confer vulnerability to alcohol abuse. *Addiction Biology* 17:202–208, 2012. PMID: 21070505

Clarke, T.K.; Laucht, M.; Ridinger, M.; et al. KCNJ6 is associated with adult alcohol dependence and involved in gene × early life stress interactions in adolescent alcohol drinking. *Neuropsychopharmacology* 36:1142–1148, 2011. PMID: 21307845

Copeland, W.E.; Sun, H.; Costello, E.J.; et al. Child mu–opioid receptor gene variant influences parent-child relations. *Neuropsychopharmacology* 36:1165–1170, 2011. PMID : 21326192

Desrivieres, S.; Lourdusamy, A.; Muller, C.; et al. Glucocorticoid receptor (NR3C1) gene polymorphisms and onset of alcohol abuse in adolescents. *Addiction Biology* 16:510–513, 2011. PMID: 20731635

Di Chiara, G., and Imperato, A. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proceedings of the National Academy of Sciences of the United States of America* 85:5274–5278, 1988. PMID: 2899326

Diana, M.; Pistis, M.; Carboni, S.; et al. Profound decrement of mesolimbic dopaminergic neuronal activity during ethanol withdrawal syndrome in rats: Electrophysiological and biochemical evidence. *Proceedings of the National Academy of Sciences of the United States of America* 90:7966–7969, 1993. PMID: 8367449

Dick, D.M.; Pagan, J.L.; Viken, R.; et al. Changing environmental influences on substance use across development. *Twin Research and Human Genetics* 10:315–326, 2007. PMID: 17564520

Dillon, D.G.; Holmes, A.J.; Birk, J.L.; et al. Childhood adversity is associated with left basal ganglia dysfunction during reward anticipation in adulthood. *Biological Psychiatry* 66:206–213, 2009. PMID: 19358974

Ducci, F.; Roy, A.; Shen, P.H.; et al. Association of substance use disorders with childhood trauma but not African genetic heritage in an African American cohort. *American Journal of Psychiatry* 166:1031–1040, 2009. PMID: 19605534

Enoch, M.A.; Hodgkinson, C.A.; Yuan, Q.; et al. The influence of GABRA2, childhood trauma, and their interaction on alcohol, heroin, and cocaine dependence. *Biological Psychiatry* 67:20–27, 2010. PMID: 19833324

Fahlke, C.; Engel, J.A.; Eriksson, C.J.; et al. Involvement of corticosterone in the modulation of ethanol consumption in the rat. *Alcohol* 11:195–202, 1994. PMID : 8060519

Fahlke, C.; Lorenz, J.G.; Long, J.; et al. Rearing experiences and stress-induced plasma cortisol as early risk factors for excessive alcohol consumption in nonhuman

primates. *Alcoholism: Clinical and Experimental Research* 24:644–650, 2000. PMID: 10832905

Finley, J.C., Jr.; O'Leary, M.; Wester, D.; et al. A genetic polymorphism of the alpha2-adrenergic receptor increases autonomic responses to stress. *Journal of Applied Physiology* 96:2231–2239, 2004. PMID: 14742450

Flint, J., and Munafo, M.R. Forum: Interactions between gene and environment. *Current Opinion in Psychiatry* 21:315–317, 2008. PMID: 18520729

Fullgrabe, M.W.; Vengeliene, V.; and Spanagel, R. Influence of age at drinking onset on the alcohol deprivation effect and stress-induced drinking in female rats. *Pharmacology, Biochemistry, and Behavior* 86:320–326, 2007. PMID: 17098280

Gass, J.T., and Olive, M.F. Glutamatergic substrates of drug addiction and alcoholism. *Biochemical Pharmacology* 75:218–265, 2008. PMID: 17706608

George, D.T.; Gilman, J.; Hersh, J.; et al. Neurokinin 1 receptor antagonism as a possible therapy for alcoholism. *Science* 319:1536–1539, 2008. PMID: 18276852

Giedd, J.N. The anatomy of mentalization: A view from developmental neuroimaging. *Bulletin of the Menninger Clinic* 67:132–142, 2003. PMID: 14604098

Gower, W.R., Jr. Mechanism of glucocorticoid action. *Journal of the Florida Medical Association* 80:697–700, 1993. PMID: 8270904

Grant, B.F., and Dawson, D.A. Age at onset of alcohol use and its association with DSM-IV alcohol abuse and dependence: Results from the National Longitudinal Alcohol Epidemiologic Survey. *Journal of Substance Abuse* 9:103–110, 1997. PMID: 9494942

Gresch, P.J.; Sved, A.F.; Zigmond, M.J.; and Finlay, J. M. Stress-induced sensitization of dopamine and norepinephrine efflux in medial prefrontal cortex of the rat. *Journal of Neurochemistry* 63:575–583, 1994. PMID: 8035182

Habib, K.E.; Gold, P.W.; and Chrousos, G. P. Neuroendocrinology of stress. *Endocrinology and Metabolism Clinics of North America* 30:695–728, 2001. PMID: 11571937

Hawkins, J.D.; Graham, J.W.; Maguin, E.; et al. Exploring the effects of age of alcohol use initiation and psychosocial risk factors on subsequent alcohol misuse. *Journal of Studies on Alcohol* 58:280–290, 1997. PMID: 9130220

Heath, A.C.; Bucholz, K.K.; Madden, P.A.; et al. Genetic and environmental contributions to alcohol dependence risk in a national twin sample: Consistency of findings in women and men. *Psychological Medicine* 27:1381–1396, 1997. PMID: 9403910

Hopfer, C.J.; Crowley, T.J.; and Hewitt, J.K. Review of twin and adoption studies of adolescent substance use. *Journal of the American Academy of Child and Adolescent Psychiatry* 42:710–719, 2003. PMID: 12921479

Huizenga, N.A.; Koper, J.W.; De Lange, P.; et al. A polymorphism in the glucocorticoid receptor gene may be associated with an increased sensitivity to glucocorticoids in vivo. *Journal of Clinical Endocrinology and Metabolism* 83:144–151, 1998. PMID: 9435432

Jahng, J.W.; Ryu, V.; Yoo, S.B.; et al. Mesolimbic dopaminergic activity responding to acute stress is blunted in adolescent rats that experienced neonatal maternal separation. *Neuroscience* 171:144–152, 2010. PMID : 20828601

Kalsbeek, A.; Voorn, P.; Buijs, R.M.; et al. Development of the dopaminergic innervation in the prefrontal cortex of the rat. *Journal of Comparative Neurology* 269:58–72, 1988. PMID: 3361004

Karvonen, S. Regional differences in drinking among Finnish adolescents. *Addiction* 90:57–64, 1995. PMID: 7888980

Kendler, K.S.; Gardner, C.; and Dick, D.M. Predicting alcohol consumption in adolescence from alcohol-specific and general externalizing genetic risk factors, key environmental exposures and their interaction. *Psychological Medicine* 41:1507–1516, 2011. PMID: 20942993

Koob, G.F. A role for brain stress systems in addiction. *Neuron* 59:11–34, 2008. PMID: 18614026

Koob, G.F. The role of CRF and CRF-related peptides in the dark side of addiction. *Brain Research* 1314:3–14, 2010. PMID: 19912996

Koob, G.F., and Le Moal, M. Addiction and the brain antireward system. *Annual Review of Psychology* 59:29–53, 2008. PMID: 18154498

Kuzhikandathil, E.V.; Yu, W.; and Oxford, G.S. Human dopamine D3 and D2L receptors couple to inward rectifier potassium channels in mammalian cell lines. *Molecular and Cellular Neurosciemces* 12:390–402, 1998. PMID: 9888991

Latendresse, S.J.; Rose, R.J.; Viken, R.J.; et al. Parenting mechanisms in links between parents' and adolescents' alcohol use behaviors. *Alcoholism: Clinical and Experimental Research* 32:322–330, 2008. PMID: 1162066

Laucht, M.; Treutlein, J.; Schmid, B.; et al. Impact of psychosocial adversity on alcohol intake in young adults: Moderation by the LL genotype of the serotonin transporter polymorphism. *Biological Psychiatry* 66:102–109, 2009. PMID: 19358979

Le, A.D.; Harding, S.; Juzytsch, W.; et al. The role of corticotrophin-releasing factor in stress-induced relapse to alcohol-seeking behavior in rats. *Psychopharmacology (Berlin)* 150:317–324, 2000. PMID: 10923760

Linkowski, P.; Van Onderbergen, A.; Kerkhofs, M.; et al. Twin study of the 24-h cortisol profile: Evidence for genetic control of the human circadian clock. *American Journal of Physiology* 264(2 Pt. 1):E173–E181, 1993. PMID: 8447383

Malcolm, R.J. GABA systems, benzodiazepines, and substance dependence. *Journal of Clinical Psychiatry* 64(Suppl. 3):36–40, 2003. PMID: 12662132

McGue, M.; Elkins, I.; and Iacono, W.G. Genetic and environmental influences on adolescent substance use and abuse. *American Journal Medical Genetics* 96:671–677, 2000. PMID: 11054776

Meikle, A.W.; Stringham, J.D.; Woodward, M.G.; and Bishop, D.T. Heritability of variation of plasma cortisol levels. *Metabolism* 37:514–517, 1988. PMID: 2967419

Moffitt, T.E. Adolescence-limited and life-course-persistent antisocial behavior: A developmental taxonomy. *Psychological Review* 100:674–701, 1993. PMID: 8255953

Moffitt, T.E.; Caspi, A.; Harrington, H.; and Milne, B.J. Males on the life-course-persistent and adolescence-limited antisocial pathways: Follow-up at age 26 years. *Development and Psychopathology* 14:179–207, 2002. PMID: 11893092

Nelson, E.C.; Agrawal, A.; Pergadia, M.L.; et al. H2 haplotype at chromosome 17q21.31 protects against childhood sexual abuse-associated risk for alcohol consumption and dependence. *Addiction Biology* 15:1–11, 2010. PMID: 19878140

Newman, T.K.; Parker, C.C.; Suomi, S.J.; et al. DRD1 5'UTR variation, sex and early infant stress influence ethanol consumption in rhesus macaques. *Genes, Brain, and Behavior* 8:626–630, 2009. PMID: 19563515

Ortiz, J.; Fitzgerald, L.W.; Lane, S.; et al. Biochemical adaptations in the mesolimbic dopamine system in response to repeated stress. *Neuropsychopharmacology* 14:443–452, 1996. PMID: 8726755

Oslin, D.W.; Berrettini, W.; Kranzler, H.R.; et al. A functional polymorphism of the mu-opioid receptor gene is associated with naltrexone response in alcohol-dependent patients. *Neuropsychopharmacology* 28:1546–1552, 2003. PMID: 12813472

Oswald, L.M., and Wand, G.S. Opioids and alcoholism. *Physiology & Behavior* 81:339–358, 2004. PMID: 15159175

Pagan, J.L.; Rose, R.J.; Viken, R.J.; et al. Genetic and environmental influences on stages of alcohol use across adolescence and into young adulthood. *Behavior Genetics* 36:483–497, 2006. PMID: 16586152

Pascual, M.; Boix, J.; Felipo, V.; and Guerri, C. Repeated alcohol administration during adolescence causes changes in the mesolimbic dopaminergic and glutamatergic systems and promotes alcohol intake in the adult rat. *Journal of Neurochemistry* 108:920–931, 2009. PMID: 19077056

Peters, J.; Bromberg, U.; Schneider, S.; et al. Lower ventral striatal activation during reward anticipation in adolescent smokers. *American Journal of Psychiatry* 168:540–549, 2011. PMID: 21362742

Philpot, R., and Kirstein, C. Developmental differences in the accumbal dopaminergic response to repeated ethanol exposure. *Annals of the New York Academy of Sciences* 1021:422–426, 2004. PMID: 15251921

Piazza, P.V.; Rouge-Pont, F.; Deminiere, J.M.; et al. Dopaminergic activity is reduced in the prefrontal cortex and increased in the nucleus accumbens of rats predisposed to develop amphetamine self-administration. *Brain Research* 567:169–174, 1991. PMID: 1726140

Pilowsky, D.J.; Keyes, K.M.; and Hasin, D.S. Adverse childhood events and lifetime alcohol dependence. *American Journal of Public Health* 99:258–263, 2009. PMID: 19059847

Prescott, C.A., and Kendler, K.S. Genetic and environmental contributions to alcohol abuse and dependence in a population-based sample of male twins. *American*

Journal of Psychiatry 156:34–40, 1999. PMID: 9892295

Ramchandani, V.A.; Umhau, J.; Pavon, F.J.; et al. A genetic determinant of the striatal dopamine response to alcohol in men. *Molecular Psychiatry* 16:809–817, 2011. PMID: 20479755

Ray, L.A., and Hutchison, K.E. A polymorphism of the mu-opioid receptor gene (OPRM1) and sensitivity to the effects of alcohol in humans. *Alcoholism: Clinical and Experimental Research* 28:1789–1795, 2004. PMID: 15608594

Rivier, C. Alcohol stimulates ACTH secretion in the rat: Mechanisms of action and interactions with other stimuli. *Alcoholism: Clinical and Experimental Research* 20:240–254, 1996. PMID: 8730214

Rivier, C., and Lee, S. Acute alcohol administration stimulates the activity of hypothalamic neurons that express corticotropin-releasing factor and vasopressin. *Brain Research* 726:1–10, 1996. PMID: 8836539

Robinson, T.E., and Berridge, K.C. The neural basis of drug craving: An incentive-sensitization theory of addiction. *Brain Research. Brain Research Reviews* 18:247–291, 1993. PMID: 8401595

Rodrigues, A.J.; Leao, P.; Carvalho, M.; et al. Potential programming of dopaminergic circuits by early life stress. *Psychopharmacology (Berlin)* 214:107–120, 2011. PMID: 21088961

Rosenberg, D.R., and Lewis, D.A. Changes in the dopaminergic innervation of monkey prefrontal cortex during late postnatal development: A tyrosine hydroxylase immunohistochemical study. *Biological Psychiatry* 36:272–277, 1994. PMID: 7986893

Saccone, S.F.; Hinrichs, A.L.; Saccone, N.L.; et al. Cholinergic nicotinic receptor genes implicated in a nicotine dependence association study targeting 348 candidate genes with 3713 SNPs. *Human Molecular Genetics* 16:36–49, 2007. PMID: 17135278

Schein, J.C.; Hunter, D.D.; and Roffler-Tarlov, S. Girk2 expression in the ventral midbrain, cerebellum, and olfactory bulb and its relationship to the murine mutation weaver. *Developmental Biology* 204:432–450, 1998. PMID: 9882481

Schmid, B.; Blomeyer, D.; Becker, K.; et al. The interaction between the dopamine transporter gene and age at onset in relation to tobacco and alcohol use among 19-year-olds. *Addiction Biology* 14:489–499, 2009. PMID: 19740369

Schmid, B.; Blomeyer, D.; Treutlein, J.; et al. Interacting effects of CRHR1 gene and stressful life events on drinking initiation and progression among 19-year-olds. *International Journal of Neuropsychopharmacology* 13:703–714, 2010. PMID: 19607758

Schuckit, M.A.; Anthenelli, R.M.; Bucholz, K.K.; et al. The time course of development of alcohol-related problems in men and women. *Journal of Studies on Alcohol* 56:218–225, 1995. PMID: 7760569

Schumann, G.; Loth, E.; Banaschewski, T.; et al. The IMAGEN study: Reinforcement-related behaviour in normal brain function and psychopathology. *Molecular Psychiatry* 15:1128–1139, 2010. PMID: 21102431

Shaham, Y., and Stewart, J. Exposure to mild stress enhances the reinforcing efficacy of intravenous heroin self-administration in rats. *Psychopharmacology (Berlin)* 114:523–527, 1994. PMID: 7855213

Siegmund, S.; Vengeliene, V.; Singer, M.V., and Spanagel, R. Influence of age at drinking onset on long-term ethanol self-administration with deprivation and stress phases. *Alcoholism: Clinical and Experimental Research* 29:1139–1145, 2005. PMID: 16046868

Simons, S.S., Jr.; Oshima, H.; and Szapary, D. Higher levels of control: Modulation of steroid hormone-regulated gene transcription. *Molecular Endocrinology* 6:995–1002, 1992. PMID: 1324423

Soderpalm, B.; Lof, E.; and Ericson, M. Mechanistic studies of ethanol's interaction with the mesolimbic dopamine reward system. *Pharmacopsychiatry* 42(Suppl. 1): S87–S94, 2009. PMID: 19434560

Spanagel, R. Alcoholism: A systems approach from molecular physiology to addictive behavior. *Physiology Reviews* 89:649–705, 2009. PMID: 19342616

Spear, L.P. The adolescent brain and age-related behavioral manifestations. *Neuroscience and Biobehavioral Reviews* 24:417–463, 2000. PMID: 10817843

Stevens, A.; Ray, D.W.; Zeggini, E.; et al. Glucocorticoid sensitivity is determined by a specific glucocorticoid receptor haplotype. *Journal of Clinical Endocrinology and Metabolism* 89:892–897, 2004. PMID: 14764810

Swanson, L.W.; Sawchenko, P.E.; Rivier, J.; and Vale, W.W. Organization of ovine corticotropin-releasing factor immunoreactive cells and fibers in the rat brain: An immunohistochemical study. *Neuroendocrinology* 36:165–186. 1983. PMID: 6601247

Szot, P.; White, S.S.; Veith, R.C.; and Rasmussen, D.D. Reduced gene expression for dopamine biosynthesis and transport in midbrain neurons of adult male rats exposed prenatally to ethanol. *Alcoholism: Clinical and Experimental Research* 23:1643–1649, 1999. PMID: 10549997

Treutlein, J.; Kissling, C.; Frank, J.; et al. Genetic association of the human corticotropin releasing hormone receptor 1 (CRHR1) with binge drinking and alcohol intake patterns in two independent samples. *Molecular Psychiatry* 11:594–602, 2006. 16550213

Tupala, E., and Tiihonen, J. Dopamine and alcoholism: Neurobiological basis of ethanol abuse. *Progress in Neuro-psychopharmacology & Biological Psychiatry* 28:1221–1247, 2004. PMID: 15588749

Uhart, M., and Wand, G.S. Stress, alcohol and drug interaction: An update of human research. *Addiction Biology* 14:43–64, 2009. PMID: 18855803

Uhart, M.; Oswald, L.; McCaul, M.E.; et al. Hormonal responses to psychological stress and family history of alcoholism. *Neuropsychopharmacology* 31:2255–2263, 2006. PMID: 16554744

Waltman, C.; McCaul, M.E.; and Wand, G.S. Adrenocorticotropin responses following administration of ethanol and ovine corticotropin-releasing hormone in the sons of alcoholics and control subjects. *Alcoholism: Clinical and Experimental Research* 18:826–830, 1994. 7978091

Weiss, F.; Parsons, L.H.; Schulteis, G.; et al. Ethanol self-administration restores withdrawal-associated deficiencies in accumbal dopamine and 5-hydroxytryptamine release in dependent rats. *Journal of Neuroscience* 16:3474–3485, 1996. PMID: 8627380

Wu, P.; Bird, H.R.; Liu, X.; et al. Trauma, posttraumatic stress symptoms, and alcohol-use initiation in children. *Journal of Studies on Alcohol and Drugs* 71:326–334, 2010. PMID: 20409425

Wust, S.; van Rossum, E.F.; Federenko, I.S.; et al. Common polymorphisms in the glucocorticoid receptor gene are associated with adrenocortical responses to psychosocial stress. *Journal of Clinical Endocrinology and Metabolism* 89:565–573, 2004. PMID: 14764763

Zhang, H.; Kranzler, H.R.; Weiss, R.D.; et al. Pro-opiomelanocortin gene variation related to alcohol or drug dependence: Evidence and replications across family- and population-based studies. *Biological Psychiatry* 66:128–136, 2009. PMID: 19217079

Zimmermann, U.; Spring, K.; Kunz-Ebrecht, S.R.; et al. Effect of ethanol on hypothalamic-pituitary-adrenal system response to psychosocial stress in sons of alcohol-dependent fathers. *Neuropsychopharmacology* 29:1156–1165, 2004*a*. PMID: 15100697

Zimmermann, U.; Spring, K.; Wittchen, H.U.; et al. Arginine vasopressin and adrenocorticotropin secretion in response to psychosocial stress is attenuated by ethanol in sons of alcohol-dependent fathers. *Journal of Psychiatric Research* 38:385–393, 2004*b*. PMID: 15203290

Genetic and Genomic Web Resources for Research on Alcohol Use and Abuse

Robert W. Williams, Ph.D.

There are two major ways of publishing scientific data and results: (1) the standard peer-reviewed paper, which dates back to volume 1 of the Philosophical Transactions of the Royal Society in 1665; and (2) online distribution of data, resources, and software using the Internet that dates back a mere 21 years to the first Web site at the European Organization for Nuclear Research (CERN) established by Tim Berners-Lee.

Today online resources for sharing scientific work abound. The National Library of Medicine's repository, PubMed, captures more than 21 million citations for biomedical literature. NIAAA can lay claim to the first URL listed in PubMed: The Portable Dictionary of the Mouse Genome (Williams 1994). This site—now called GeneNetwork.org—has been supported by NIAAA for more than a decade as part of the Integrative Neuroscience of Initiative on Alcoholism (INIA).

There are hundreds of sophisticated Web services and resources that can be exploited by students and researchers interested in alcoholism and other substance use disorders. These resources can be used like publications, but a better way to think about them is as a second "dry" laboratory in which it is possible to carry out experiments and to either generate or test ideas by reusing data that often have been rescued from the classic literature.

Below is a short list of both well-known and more esoteric resources, many of which have been supported by NIAAA, that can be used as a complement to the set of reviews in this special issue. There are two major categories of sites in this list: (1) those that provide deep data along with software that can be used to perform analysis, (2) those that can provide physical resources such as samples, clones, and powerful experimental murine models. The first category is easy to browse directly from the links below; whereas the second category is geared more to students and scientists in need of a jump-start to understand the function of specific genes.

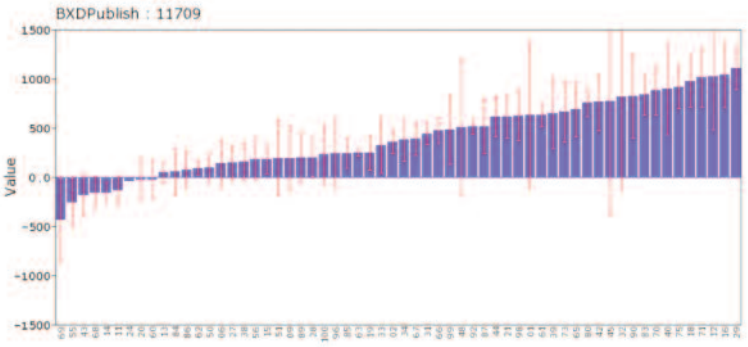


Figure 1A Effects of alcohol taken from mice. This plot is taken directly from GeneNetwork and the work of Philip and colleagues (2010). It illustrates the high variability in the response to alcohol among 60 strains of mice (adult females, GeneNetwork Trait Mouse BXD Published Phenotype 11709). Differences in the height of the blue bars highlight the substantial genetic variation in activity levels (sedation to the left, activation to the right) among strains. The red error bars highlight the important role of non-genetic factors. Data of this type can be computationally compared to single-nucleotide polymorphism (SNP) genetic markers to determine which regions of the mouse genome modulate sedative vs. excitatory effects of ethanol (2.25 mg/kg) in this family.

Category 1: Web Resources for Online Analysis of the Genetics of Alcoholism and More

GeneNetwork (www.genenetwork.org): This is a comprehensive resource for learning about genetics, but users may need to read the help files, FAQs, or one of the references (Chesler et al., 2003; Grisham et al., 2010, www.lifescied.org/content/9/2/98.full.pdf). GeneNetwork is one of an interlinked trio of sites built up by NIAAA (GeneWeaver and WebGestalt are the other two) to house extensive data for human, monkey, rat, mouse, and fruit fly. It includes hundreds of data sets on responses to alcohol, particularly in a family of mice called the BXDs. Data are linked with powerful gene analysis and mapping tools. Think of it as a free suite of genetics and statistics programs that happen to be loaded with genetic and genomic data sets, along with complimentary data on

Robert W. Williams, Ph.D., *is a professor in the Center for Integrative and Translational Genomics, The University of Tennessee Health Science Center, Memphis, Tennessee.*

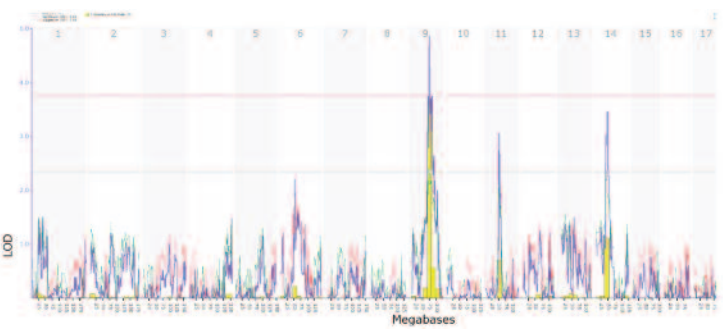


Figure 1B A genetic map of alcohol sedation/activation effects. The horizontal x-axis lists mouse chromosomes, from 1 to the left to chromosome X to the right. The large peak on chromosome 9—a so-called quantitative trait locus (QTL)—is the principal part of the mouse genome that modulates activation levels in females of this BXD family. This sharp peak (high LOD score of 5.0 on the y-axis) can be expanded in GeneNetwork and reveals approximately 160 genes at the peak between 67 and 87 megabases. This set of genes can then be analyzed in GeneWeaver, WebGestalt, COGA, and many other Web resources to evaluate which subsets are most likely to cause differences in response to alcohol, including the suspected alcohol candidate genes, serotonin 1B receptor (5HT1B) and RAB27A. Other features of this genetic map are explained on the Web site.

biological responses to alcohol and many other drugs (Philip et al., 2010). (For more information, see figure 1A and B)

GeneWeaver (www.geneweaver.org): This is another NIAAA-funded project that offers a powerful tool for the integrative analysis of collections of lists of genes and their functional relationships (Baker et al., 2012). This resource-and-analysis tool provides a way of making sense of a large group of related genomic studies. Excellent user interface and tutorials make this a starting point for those with large gene expression data sets. It also is a straightforward of performing analyses of many curated gene sets in the GeneWeaver database (see figure 2).

WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt): Like GeneWeaver this is a sophisticated tool for the analysis of sets of genes. It includes species as diverse as yeast, worms, and humans (Duncan et al., 2010). WebGestalt often is used to perform pathway analysis and gene ontology analysis—a computationally demanding categorization of genes based on their known functions.

Allen Brain Atlas (www.brain-map.org): This site is a noteworthy philanthropic contribution from Paul Allen to brain research. The site started with a focus on gene expression patterns in the brain of the mouse; however, within the last year, it has expanded rapidly and now also covers gene expression in humans and non-human primates. Scientists interested in brain research should visit this site at least once to see the full power of Web services and Web science—it puts a massive research lab at your fingertips.

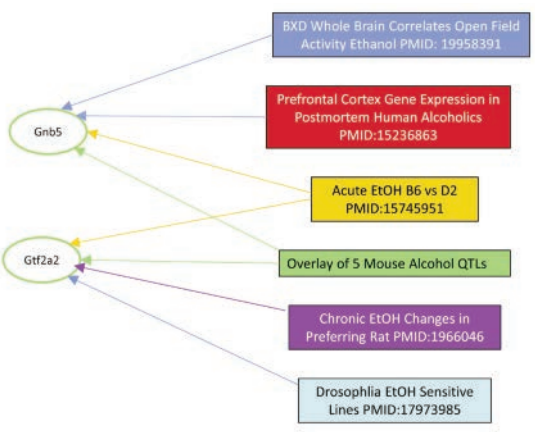


Figure 2 GeneWeaver output graph illustrating this program's ability to find the connections between six data sets listed to the right in boxes with two interesting candidate genes—GNB5 and GTF2A2 (left side in ovals). These two candidate genes are both located in the region highlighted in the QTL map of Figure 1B.

Genetic and Genomic Web Resources for Research on Alcohol Use and Abuse *continued*

GeneMania (www.genemania.org) and Gemma (www.chibi.ubc.ca/Gemma): These are two Web sites used for the systematic analysis of molecular networks. Both of these Canadian sites have powerful and user-friendly interfaces.

Mouse Phenome Database (http://phenome.jax.org): This database offers medical records for mice, including mice that will voluntarily drink to intoxication. The site includes hundreds of valuable phenotype data for generations of mice (Maddatu et al., 2012). It includes links to matched genetic data.

PhenoGen Informatics (http://phenogen.ucdenver.edu): This site provides deep and total access to many massive microarray data sets, many with a focus on the genetics and genomics of alcoholism (Bhave et al., 2007). The site can be used to analyze scientists' own data sets, particularly microarray data.

Collaborative Study on the Genetics of Alcoholism (http://zork.wustl.edu/niaaa/): COGA might be considered the "mother lode" of studies on the genetics of alcoholism, with more than 300 families and 3,000 individuals. This remains an active research program. Because of patient confidentiality it is not possible to directly access key data, as is the case for mouse and rat resources. Still this site provides a comprehensive overview of the data that have been generated and links to virtually all of the associated research papers.

Portland Alcohol Research Center (http://www.ohsu.edu/parc/by_phen.shtml): This site contains a useful and extensive list of gene regions known to modulate response to alcohol in mouse models. A number of these loci have been "converted" into single causal gene variants and many more should now be aligned to corresponding regions in the mouse genome. (See the next resource—the ERGR—for online comparison of the human genome to those of other species.)

Ethanol-Related Gene Resource (http://bioinfo.mc.vanderbilt.edu/ERGR/): This is an excellent site for reviewing what is known about the genetics of alcoholism in many different types of organism—offering a comparative approach (Guo et al., 2009).

Category 2: Web Resources for Tissues, Clones, and Mouse Models

MATRR (https://gleek.ecs.baylor.edu/): The Monkey Alcohol Tissue Research Resource is a new NIAAA-supported site intended for experts in the field who need to understand the causes and impact of alcoholism. Non-human primates have proved to be strikingly faithful models of many aspects of alcoholism in humans, and they provide far better experimental control.

Gene Knockout Project (KOMP and EUCOMM, at www.knockoutmouse.org): This is an international initiative that is making major mutations (knock-outs) in every one of about 19,000 genes in the mammalian (murine) genome. NIAAA contributes to this effort, with a special focus on those genes known to be involved in brain function and suspected to modulate risk of alcoholism.

Cre-driver Network (www.credrivermice.org): This is a companion to the knockout project (above). It provides information on how to obtain lines of mice that can be used to turn genes off in specific types of cells at different points in life.

References

Baker, E.J.; Jay, J.J.; Bubier, J.A.; Langston, M.A.; Chesler, E.J. GeneWeaver: a web-based system for integrative functional genomics. *Nucleic Acids Research* 2012 40(Database issue):D1067–D1076, 2012.

Bhave, S.V.; Hornbaker, C.; Phang, T.L; et al. The PhenoGen informatics website: tools for analyses of complex traits. *BMC Genetics* 8:59, 2007.

Chesler, E.J.; Wang, J.; LU, L; et al. Genetic correlates of gene expression in recombinant inbred strains: a relational model to explore for neurobehavioral phenotypes. *Neuroinformatics* 1: 343–357, 2003

Duncan, D.T.; Prodduturi, N.; Zhang, B. WebGestalt2: an updated and expanded version of the Web-based Gene Set Analysis Toolkit. *BMC Bioinformatics* 11(Suppl 4): P10, 2010.

Grisham, W.; Schottler, N.A.; Valli-Marill, J.; et al. Teaching bioinformatics and neuroinformatics by using free web-based tools. CBE—*Life Sciences Education* 9: 98–107, 2010.

GUO, A.Y.; Webb, B.T.; Miles, M.F.; et al. ERGR: An ethanol-related gene resource. *Nucleic Acids Research* 37 (Database issue):D840–D845, 2009.

Maddatu, T.P.; Grubb, S.C.; Bult, C.J.; And Bogue, M.A. Mouse Phenome Database (MPD). *Nucleic Acids Research* 40(Database issue):D887–894, 2012.

Philip, V.M.; Duvvuro, S.; Gomero, B.; et al. High-throughput behavioral phenotyping in the expanded panel of BXD recombinant inbred strains. *Genes Brain Behavior* 9:129–59, 2010.

Williams, R.W. The portable dictionary of the mouse genome: a personal database for mapping and molecular biology. *Mammalian Genome* 5:372–375, 1994.

Genetics Glossary

Agonist: An agent that mimics the actions or effects of another agent at a receptor (e.g., a drug that mimics the effects of a neurotransmitter).

Allele: One of two or more forms of a gene that reside at the same position on a pair of chromosomes; different alleles of a gene may serve the same function (e.g., code for an enzyme that breaks down alcohol) but may result in proteins with different levels of activity (e.g., rapid or slow alcohol metabolism); alternatively, an allelic variant may not produce a functional protein.

Antagonist: An agent that blocks or reverses the actions or effects of another agent at a receptor (e.g., a drug that blocks the effects of a neurotransmitter).

Astroglia: Characteristic star-shaped cells in the brain and spinal cord that are a type of glial support cell; also called astrocytes; astrocytes and other glial support cells perform many functions to support neurons and thus, normal brain functioning.

Ataxia: Inability to coordinate muscle movements.

Candidate gene: A gene that has been implicated in causing or contributing to a particular trait (e.g., a disease); can be identified by its association with the trait and by performing linkage analyses to identify regions of the *genome* where such genes reside.

Chemokine: Member of a subgroup of proteins (i.e., *cytokines*) that are released by cells and can attract nearby target cells; chemokines are involved in several disease processes, including inflammation.

Conditional inactivation: Inactivation of a gene or protein only under certain conditions (e.g., in the presence of another chemical).

Cre-driver lines: Genetically engineered lines of laboratory animals in which the expression of a certain regulatory protein (i.e., the *Cre recombinase*) is controlled by cell-specific regulatory elements; these animal lines allow for the expression of other studied genes only in certain tissues or under certain conditions.

Cre recombinase: A bacterial enzyme that recognizes a specific DNA sequence called a loxP site and cuts out DNA segments that are located between two loxP sequences; animals expressing Cre recombinase in the central nervous system are used to perform site-specific gene deletions in the brain.

Cytokines: A family of molecules, produced primarily by cells of the immune system, that regulate cellular interactions and other functions; some cytokines play important roles in initiating and regulating inflammation.

Embryonic stem (ES) cells: Cells derived from early-stage embryos that still retain the ability to develop into all the types of cells that make up an organism.

Endocytosis: A process by which cells absorb molecules (e.g., proteins) by engulfing them.

Endophenotype: A trait or characteristic that is not a direct symptom of the condition under investigation but has been shown to be associated with the condition and shares a genetic cause with the condition; for example, reduced initial sensitivity to some effects of alcohol has been noted in people with greater genetic risk for alcohol use disorders and may be used as an endophenotype to identify people at risk for such disorders.

Epigenetic: A change in gene function that occurs without a change in DNA sequence; epigenetic changes can alter *gene expression* and may be heritable.

Epigenome: The entirety of all chemical modifications that occur within a *genome* without changing the DNA sequence.

Epistasis: The interaction of genes; the effect of one gene is modified by the *allele* that exists at a different site in the genome.

Excitatory neurotransmitter: Any neurotransmitter that in the brain acts to enhance the activity of the signal-receiving neuron.

Expression quantitative trait locus (eQTL): A *quantitative trait locus* (QTL) that controls the expression of *a candidate gene.*

Functional magnetic resonance imaging (fMRI): A type of specialized MRI used to assess brain activity during mental operations by measuring changes in blood flow that result from nerve cell activity in the brain.

Gene expression: The process by which the genetic information encoded in a gene is used to direct the creation of a gene product (i.e., protein).

Genetics Glossary *continued*

Gene network: A group of genes (and the products they encode) that interact with each other to influence a certain outcome or trait.

Gene targeting: A genetic technique used to exchange a specific gene (the targeted gene) within an organism with a modified version of that gene to obtain information about the function of the targeted gene.

Gene trapping: A high-throughput genetic engineering approach to randomly insert a new DNA sequence into genes across the *genome;* results in the expression of the inserted, "trapped" gene and the inactivation of the endogenous gene within which the insertion has occurred.

Genetical genomics: A genetic research approach that combines traditional genetic analyses and *gene expression* information to identify the genetic basis of *gene expression* (i.e., *expression quantitative trait loci* [*eQTLs*]).

Genome: The total genetic information of an organism, cell, or species.

Genotype: The genetic makeup of an organism determined by the particular combination of *alleles* at one or more specific locations (loci) on one or more paired chromosomes.

Haplotype: A set of closely linked genes or genetic markers present on one chromosome that tend to be inherited together.

Heterozygous: Carrying two different *alleles* for a particular gene.

Homozygous: Carrying two copies of the same *allele* for a particular gene.

Knockin mice: Genetically engineered mice in which the gene under investigation has been inserted at a particular site (locus) in the mouse's chromosome.

Knockout mice: Genetically engineered mice in which the gene under investigation has been inactivated by replacing it with a mutated version of the gene that does not code for a functional protein.

Long-term depression: Long-lasting mechanism contributing to *neuroplasticity,* whereby (depending on cell type) an episode of either very strong or very weak signal transmission at a synapse leads to a decreased effectiveness of subsequent signal transmission across that synapse.

Mesocorticolimbic reward pathway: System of interconnected brain regions that includes the ventral tegmental area, nucleus accumbens, amygdala, hippocampus, and frontal cortical regions and which is thought to mediate the rewarding effects of alcohol and other drugs, use as well as natural rewards (e.g., sweets); several neurotransmitters factor prominently in this pathway, but the role of dopamine has been most widely studied.

Messenger RNA (mRNA): Key intermediary molecule generated when a gene is expressed (i.e., when the information encoded in the gene is converted into a protein product); mRNA levels for a gene are used as an indicator of how "active" the gene is (i.e., how much of the protein is likely to be produced).

Metabolite: Intermediary product generated during the break-down (i.e., metabolism) of a particular molecule.

Microarray: A microscopic chip made from glass, plastic, or other type of support material onto which a large number of minute amounts of samples (e.g., proteins or DNA) are affixed in an orderly manner for performing automated assays of protein interactions or *gene expression* or for genotyping.

Microglia: Type of glial cells that act as the first and main form of active immune defense in the central nervous system (i.e., brain and spinal cord); they clear debris such as dead neurons through a process called phagocytosis, in which these cells are engulfed by the microglia and then destroyed.

microRNAs (miRNAs): a class of RNAs that do not encode proteins (i.e, noncoding RNAs) and which are approximately 21 to 23 building blocks (i.e., nucleotides) in length; they are naturally produced in cells and can interact with complementary sequences on *mRNA* molecules, thereby interfering with the further use of these *mRNAs* for protein production.

Mitochondria: Membrane-enclosed structures within cells that generate most of the cells' energy through the production of adenosine triphosphate, a molecule that provides the energy needed for many key metabolic reactions.

Monocytes: A type of white blood cells that are part of the innate immune system and which play multiple roles in immune function.

Genetics Glossary *continued*

Mutagenesis: A process by which the genetic information of an organism is changed in a stable manner, resulting in a mutation that may disrupt or alter gene function.

Neuropeptide: Protein-like molecules used by neurons to communicate with each other.

Neuroplasticity: Ability of the nervous system to change and reorganize itself throughout life by forming new connections among nerve cells or altering the activities of existing nerve cells and connections; is the basis for the ability to learn throughout life; neuroplastic changes also may underlie long-term effects of alcohol or drug exposure.

Next-generation sequencing: High-throughput technique to determine the DNA sequence of entire genomes; allows investigators to determine the order (i.e., sequence) of several hundred billion DNA building blocks per week at a dramatically reduced cost compared with traditional *sequencing* approaches.

Oxidase: Any enzyme that increases the rate of (i.e., catalyzes) an oxidation–reduction reaction involving molecular oxygen (O_2).

Oxidative stress: An imbalance between oxidants (e.g., free radicals or reactive oxygen species) and agents that can detoxify these oxidants (i.e., antioxidants) that can lead to excessive oxidation and cell damage.

Phenotype: The observable structural or functional (e.g., behavioral, physiological, biochemical) characteristics of an individual organism; each phenotype is determined to a varying degree by the *genotype* and environmental factors.

Polymorphism: The presence of two or more *alleles* of a gene or other DNA sequence at a particular locus in a population.

Protease: An enzyme that cuts (i.e., cleaves) proteins into smaller pieces (i.e., peptides); many proteases (e.g., trypsin) cleave the proteins only at specific sites characterized by a specific sequence of amino acids.

Quantitative trait: A *phenotype* that varies in the degree or magnitude to which it is present (e.g., sensitivity to alcohol or height) and which typically is influenced by more than one gene.

Quantitative trait locus (QTL): A DNA region that is associated with a *quantitative trait* and which may contain one or more of the genes contributing to variation in that trait.

Recombinant inbred (RI) mice: A strain of genetically identical animals produced by mating successive generations of sibling animals initially descended from the offspring of a cross (i.e., a recombination) between two distinct inbred strains.

RNA interference (RNAi): A RNA *transcript* produced in the body that does not encode a protein but which can alter the expression of other protein-coding mRNA transcripts; also refers to the process of quelling *gene expression* through the use of these RNA molecules.

Sequencing: Determining the order of the building blocks (i.e., nucleotides) in a DNA or RNA segment or of amino acids in a protein.

Single-nucleotide polymorphism (SNP): A DNA sequence variation at a single nucleotide between members of a species or between paired chromosomes in an individual.

Synaptic plasticity: The ability of the connection between two neurons to change in strength in response to either use or disuse of signal transmission across the site where the two neurons interact (i.e., the synapse).

Syntenic: Pertaining to synteny-the phenomenon that the gene order along chromosomes of different species often is conserved; for example, certain genes located next to each other on mouse chromosome 4 also are located next to each other (i.e., are syntenic) on human chromosome 9.

Transcript: The product of transcription—the process by which the genetic information contained in DNA is converted into an exactly complementary sequence of RNA; used synonymously with *mRNA.*

Transcriptome: The entirety of all transcription products (*transcripts* or *mRNA.*) present in a cell, tissue, or organism.

Whole-genome expression profiling: High-throughput analytical approach to identify genes in a cell that are expressed at a given point in time as well as the level of *gene expression.*

Discovering Genes Involved in Alcohol Dependence and Other Alcohol Responses

Role of Animal Models

Kari J. Buck, Ph.D.; Lauren C. Milner, Ph.D.; Deaunne L. Denmark, M.D., Ph.D.; Seth G.N. Grant, Ph.D.; and Laura B. Kozell, Ph.D.

The genetic determinants of alcoholism still are largely unknown, hindering effective treatment and prevention. Systematic approaches to gene discovery are critical if novel genes and mechanisms involved in alcohol dependence are to be identified. Although no animal model can duplicate all aspects of alcoholism in humans, robust animal models for specific alcohol-related traits, including physiological alcohol dependence and associated withdrawal, have been invaluable resources. Using a variety of genetic animal models, the identification of regions of chromosomal DNA that contain a gene or genes which affect a complex phenotype (i.e., quantitative trait loci [QTLs]) has allowed unbiased searches for candidate genes. Several QTLs with large effects on alcohol withdrawal severity in mice have been detected, and fine mapping of these QTLs has placed them in small intervals on mouse chromosomes 1 and 4 (which correspond to certain regions on human chromosomes 1 and 9). Subsequent work led to the identification of underlying quantitative trait genes (QTGs) (e.g., *Mpdz*) and high-quality QTG candidates (e.g., *Kcnj9* and genes involved in mitochondrial respiration and oxidative stress) and their plausible mechanisms of action. Human association studies provide supporting evidence that these QTLs and QTGs may be directly relevant to alcohol risk factors in clinical populations. **KEY WORDS: Alcoholism; alcohol dependence; alcohol withdrawal; risk factors; genetic factors; genetic theory of AODU; genetic trait; DNA; QTL mapping; quantitative trait loci (QTLs); quantitative trait genes (QTGs); animal models**

A host of biological (i.e., genetic) and environmental factors interact throughout the addictive process to influence alcohol use and abuse. These processes are accompanied by a number of behavioral and neural events that include, but are not limited to, changes in the motivational effects of ethanol (both rewarding and aversive), tolerance to some effects of ethanol, and withdrawal when ethanol use is discontinued (Koob and Le Moal 2008). The role of genetics in individual differences in the degree and/or the rate of development of all such changes clearly has been illustrated in human genetic studies as well as studies using genetic animal models (for reviews, see Crabbe 2008 and references therein; Heath et al. 2011 and references therein). Although a few genes consistently have demonstrated a role in alcoholism or associated characteristics (i.e., subclinical markers known as endophenotypes) in human studies, their identification has relied heavily on prior knowledge of the physiological responses to alcohol. Thus, these genes primarily encode alcohol-metabolizing enzyme isoforms and neurotransmitter receptor subunits, both of which already had been known to be affected by alcohol (for more information, see the articles by Hurley and Edenberg, pp. 339–344, and by Borghese and Harris, pp. 345–354, in this issue). Moreover, the genetic variants identified to date do not wholly explain the complex genetic susceptibility to alcoholism. Accordingly, researchers need unbiased, systematic approaches to gene discovery in order to discover novel genes and mechanisms and translate them into improved treatment and prevention approaches. One promising approach to achieving this is to conduct genome-wide association studies (GWASs). However, human GWASs require large sample sizes to identify alcoholism susceptibility genes, and the studies published to date have been underpowered and show limited replicability (for a review, see Treutlein and Reitschel 2011 and references therein).

The use of preclinical (i.e., animal) models that closely approximate the clinical situation has been essential for elucidating genetic factors involved in the response to alcohol. Although no animal model can exactly duplicate alcoholism in humans, robust animal models for specific alcohol-related traits are useful for identifying potential determinants of liability in humans. These models include, but certainly are not limited to, the following:

Kari J. Buck, Ph.D., *is a professor of behavioral neuroscience at the Oregon Health & Science University and a research scientist at the Department of Veterans Affairs Medical Center, Portland, Oregon.*

Deaunne L. Denmark, M.D., Ph.D., *and* **Laura B. Kozell, Ph.D.,** *are senior research associates, both at the Portland Alcohol Research Center and Department of Behavioral Neuroscience, Department of Veterans Affairs Medical Center and Oregon Health & Science University, Portland, Oregon.*

Lauren C. Milner, Ph.D., *is a postdoctoral fellow at Stanford University, Palo Alto, California.*

Seth G.N. Grant, Ph.D., *is a professor of molecular neuroscience at the Wellcome Trust Sanger Institute, Cambridge, United Kingdom.*

- Animal models of ethanol self-administration, given that excessive ethanol consumption is a hallmark of alcohol use disorders in humans;
- Models of conditioned place preference and conditioned taste aversion to assess the motivational (i.e., rewarding and aversive) effects of ethanol;
- Models of ethanol sensitivity, because evidence from human studies indicates that sensitivity to the intoxicating effects of ethanol is a marker of genetic susceptibility to develop alcohol dependence; and
- Models of withdrawal, because physiological dependence on alcohol and associated withdrawal symptoms are thought to constitute a motivational force that perpetuates alcohol use and abuse.

For recent publications that address potential consilience between human alcohol dependence and animal models in more depth, the reader is referred to Crabbe and colleagues (2010), Ehlers and colleagues (2010), and Sher and colleagues (2010).

Studies using robust animal, and particularly mouse, models have been fundamental to unbiased searches for genetic determinants of ethanol responses. For example, researchers have used such models to detect and map quantitative trait loci (QTLs)—chromosomal regions containing or linked to the genes that underlie a quantitative, complex trait. These approaches have identified significant and suggestive QTLs for ethanol sensitivity (e.g., Bennett et al. 2006; Downing et al. 2006; Palmer et al. 2006), consumption (e.g., Belknap and Atkins 2001; Boyle and Gill 2008; Hitzemann et al. 2009; Phillips et al. 1998, 2010;Tarantino et al. 1998), withdrawal (Buck et al. 1997, 2002), conditioned aversion (Risinger and Cunningham 1998), conditioned place preference (Cunningham 1995), and tolerance (e.g., Bennett et al. 2007; Crabbe et al. 1994; Drews et al. 2010; Kirstein et al. 2002). The identification of specific genes (i.e., quantitative trait genes [QTGs], which carry allelic variations in the DNA that affect their expression and/or the structure of the product that they code for) that underlie QTL phenotypic effects and elucidation of their mechanisms of action is a crucial next step in the translation of such preclinical research.

Successful strategies to identify genes involved in alcohol dependence and other alcohol-related responses most often have relied upon evidence from several sources (e.g., Shirley et al. 2004). This article summarizes the detection, confirmation, and fine mapping of several QTLs that have large effects on ethanol withdrawal in mice using a variety of approaches, including robust behavioral models of physiological dependence and associated withdrawal following acute and chronic ethanol exposure, positional cloning, sequence and expression analyses, and novel genetic animal models. In addition, the article discusses progress toward the identification of the underlying QTGs, their mechanisms of action, and their potential broader effects on behavioral responses to ethanol.

QTLs and QTGs Associated With Alcohol Dependence and Other Alcohol Effects

The Mpdz Gene

Using a robust behavioral model of physiological dependence, Buck and colleagues (1997) identified a QTL on chromosome 4 that accounts for up to 26 percent of the genetic variance in withdrawal-associated convulsions following acute and chronic ethanol exposure in mice. Positional cloning using novel interval-specific congenic mouse strains[1] narrowed this QTL to a 1.8-Mb interval with conserved colocalization of genes (i.e., shared synteny) on human chromosome 9p23–p22.3.[2] Detailed expression and sequence analyses subsequently identified a single QTG candidate, *Mpdz*, which encodes a protein called multi-PDZ domain protein (MPDZ/MUPP1) (Shirley et al. 2004). Standard inbred and congenic strain analyses suggested that lower *Mpdz* expression and/or certain variations in the amino acid sequence of the encoded MPDZ protein were associated with more severe ethanol withdrawal (Fehr et al. 2002, 2004; Shirley et al. 2004). However, direct evidence that *Mpdz* affects ethanol withdrawal behavior in the intact organism (i.e., in vivo) currently is lacking. Investigators now are addressing this issue in ongoing studies using novel animal models in which either a foreign *Mpdz* gene has been introduced (i.e., MPDZ transgenic [MPDZ-TG] mice) or in which *Mpdz* expression has been reduced (i.e., knockout [MPDZ-KO] mice). The MPDZ-TG animals show increased *Mpdz* expression compared with their wild-type (WT), non-TG littermates. Ongoing studies suggest that withdrawal-related hyperexcitability of the central nervous system (CNS), which can be assessed using handling-induced convulsions, may be less severe in MPDZ-TG mice than in WT littermates—in other words, the animals with increased MPDZ expression experience less severe withdrawal. Conversely, ongoing studies indicate that MPDZ-KO heterozygotes, which exhibit reduced MPDZ expression, may show more severe ethanol withdrawal than WT littermates. Thus, it seems that an inverse relationship exists between *Mpdz* expression and withdrawal severity. However, other

[1] Congenic and interval-specific congenic strains refer to genetic animal models that are genetically identical to noncongenic (wild type) counterparts except for a limited genetic region (the congenic interval, which can range in size from moderately large to as small as 1 megabase). Through specific breeding strategies, a defined DNA segment from a donor strain's genome is transferred to a different recipient strain. By analyzing a panel of interval-specific congenic strains, each with a different congenic interval, and determining which of the congenic strains "capture" the trait of interest (i.e., differ from noncongenic animals) and which do not, researchers can identify a small DNA segment that influences a trait of interest. This is an important step toward identifying the gene or genes within the DNA segment that affect the trait of interest.

[2] When human chromosomes are prepared for microscopic examination, they take on an x-shaped form, with the shorter arm being labeled the p arm and the longer one the q arm. In addition, the chromosomes are stained with a special dye, giving them a characteristic pattern of light and dark bands that are numbered from the center of the chromosome outwards. Thus, a label such as 9p23 refers to a specific band on the short arm of chromosome 9.

studies have suggested that different variants of the *Mpdz* gene exist, resulting in variations in MPDZ protein structure that also may affect withdrawal severity (Fehr et al. 2002). Thus, it still is unclear whether one or both of these mechanisms underlie the effect of *Mpdz* on alcohol withdrawal. Nevertheless, because the strengths of the MPDZ-TG approach complement the limitations of the MPDZ-KO approach and vice versa, the finding that both approaches support a role for MPDZ in ethanol withdrawal is compelling.

Currently, little is known about how MPDZ function relates to alcohol dependence and withdrawal. PDZ-domain proteins regulate numerous aspects of the fate of proteins in the cell (e.g., targeting them to their appropriate location in the cell, stabilization in membranes, retention in a cell component called the endoplasmic reticulum, and endocytosis). MPDZ physically associates with certain proteins involved in brain signaling (i.e., neurotransmitter) systems. These include the following:

- Receptors for the neurotransmitter serotonin (5-HT) (e.g., the 5-HT_{2C} receptor) (Becamel et al. 2001);
- A receptor for the neurotransmitter γ-aminobutyric acid (GABA) (i.e., the $GABA_B$ receptor) (Balasubramanian et al. 2007); and
- A protein called synaptic GTPase-activating protein (SynGAP). Through this association, MPDZ is involved in regulating the functions of the neurotransmitter glutamate, because after binding to SynGAP, MPDZ interacts (i.e., complexes) with one type of glutamate receptor (i.e., NR2B-containing NMDA receptors) to regulate the function of another type of glutamate receptor (i.e., synaptic AMPA receptors) (Krapivinsky et al. 2004).

MPDZ may affect withdrawal by altering the rate and/or fidelity of signal transduction mediated by one or more of the proteins with which it associates, particularly through its effects in a brain region(s) relevant to withdrawal. The striatum appears to be one such region, as ethanol-withdrawal–associated activation of this brain region is related to MPDZ status (Chen and Buck 2010). Within the striatum, virtually all neurons activated in alcohol-withdrawn mice produce both MPDZ and the 5-HT_{2C} receptor, and 30 percent of neurons also produce SynGAP (Chen and Buck 2010). Analyses of inbred mouse strains have indicated that MPDZ status is genetically correlated with convulsions induced by certain chemicals, with some of the strongest genetic correlations observed for chemicals affecting signaling pathways involving glutamate (Fehr et al. 2004). For example, two mouse strains known as C57BL/6J and DBA/2J differ both in the MPDZ variant they carry, affecting both MPDZ expression and structure (Shirley et al. 2004; Fehr et al. 2002), and markedly in handling-induced convulsions in response to agents that modify glutamate signaling (Fehr et al. 2004). Further, MPDZ congenic mice, which possess the QTL interval containing *Mpdz* from the C57BL/6J strain in a genetic background that is more than 99 percent DBA/2J DNA, demonstrate less severe ethanol withdrawal (Fehr et al. 2002) and less severe handling-induced convulsions than DBA/2J mice in response to a 5-HT_{2C} receptor blocker (i.e., SB242084) and a drug that activates $GABA_B$ receptors (i.e., baclofen), but not in response to a $GABA_A$ receptor channel blocker (Reilly et al. 2008). These findings indicate that MPDZ does not regulate seizure susceptibility in general and suggest that MPDZ may affect ethanol-withdrawal–associated CNS hyperexcitability through its effects on glutamate, 5-HT_{2C}, and/or $GABA_B$ receptor function. Ongoing neurophysiological studies using MPDZ genetic models can address this issue to provide mechanistic information.

The Kcnj9 Gene

Buck and colleagues (1997, 2002) also identified a QTL on mouse chromosome 1 that accounts for 26 percent of the genetic variance in ethanol withdrawal convulsions in mice. Positional cloning using interval-specific congenic strains narrowed this QTL to a 0.44-Mb interval syntenic with human chromosome 1q23.2 (Kozell et al. 2009). This chromosome region contains a gene called *Kcnj9* which may be the QTG underlying this QTL. DBA/2J and chromosome 1 congenic mice (which possess a small QTL interval containing *Kcnj9* from the DBA/2J strain in a genetic background that is more than 99 percent C57BL/6J DNA) exhibit significantly more severe withdrawal from ethanol and other sedative drugs of abuse than C57BL/6J mice. Further, *Kcnj9* expression in the brain is significantly greater in chromosome 1 congenic and DBA/2J mice compared with C57BL/6J mice (Kozell et al. 2009). Additionally, mice in which the *Kcnj9* gene has been knocked out demonstrate less severe withdrawal than their WT littermates (Kozell et al. 2009). *Kcnj9* encodes a protein called GIRK3, which forms a subunit of a family of ion channels (i.e., G-protein–dependent inwardly-rectifying K+ channels) that are gated by G-proteins. GIRK channels primarily mediate the inhibitory effects of certain G-protein–coupled neurotransmitter receptors (Luscher et al. 1997).

Currently, little is known about GIRK3 function in the brain and the mechanism by which it can affect withdrawal. GIRK3 is widely expressed in the brain and forms ion channels together with the related GIRK2 protein subunit (Koyrakh et al. 2005; Labouebe et al. 2007; Torrecilla et al. 2002). One plausible mechanism by which GIRK3 may influence withdrawal is via its role in $GABA_B$ receptor signaling. $GABA_B$ receptor activation usually suppresses withdrawal symptoms in ethanol-dependent rats (Colombo et al. 2000; Knapp et al. 2007) and humans (Addolorato et al. 2006), and GIRK3-KO mice show increased GIRK-$GABA_B$ receptor coupling efficiency in the brain compared with WT mice (Labouebe et al. 2007). Further, ethanol enhances $GABA_B$ receptor mediated inhibitory transmission in brain neurons by facilitating GIRK currents (Federici et al. 2009; McDaid et al. 2008) and enhances GIRK currents evoked by $GABA_B$ receptor activation (Kobayashi et al. 1999; Lewohl et al. 1999). In addition to altered $GABA_B$ receptor function,

GIRK3-KO mice have blunted behavioral responses to drugs that act at other $G_{i/o}$-coupled receptors, including μ-opioid (Marker et al. 2002; but see Smith et al. 2008), cannabinoid and α_2-adrenergic receptors (Smith et al. 2008). This suggests the potential involvement of $GABA_B$ and additional G-coupled receptors in mediating GIRK3 effects on behavior. Of course, it should be kept in mind that deletion of a specific gene (in this case the *Kcnj9* gene) in KO animals may lead to developmental changes in other systems to compensate for this loss, and these compensatory changes can confound the interpretation of knockout studies. Future studies will be needed to assess GIRK coupling to $GABA_B$ and similar receptors and its role in ethanol physiological dependence and withdrawal.

QTLs and QTGs Related to Mitochondrial Respiration and Oxidative Stress

Additional studies using the interval-specific congenic mice carrying short segments of DBA/2J DNA in a background of C57BL/6J DNA detected, confirmed, and finely mapped a second QTL on mouse chromosome 1 with large effects on the predisposition to withdrawal following chronic and acute alcohol exposure (Kozell et al. 2008). This QTL maps to a 1.1-Mb interval syntenic with human 1q23.2–23.3. Although considerable evidence indicates that some genetic factors influence vulnerability to withdrawal from a variety of sedative drugs, this QTL does not influence pentobarbital withdrawal (Kozell et al. 2009) and provides a crucial clue as to the identity of the underlying QTG(s).

Detailed molecular analyses of this QTL interval have shown that it harbors 17 genes that exhibit genotype-dependent transcript expression between chromosome 1 congenic and C57BL/6J background strain mice and/or nonsynonymous sequence variation that changes the structure of the protein coded for by the gene, either one or both of which may underlie the QTL's influence (Denmark and Buck 2008). Three of these genes (called *Sdhc*, *Ndufs2*, and *Ppox*) encode proteins found in cell organelles called mitochondria. Mitochondria supply most cellular energy and also are involved in pathways that help the cells avoid or deal with oxidative stress. This is notable because studies both in cultured cells (i.e., in vitro) and in vivo found that ethanol exposure introduces intense oxidative stress, largely through its effects on the mitochondria (Bailey 2003; Sun and Sun 2001).[3] The protein products of *Sdhc* and *Ndufs2* are integral subunits of certain components (i.e., respiratory complexes I and II) involved in a series of biochemical reactions known as the mitochondrial electron transport chain. The protein encoded by *Ppox* catalyzes the penultimate step in the biosynthesis of heme, which is required for the function of cytochrome molecules that play a central role in mitochondrial respiratory function. Consistent with the hypothesis that these mitochondrial proteins could be related to ethanol withdrawal, studies found that mutations in the human genes *NDUFS2*[4] (Ugalde et al. 2004) and *PPOX* (Gonzalez-Arriaza and Bostwick 2003) cause several inherited diseases that include seizures as a prominent symptom. Similar mutations in the gene corresponding to *Ndufs2* in the roundworm *Chromatia elegans* (which is called *gas-1*) creates ethanol hypersensitivity and increased oxidative stress (Kayser et al. 2001, 2003; Morgan and Sedensky 1995). Evidence from rat models suggests that ethanol withdrawal is accompanied by increases in oxygen-containing molecules that increase oxidative stress (i.e., reactive oxygen species) and that these increases correlate with withdrawal severity (Dahchour et al. 2005; Vallett et al. 1997).

Because the DBA/2J and C57BL/6J mice that were used to generate the interval-specific congenic strains differ significantly in the brain levels of some oxidative stress markers (Rebrin et al. 2007), this model system is highly attractive for conducting subsequent QTG and mechanistic analyses. Ongoing studies to address these issues suggest that a gene(s) within this QTL interval may affect mitochondrial respiratory complex structure in the brain and that antioxidant administration may reduce ethanol withdrawal severity in mice.

Potential Roles of MPDZ and GIRK3 in Additional Responses to Ethanol

Alcohol consumption and preference show significant genetic correlation with ethanol withdrawal convulsion severity in independently tested groups of mice (Metten et al. 1998), suggesting that ethanol withdrawal and consumption/preference may share specific genetic contributions, which may include the *Mpdz* and *Kcnj9* genes and their encoded proteins. Consistent with this hypothesis, the same chromosomal region that contains *Kcnj9* also harbors QTLs for ethanol drinking (Tarantino et al. 1998) and for ethanol-conditioned aversion (Risinger and Cunningham 1998) and acute sensitivity to ethanol (Crabbe et al. 1994; Demarest et al. 1999). Moreover, analyses of standard and recombinant inbred animal strains have suggested that *Mpdz* status and/or expression may be genetically correlated with ethanol consumption and preference (behavioral data from Belknap et al. 1993; Fernandez et al. 1999; Gill et al. 1996; Rodriguez et al. 1994), ethanol-conditioned place preference and taste aversion (behavioral data from Broadbent et al. 2002; Cunningham 1995), and sensitivity and tolerance to ethanol-induced motor incoordination (behavioral data from Gallaher et al. 1996; Phillips et al. 1996; Rustay et al. 2003). These results suggest that *Mpdz* and *Kcnj9* may have roles in a wide variety of ethanol-related behaviors. The possibility that these QTGs play important roles in diverse responses to ethanol makes them important targets. Ongoing analyses using MPDZ and GIRK3 genetic models can begin to address their potential roles in ethanol consumption, preference, and other related behaviors.

[3] In contrast, the sedative pentobarbital seems to have a neutral or anti-oxidative effect in the brain (Smith et al. 1980; Ueda et al. 2007).

[4] By convention, gene names in animals are written in uppercase and lowercase and italicized. Gene names in humans are written in all caps and are italicized, whereas the acronyms for the encoded proteins are all caps but not italicized.

Human Relevance of QTGs Identified in Mice

As reviewed here, studies in mouse models of various aspects of alcohol dependence have identified several QTLs with large effects on ethanol physiological dependence and associated withdrawal, have reduced these QTLs to small intervals of chromosomes 1 and 4 (which are syntenic to human chromosomes 1q23.2-23.3 and 9p23-p22.3, respectively), and have led to the description of a QTG or high-quality QTG candidate(s). Human association studies have provided evidence that the QTLs and QTGs identified in mice may be relevant to risk factors for alcoholism in clinical populations. For example, two studies have identified DNA regions associated with alcohol dependence on human chromosome 1q (LOD>3), and additional studies have provided supporting evidence for the association of 1q markers with alcoholism (for reviews, see Edenberg et al. 2010; Ehlers et al. 2010; Hansell et al. 2009; Heath et al. 2011; Joslyn et al. 2010) (see figure 1). These loci potentially are syntenic with the identified mouse chromosome 1 QTLs for alcohol consumption and withdrawal (Ehlers et al. 2010).

Several studies also have provided evidence for an association of markers on human chromosome 9p with alcoholism, but these associations only remain suggestive (Edenberg et al. 2010; Joslyn et al. 2010; Long et al. 1998; Williams et al. 2005). These markers potentially are syntenic with the mouse chromosome 4 QTL for which *Mpdz* has been proposed as a QTG (figure 2). In addition, limited human association studies using only small populations have implicated *MPDZ* as potentially involved in excessive alcohol consumption and risk for alcoholism (Karpyak et al. 2009; Tabakoff et al. 2009). Thus, this gene is a promising translational candidate for future work toward improving prevention and treatment of alcohol abuse in dependent individuals.

These findings suggest that QTL/QTG research in mice may help in the identification of new targets for improved therapeutic approaches and individualized strategies to treat and prevent dependence in humans. In some cases, a QTG can be the same in mice and humans, as has been found for some disorders (Mogil et al. 2003) and also may be the case for one or more of the examples above. In other cases, animal models may identify a relevant network operating in both species, within which many potential targets

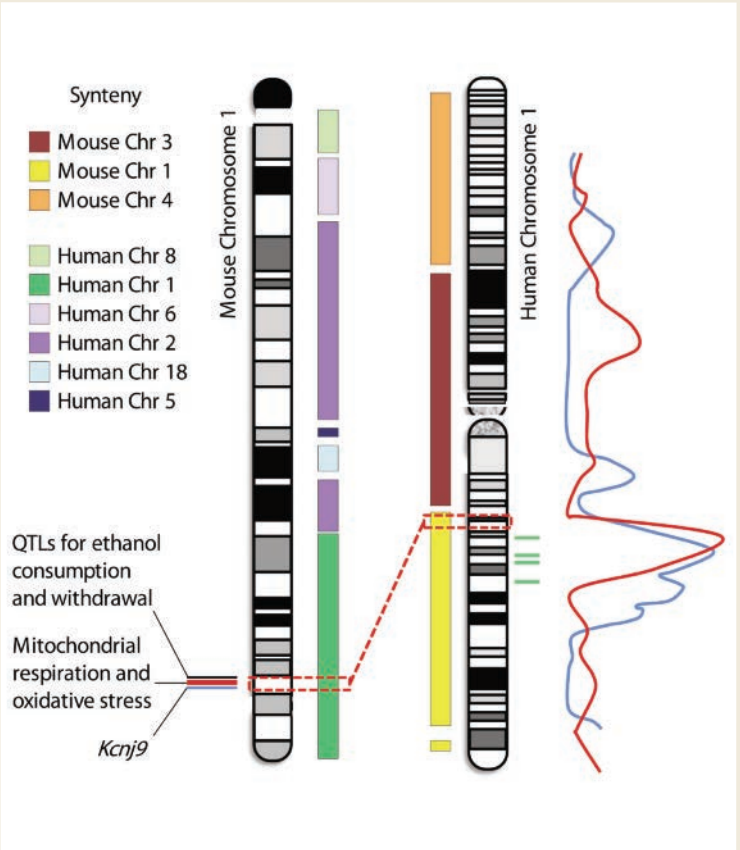


Figure 1 Potential synteny between mouse chromosome 1 and human chromosome 1 quantitative trait loci (QTLs). Human chromosome 1 shares primary conserved regions with mouse chromosomes 1, 3, and 4. Conversely, mouse chromosome 1 shares regions syntenic with human chromosomes 1, 2, 5, 6, 8, and 18. For both mouse and human chromosomes, additional smaller syntenic regions exist (not shown). Mouse chromosome 1 carries significant QTLs for physiological dependence and associated withdrawal following chronic and acute ethanol exposure, two of which have been finely mapped to small DNA intervals of 0.44 and 1.7 Mb (see blue and red lines next to mouse chromosome 1). High-quality quantitative trait gene (QTG) candidates have been identified within these two intervals, including *Kcnj9* and genes involved in mitochondrial respiration and/or oxidative stress. (Denmark and Buck 2009; Kozell et al. 2009). Another QTL for ethanol consumption and withdrawal has also been detected nearby (see black line) but has not yet been finely mapped. The dashed red boxes and line denote the two finely mapped mouse QTL intervals and the syntenic region of human chromosome 1 (1q23.2–1q23.3). Two human QTL studies have determined peak log of the odds of linkage (LOD) scores for alcoholism (red line; Hill et al. 2004) and for age of onset of drinking, harm avoidance, novelty seeking, and alcohol dependence (blue line; Dick et al. 2002) in this human chromosome 1 region. Four genetic markers (rs1229430, rs2001270, rs3753563, and rs84465) that are associated ($P < 0.0001$) with heaviness of drinking, alcohol use disorder, and/or alcohol dependence (Heath et al. 2011) are located in that same region (green lines). Thus, one or more human QTLs may be narrowed to a small syntenic interval of human chromosome 1 that harbors the homologs of high-quality QTG candidates identified in mice.

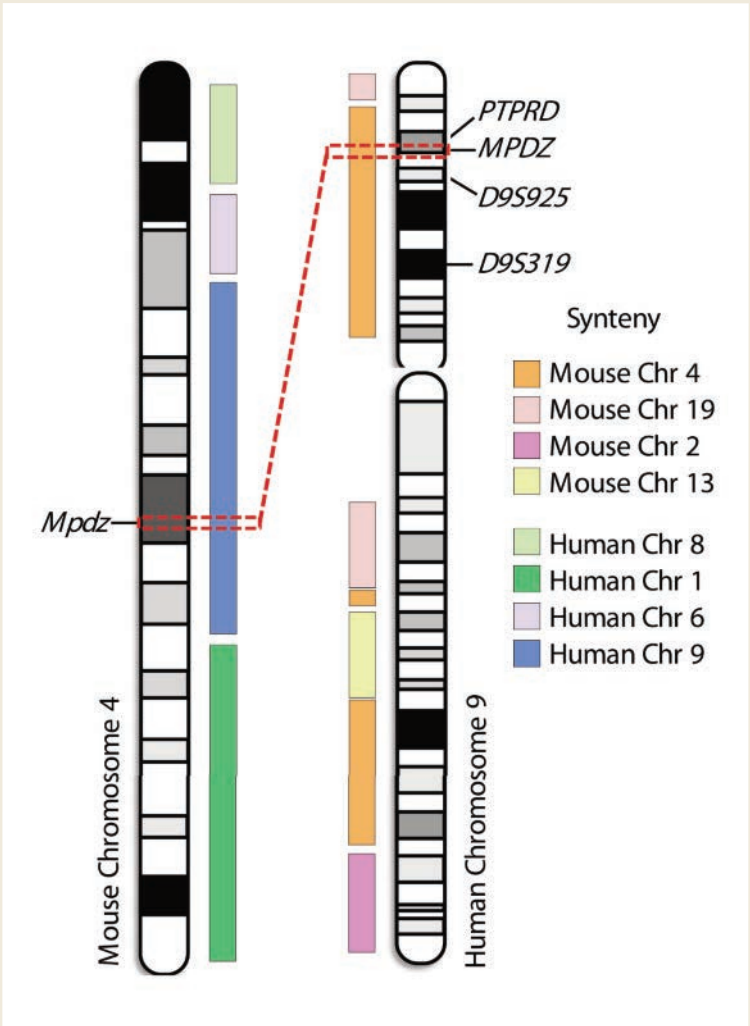


Figure 2 Potential synteny between mouse chromosome 4 and human chromosome 9 quantitative trait loci (QTLs). Human chromosome 9 shares primary conserved regions with mouse chromosomes 1, 4, 19, 2, and 13, and mouse chromosome 4 shares regions syntenic with human chromosomes 9, 8, 1, and 6. For both mouse and human chromosomes additional smaller syntenic regions exist (not shown). Mouse chromosome 4 carries a significant QTL for ethanol withdrawal that has been mapped to 1.8-Mb interval. Within this interval, a gene called *Mpdz* has been identified as a quantitative trait gene (QTG) candidate for ethanol withdrawal (Shirley et al. 2004). The dashed red boxes and line denote this 1.8-Mb QTL interval and syntenic region on human chromosome 9 (9p23–p22.3). A recent human association study for alcohol consumption (Tabakoff et al. 2010) found significant association with a DNA variation (i.e., single nucleotide polymorphism [SNP]) within the human gene *MPDZ* ($P < 0.0001$). Sequence variations in human *MPDZ* also may be associated with alcohol dependence (Karpyak et al. 2010). Thus, this gene has been implicated in studies using animal model and clinical populations. Additional markers near this region of human chromosome 9 may be associated with alcohol-related phenotypes, including age of onset of use (*D9S925;* Williams et al. 2005), predisposition to alcohol dependence (*D9S319;* Long et al. 1998), and alcohol response (*PTPRD;* Joslyn et al. 2010).

for pharmacologic interventions may exist. In either case, a comprehensive understanding of genetic variation, both in humans and informative animal models, is crucial to establishing its relationship to biological function (Collins et al. 2003). As more information becomes available, the mechanisms by which QTGs affect response to ethanol, and their potential role in alcohol dependence in humans, will become apparent. ■

Acknowledgments

This work was supported by Department of Veterans Affairs and National Institutes of Health grants AA–011114, DA–005228, and AA–010760 (KB); AA–017342 (DD); AA–007468 (to Deaunne L. Denmark and Lauren C. Milner); AA–001731 (to Lauren C. Milner); and DA–007262 and by the Wellcome Trust (to Seth G.N. Grant). We thank Nikki Walter and Renee Shirley for their assistance.

Financial Disclosure

The authors declare that they have no competing financial interests.

References

Addolorato, G.; Leggio, L.; Abenavoli, L.; et al. Baclofen in the treatment of alcohol withdrawal syndrome: A comparative study vs diazepam. *American Journal of Medicine* 119(3): 276.e213–278, 2006. PMID: 16490478

Bailey, S.M. A review of the role of reactive oxygen and nitrogen species in alcohol-induced mitochondrial dysfunction. *Free Radical Research* 37(6):585– 596, 2003. PMID: 12868485

Balasubramanian, S.; Fam, S.R.; and Hall, R.A. $GABA_B$ receptor association with the PDZ scaffold Mupp1 alters receptor stability and function. *Journal of Biological Chemistry* 282(6): 4162–4171, 2007. PMID: 17145756

Becamel, C.; Figge, A.; Poliak, S.; et al. Interaction of serotonin 5-hydroxytryptamine type 2C receptors with PDZ10 of the multi-PDZ domain protein MUPP1. *Journal of Biological Chemistry* 276(16):12974– 12982, 2001. PMID: 11150294

Belknap, J.K., and Atkins, A.L. The replicability of QTLs for murine alcohol preference drinking behavior across eight independent studies. *Mammalian Genome* 12(12): 893–899, 2001. PMID: 11707775

Belknap, J.K.; Crabbe, J.C.; and Young, E.R. Voluntary consumption of ethanol in 15 inbred mouse strains. *Psychopharmacology (Berl)* 112(4):503–510, 1993. PMID: 7871064

Bennett, B.; Carosone-Link, P.; Zahniser, N.R.; and Johnson, T.E. Confirmation and fine mapping of ethanol sensitivity quantitative trait loci, and candidate gene testing in the LXS recombinant inbred mice. *Journal of Pharmacology and Experimental Therapeutics* 319(1):299–307, 2006. PMID: 16803863

Bennett, B.; Downing, C.; Carosone-Link, P.; et al. Quantitative trait locus mapping for acute functional tolerance to ethanol in the L × S recombinant inbred panel. *Alcoholism: Clinical and Experimental Research* 31(2):200–208, 2007. PMID: 17250610

Boyle, A.E., and Gill, K.J. Confirmation of provisional quantitative trait loci for voluntary alcohol consumption: Genetic analysis in chromosome substitution strains and F2 crosses derived from A/J and C57BL/6J progenitors. *Pharmacogenetics and Genomics* 18(12):1071–1082, 2008. PMID: 19008751

Broadbent, J.; Muccino, K.J.; and Cunningham, C.L. Ethanol-induced conditioned taste aversion in 15 inbred mouse strains. *Behavioral Neuroscience* 116(1):138–148, 2002. PMID: 11895176

Buck, K.J.; Metten, P.; Belknap, J.K.; and Crabbe, J.C. Quantitative trait loci involved in genetic predisposition to acute alcohol withdrawal in mice. *Journal of Neuroscience* 17(10):3946–3955, 1997. PMID: 9133412

Buck, K.J.; Rademacher, B.S.; Metten, P.; and Crabbe, J.C. Mapping murine loci for physical dependence on ethanol. *Psychopharmacology (Berl)* 160(4):398–407, 2002. PMID: 11919667

Chen, G., and Buck, K.J. Rostroventral caudate putamen involvement in ethanol withdrawal is influenced by a chromosome 4 locus. *Genes, Brain, and Behavior* 9(7):768–776, 2010. PMID: 20608999

Collins, F.S.; Green, E.D.; Guttmacher, A.E.; et al. A vision for the future of genomics research. *Nature* 422(6934):835–847, 2003. PMID: 12695777

Colombo, G.; Agabio, R.; Carai, M.A.; et al. Ability of baclofen in reducing alcohol intake and withdrawal severity: I—Preclinical evidence. *Alcoholism: Clinical and Experimental Research* 24(1):58–66, 2000. PMID: 10656194

Crabbe, J.C. Neurogenetic studies of alcohol addiction. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 363(1507):3201–3211, 2008. PMID: 18640917

Crabbe, J.C.; Belknap, J.K.; Mitchell, S.R.; and Crawshaw, L.I. Quantitative trait loci mapping of genes that influence the sensitivity and tolerance to ethanol-induced hypothermia in BXD recombinant inbred mice. *Journal of Pharmacology and Experimental Therapeutics* 269(1):184–192, 1994. PMID: 8169823

Crabbe, J.C.; Bell, R.L.; and Ehlers, C.L. Human and laboratory rodent low response to alcohol: Is better consilience possible? *Addiction Biology* 15(2):125–144, 2010. PMID: 20148776

Cunningham, C.L. Localization of genes influencing ethanol-induced conditioned place preference and locomotor activity in BXD recombinant inbred mice. *Psychopharmacology (Berl)* 120(1):28–41, 1995. PMID: 7480533

Dahchour, A.; Lallemand, F.; Ward, R.J.; and De Witte, P. Production of reactive oxygen species following acute ethanol or acetaldehyde and its reduction by acamprosate in chronically alcoholized rats. *European Journal of Pharmacology* 520(1–3):51–58, 2005. PMID: 16135364

Demarest, K.; McCaughran, J., Jr.; Mahjubi, E.; et al. Identification of an acute ethanol response quantitative trait locus on mouse chromosome 2. *Journal of Neuroscience* 19(2):549–561, 1999. PMID: 9880575

Denmark, D.L., and Buck, K.J. Molecular analyses and identification of promising candidate genes for loci on mouse chromosome 1 affecting alcohol physical dependence and associated withdrawal. *Genes, Brain, and Behavior* 7(5):599–608, 2008. PMID: 18363851

Dick, D.M.; Nurnberger, J., Jr.; Edenberg, H.J.; et al. Suggestive linkage on chromosome 1 for a quantitative alcohol-related phenotype. *Alcoholism: Clinical and Experimental Research* 26(10):1453–1460, 2002. PMID: 1239277

Downing, C.; Carosone-Link, P.; Bennett, B.; and Johnson, T. QTL mapping for low-dose ethanol activation in the LXS recombinant inbred strains. *Alcoholism: Clinical and Experimental Research* 30(7):1111–1120, 2006. PMID: 16792557

Drews, E.; Racz, I.; Lacava, A.D.; et al. Quantitative trait loci contributing to physiological and behavioural ethanol responses after acute and chronic treatment. *International Journal of Neuropsychopharmacology* 13(2):155–169, 2010. PMID: 19691874

Edenberg, H.J.; Koller, D.L.; Xuei, X.; et al. Genome-wide association study of alcohol dependence implicates a region on chromosome 11. *Alcoholism: Clinical and Experimental Research* 34(5):840–852, 2010. PMID: 20201924

Ehlers, C.L.; Walter, N.A.; Dick, D.M.; et al. A comparison of selected quantitative trait loci associated with alcohol use phenotypes in humans and mouse models. *Addiction Biology* 15(2):185–199, 2010. PMID: 20148779

Federici, M.; Nistico, R.; Giustizieri, M.; et al. Ethanol enhances $GABA_B$-mediated inhibitory postsynaptic transmission on rat midbrain dopaminergic neurons by facilitating GIRK currents. *European Journal of Neuroscience* 29(7):1369–1377, 2009. PMID: 19309316

Fehr, C.; Shirley, R.L.; Belknap, J.K.; et al. Congenic mapping of alcohol and pentobarbital withdrawal liability loci to a <1 centimorgan interval of murine chromosome 4: Identification of *Mpdz* as a candidate gene. *Journal of Neuroscience* 22(9):3730–3738, 2002. PMID: 11978849

Fehr, C.; Shirley, R.L.; Metten, P.; et al. Potential pleiotropic effects of *Mpdz* on vulnerability to seizures. *Genes, Brain, and Behavior* 3(1):8–19, 2004. PMID: 14960011

Fernandez, J.R.; Vogler, G.P.; Tarantino, L.M.; et al. Sex-exclusive quantitative trait loci influences in alcohol-related phenotypes. *American Journal of Medical Genetics* 88(6):647–652, 1999. PMID: 10581484

Gallaher, E.J.; Jones, G.E.; Belknap, J.K.; and Crabbe, J.C. Identification of genetic markers for initial sensitivity and rapid tolerance to ethanol-induced ataxia using quantitative trait locus analysis in BXD recombinant inbred mice. *Journal of Pharmacology and Experimental Therapeutics* 277(2):604–612, 1996. PMID: 8627537

Gill, K.; Liu, Y.; and Deitrich, R.A. Voluntary alcohol consumption in BXD recombinant inbred mice: Relationship to alcohol metabolism. *Alcoholism: Clinical and Experimental Research* 20(1):185–190, 1996. PMID: 8651451

Gonzalez-Arriaza, H.L., and Bostwick, J.M. Acute porphyrias: A case report and review. *American Journal of Psychiatry* 160(3):450–459, 2003. PMID: 12611823

Hansell, N.K.; Agrawal, A.; Whitfield, J.B.; et al. Linkage analysis of alcohol dependence symptoms in the community. *Alcoholism: Clinical and Experimental Research* 34(1):158–163, 2010. PMID: 19860796

Heath, A.C.; Whitfield, J.B.; Martin, N.G.; et al. A quantitative-trait genome-wide association study of alcoholism risk in the community: Findings and implications. *Biological Psychiatry* 70(6):513–518, 2011. PMID: 21529783

Hill, S.Y.; Shen, S.; Zezza, N.; et al. A genome wide search for alcoholism susceptibility genes. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics* 128B(1):102–113, 2004. PMID: 15211641

Hitzemann, R.; Edmunds, S.; Wu, W.; et al. Detection of reciprocal quantitative trait loci for acute ethanol withdrawal and ethanol consumption in heterogeneous stock mice. *Psychopharmacology (Berl)* 203(4):713–722, 2009. PMID: 19052728

Joslyn, G.; Ravindranathan, A.; Brush, G.; et al. Human variation in alcohol response is influenced by variation in neuronal signaling genes. *Alcoholism: Clinical and Experimental Research* 34(5):800–812, 2010. PMID: 20201926

Karpyak, V.M.; Kim, J.H.; Biernacka, J.M.; et al. Sequence variations of the human MPDZ gene and association with alcoholism in subjects with European ancestry. *Alcoholism: Clinical and Experimental Research* 33(4):712–721, 2009. PMID:19175764

Kayser, E.B.; Hoppel, C.L.; Morgan, P.G.; and Sedensky, M.M. A mutation in mitochondrial complex I increases ethanol sensitivity in *Caenorhabditis elegans*. *Alcoholism: Clinical and Experimental Research* 27(4):584–592, 2003. PMID: 12711920

Kayser, E.B.; Morgan, P.G.; Hoppel, C.L.; and Sedensky, M.M. Mitochondrial expression and function of GAS-1 in *Caenorhabditis elegans*. *Journal of Biological Chemistry* 276(23):20551–20558, 2001. PMID: 11278828

Kirstein, S.L.; Davidson, K.L.; Ehringer, M.A.; et al. Quantitative trait loci affecting initial sensitivity and acute functional tolerance to ethanol-induced ataxia and brain cAMP signaling in BXD recombinant inbred mice. *Journal of Pharmacology and Experimental Therapeutics* 302(3):1238–1245, 2002. PMID: 12183685

Knapp, D.J.; Overstreet, D.H.; Breese, G.R. Baclofen blocks expression and sensitization of anxiety-like behavior in an animal model of repeated stress and ethanol withdrawal. *Alcoholism: Clinical and Experimental Research* 31(4):582–595, 2007. PMID: 17374037

Kobayashi, T.; Ikeda, K.; Kojima, H.; et al. Ethanol opens G-protein-activated inwardly rectifying K^+ channels. *Nature Neuroscience* 2(12):1091–1097, 1999. PMID: 10570486

Koob, G.F., and Le Moal, M. Neurobiological mechanisms for opponent motivational processes in addiction. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 363(1507):3113–3123, 2008. PMID: 18653439

Koyrakh, L.; Lujan, R.; Colon, J.; et al. Molecular and cellular diversity of neuronal G-protein-gated potassium channels. *Journal of Neuroscience* 25(49):11468–11478, 2005. PMID: 16339040

Kozell, L.; Belknap, J.K.; Hofstetter, J.R.; et al. Mapping a locus for alcohol physical dependence and associated withdrawal to a 1.1 Mb interval of mouse chromosome 1 syntenic with human chromosome 1q23.2-23.3. *Genes, Brain, and Behavior* 7(5):560–567, 2008. PMID: 18363856

Kozell, L.B.; Walter, N.A.; Milner, L.C.; et al. Mapping a barbiturate withdrawal locus to a 0.44 Mb interval and analysis of a novel null mutant identify a role for *Kcnj9* (GIRK3) in withdrawal from pentobarbital, zolpidem, and ethanol. *Journal of Neuroscience* 29(37):11662–11673, 2009. PMID: 19759313

Krapivinsky, G.; Medina, I.; Krapivinsky, L.; et al. SynGAP-MUPP1-CaMKII synaptic complexes regulate p38 MAP kinase activity and NMDA receptor-dependent synaptic AMPA receptor potentiation. *Neuron* 43(4):563–574, 2004. PMID: 15312754

Labouebe, G.; Lomazzi, M.; Cruz, H.G.; et al. RGS2 modulates coupling between $GABA_B$ receptors and GIRK channels in dopamine neurons of the ventral tegmental area. *Nature Neuroscience* 10(12):1559–1568, 2007. PMID: 17965710

Lewohl, J.M.; Wilson, W.R.; Mayfield, R.D.; et al. G-protein-coupled inwardly rectifying potassium channels are targets of alcohol action. *Nature Neuroscience* 2(12):1084–1090, 1999. PMID: 10570485

Long, J.C.; Knowler, W.C.; Hanson, R.L.; et al. Evidence for genetic linkage to alcohol dependence on chromosomes 4 and 11 from an autosome-wide scan in an American Indian population. *American Journal of Medical Genetics* 81(3):216–221, 1998. PMID: 9603607

Luscher, C.; Jan, L.Y.; Stoffel, M.; et al. G protein-coupled inwardly rectifying K^+ channels (GIRKs) mediate postsynaptic but not presynaptic transmitter actions in hippocampal neurons. *Neuron* 19(3):687–695, 1997. PMID: 9331358

Marker, C.L.; Cintora, S.C.; Roman, M.I.; et al. Hyperalgesia and blunted morphine analgesia in G protein-gated potassium channel subunit knockout mice. *Neuroreport* 13(18):2509–2513, 2002. PMID: 12499858

McDaid, J.; McElvain, M.A.; and Brodie, M.S. Ethanol effects on dopaminergic ventral tegmental area neurons during block of I_h: involvement of barium-sensitive potassium currents. *Journal of Neurophysiology* 100(3): 1202–1210, 2008. PMID: 18614756

Metten, P.; Phillips, T.J.; Crabbe, J.C.; et al. High genetic susceptibility to ethanol withdrawal predicts low ethanol consumption. *Mammalian Genome* 9(12):983–990, 1998. PMID: 9880664

Mogil, J.S.; Wilson, S.G.; Chesler, E.J.; et al. The melanocortin-1 receptor gene mediates female-specific mechanisms of analgesia in mice and humans. *Proceedings of the National Academy of Sciences of the United States of America* 100(8):4867–4872, 2003. PMID: 12663858

Morgan, P.G., and Sedensky, M.M. Mutations affecting sensitivity to ethanol in the nematode, *Caenorhabditis elegans*. *Alcoholism: Clinical and Experimental Research* 19(6):1423–1429, 1995. PMID: 8749805

Palmer, A.A.; Lessov-Schlaggar, C.N.; Ponder, C.A.; et al. Sensitivity to the locomotor-stimulant effects of ethanol and allopregnanolone: A quantitative trait locus study of common genetic influence. *Genes, Brain, and Behavior* 5(7):506–517, 2006. PMID: 17010097

Phillips, T.J.; Brown, K.J.; Burkhart-Kasch, S.; et al. Alcohol preference and sensitivity are markedly reduced in mice lacking dopamine D2 receptors. *Nature Neuroscience* 1(7):610–615, 1998. PMID: 10196569

Phillips, T.J.; Lessov, C.N.; Harland, R.D.; and Mitchell, S.R. Evaluation of potential genetic associations between ethanol tolerance and sensitization in BXD/Ty recombinant inbred mice. *Journal of Pharmacology and Experimental Therapeutics* 277(2):613–623, 1996. PMID: 8627538

Phillips, T.J.; Reed, C.; Burkhart-Kasch, S.; et al. A method for mapping intralocus interactions influencing excessive alcohol drinking. *Mammalian Genome* 21(1–2):39–51, 2010. PMID: 20033183

Rebrin, I.; Forster, M.J.; and Sohal, R.S. Effects of age and caloric intake on glutathione redox state in different brain regions of C57BL/6 and DBA/2 mice. *Brain Research* 1127(1):10–18, 2007. PMID: 17113050

Reilly, M.T.; Milner, L.C.; Shirley, R.L.; et al. $5\text{-}HT_{2C}$ and $GABA_B$ receptors influence handling-induced convulsion severity in chromosome 4 congenic and DBA/2J background strain mice. *Brain Research* 1198:124–131, 2008. PMID: 18262506

Risinger, F.O., and Cunningham, C.L. Ethanol-induced conditioned taste aversion in BXD recombinant inbred mice. *Alcoholism: Clinical and Experimental Research* 22(6):1234–1244, 1998. PMID: 9756038

Rodriguez, L.A.; Plomin, R.; Blizard, D.A.; et al. Alcohol acceptance, preference, and sensitivity in mice. I. Quantitative genetic analysis using BXD recombinant inbred strains. *Alcoholism: Clinical and Experimental Research* 18(6):1416–1422, 1994. PMID: 7695038

Rustay, N.R.; Wahlsten, D.; and Crabbe, J.C. Assessment of genetic susceptibility to ethanol intoxication in mice. *Proceedings of the National Academy of Sciences of the United States of America* 100(5):2917–2922, 2003. PMID: 12584362

Sher, K.J.; Dick, D.M.; Crabbe, J.C.; et al. Consilient research approaches in studying gene x environment interactions in alcohol research. *Addiction Biology* 15(2):200–216, 2010. PMID: 20148780

Shirley, R.L.; Walter, N.A.; Reilly, M.T.; et al. *Mpdz* is a quantitative trait gene for drug withdrawal seizures. *Nature Neuroscience* 7(7):699–700, 2004. PMID: 15208631

Smith, D.S.; Rehncrona, S.; and Siesjo, B.K. Barbiturates as protective agents in brain ischemia and as free radical scavengers in vitro. *Acta Physiologica Scandinavica. Supplementum* 492:129–134, 1980. PMID: 6939303

Smith, S.B.; Marker, C.L.; Perry, C.; et al. Quantitative trait locus and computational mapping identifies *Kcnj9* (GIRK3) as a candidate gene affecting analgesia from multiple drug classes. *Pharmacogenetics and Genomics* 18(3):231–241, 2008. PMID: 18300945

Sun, A.Y., and Sun, G.Y. Ethanol and oxidative mechanisms in the brain. *Journal of Biomedical Science* 8(1):37–43, 2001. PMID: 11173974

Tabakoff, B.; Saba, L.; Printz, M.; et al. Genetical genomic determinants of alcohol consumption in rats and humans. *BMC Biology* 7:70, 2009. PMID: 19874574

Tarantino, L.M.; McClearn, G.E.; Rodriguez, L.A.; and Plomin, R. Confirmation of quantitative trait loci for alcohol preference in mice. *Alcoholism: Clinical and Experimental Research* 22(5):1099–1105, 1998. PMID: 9726281

Torrecilla, M.; Marker, C.L.; Cintora, S.C.; et al. G-protein-gated potassium channels containing Kir3.2 and Kir3.3 subunits mediate the acute inhibitory effects of opioids on locus ceruleus neurons. *Journal of Neuroscience* 22(11):4328–4334, 2002. PMID: 12040038

Treutlein, J., and Reitschel, M. Genome-wide association studies of alcohol dependence and substance use disorders. *Current Psychiatry Reports* 13(2):147–155, 2011. PMID: 21253885

Ueda, Y.; Doi, T.; Nagatomo, K.; and Nakajima, A. Protective role of pentobarbital pretreatment for NMDA-R activated lipid peroxidation is derived from the synergistic effect on endogenous anti-oxidant in the hippocampus of rats. *Neuroscience Letters* 417(1):46–49, 2007. PMID: 17360116

Ugalde, C.; Janssen, R.J.; van den Heuvel, L.P.; et al. Differences in assembly or stability of complex I and other mitochondrial OXPHOS complexes in inherited complex I deficiency. *Human Molecular Genetics* 13(6):659–667, 2004. PMID: 14749350

Vallett, M.; Tabatabaie, T.; Briscoe, R.J.; et al. Free radical production during ethanol intoxication, dependence, and withdrawal. *Alcoholism: Clinical and Experimental Research* 21(2):275–285, 1997. PMID: 9113264

Williams, A.H.; Brown, W.M.; and Langefeld, C.D. Multilocus and interaction-based genome scan for alcoholism risk factors in Caucasian Americans: The COGA study. *BMC Genetics* 6(Suppl. 1):S37, 2005. PMID: 16451547

Circadian Genes, the Stress Axis, and Alcoholism

Dipak K. Sarkar, Ph.D., D.Phil.

The body's internal system to control the daily rhythm of the body's functions (i.e., the circadian system), the body's stress response, and the body's neurobiology are highly interconnected. Thus, the rhythm of the circadian system impacts alcohol use patterns; at the same time, alcohol drinking also can alter circadian functions. The sensitivity of the circadian system to alcohol may result from alcohol's effects on the expression of several of the clock genes that regulate circadian function. The stress response system involves the hypothalamus and pituitary gland in the brain and the adrenal glands, as well as the hormones they secrete, including corticotrophin-releasing hormone, adrenocorticotrophic hormone, and glucocorticoids. It is controlled by brain-signaling molecules, including endogenous opioids such as β-endorphin. Alcohol consumption influences the activity of this system and vice versa. Finally, interactions exist between the circadian system, the hypothalamic–pituitary–adrenal axis, and alcohol consumption. Thus, it seems that certain clock genes may control functions of the stress response system and that these interactions are affected by alcohol. **KEY WORDS: Alcohol consumption; alcohol use, abuse and dependence; alcohol and other drug use pattern; genetics; genetic factors; circadian system; clock genes; stress; stress response; biological adaptation to stress; neurobiology; hypothalamic– pituitary–adrenal axis**

Alcohol abuse and dependence are estimated to affect 1 in 8 adults in the United States and several hundred million people worldwide (Grant et al. 2004). To define at-risk populations and develop better treatments, it is important to further identify the genetic and environmental factors that contribute to alcohol addiction. Recent evidence suggests that the body's internal system that helps control the daily rhythm of the body's activities (i.e., the circadian system), the body's stress response system, and the body's neurobiology of alcohol are extensively intertwined. This article explores some of these interactions.

The Circadian System and Alcohol's Effects on It

The circadian system—or the body's internal clock—is a naturally present regulatory system that helps the body maintain an approximately 24-hour cycle in biochemical, physiological, or behavioral processes, thereby allowing the organism to anticipate and prepare for regular environmental changes (i.e., the day–night cycle). For example, circadian rhythms maintain not only sleeping and feeding patterns but also physiological processes such as body temperature, brainwave activity, hormone production, and cell regeneration. The circadian clockwork results from the interaction of specific clock genes, including genes known as *Period* (*Per1, Per2,* and *Per3*), *Clock*, *Bmal1*, and *Cryptochrome* (*Cry1* and *Cry2*), and others.[1] The activity of these genes is controlled by two tightly coupled transcriptional and translational feedback loops that sustain a near 24-hour periodicity of cellular activity. Expression of these clock genes, in turn, regulates the expression of other clock-controlled genes (Ko and Takahashi 2006).

In both humans and animal models, complex bidirectional relationships seem to exist between alcohol intake or exposure and circadian clock systems. The impact of the circadian system on alcohol use is shown by the fact that both preference for and consumption of alcohol are modulated by time of day, and studies found that genetic interactions link core circadian clock genes with alcohol drinking (Spanagel et al. 2005*a, b*). In addition, disruption of the normal circadian rhythm (i.e., circadian desynchronization) seems to increase the use of alcohol, as seen in frequent travelers and rotating-shift workers, possibly because it frequently activates the body's stress response (i.e., increases the allostatic load[2]) (Rosenwasser et al. 2010; Trinkoff and Storr 1998). At the same time, a strong relationship seems to exist between alcohol drinking and altered circadian functions. For example, alcohol intake can alter the following circadian responses:

- Circadian rhythms in blood pressure, core body temperature, and hormone release in humans (Danel et al. 2009; Devaney et al. 2003; Nakashita et al. 2009);
- Shifts in the normal circadian rhythm (i.e., circadian phase shifting) and in the free-running period[3] in mice (Prosser et al. 2008; Seggio et al. 2009);
- Return to a normal circadian rhythm after a disruption (i.e., circadian phase resetting) and nocturnal activity patterns in hamsters (Ruby et al. 2009; Seggio et al. 2007); and

[1] By convention, gene names in animals are written in uppercase and lowercase and italicized. Gene names in humans are written in all caps and are italicized, whereas the acronyms for the encoded proteins are all caps but not italicized.

[2] The term allostatic load refers to the physiological consequences of chronic exposure to fluctuating or heightened hormonal responses resulting from repeated or chronic stress.

[3] Free-running period is a period that is not adjusted or entrained to the 24-hour cycle in nature or to any artificial cycle.

Dipak K. Sarkar, Ph.D., D.Phil., *is the director of and a professor in the Endocrinology Program and Department of Animal Sciences, Rutgers, The State University of New Jersey, New Brunswick, New Jersey.*

- Rhythmicity in the activity of certain brain cells (i.e., proopiomelanocortin [POMC][4]-producing neurons) in a brain region called the hypothalamus (which is involved in the body's stress system) in rats (Chen et al. 2004).

Even alcohol exposure before birth can interfere with circadian systems. Thus, prenatal ethanol exposure in rats can alter core body temperature and phase-shifting ability (Sakata-Haga et al. 2006); rhythmic activity of the pituitary gland and the adrenal gland, both of which are part of the body's stress response system (Taylor et al. 1982); the rhythmic release of the main stress hormone (i.e., corticosterone) (Handa et al. 2006); immune cell rhythms (Arjona et al. 2006); and circadian expression of POMC in the hypothalamus (Chen et al. 2006).

Why Is the Body's Circadian System So Vulnerable to Alcohol Toxicity?

One logical explanation for the sensitivity of the circadian system to alcohol suggests that alcohol specifically targets one or more of the genes that regulate circadian functions. Using different experimental designs, researchers have demonstrated that alcohol exposure significantly alters the expression of several core clock genes. For example, in chronic alcohol-drinking rats, circadian expression of *Per1* and *Per2* is significantly disrupted in the hypothalamus (Chen et al. 2006). Likewise, prenatal alcohol exposure alters circadian expression of *Per1* and *Per2* genes in the hypothalamus and in tissues in other parts of the body in rats and mice (Arjona et al. 2006; Chen et al. 2004; Ko and Takahashi 2006). In addition, neonatal alcohol exposure reduces *Cry1* expression in a brain region called the suprachiasmatic nucleus and advances the phase of the *Per2* rhythm in the cerebellum and liver (Farnell et al. 2008). In human studies, the expression of clock genes (*PER*, *CRY,* and *BMAL1*) is reduced in white blood cells of male alcoholic patients (i.e., after chronic alcohol exposure) (Huang et al. 2010), whereas alcohol drinking in healthy males (i.e., acute exposure) increases *BMAL1* expression in these cells (Ando et al. 2010). Finally, variations of the *PER2* gene in which individual DNA building blocks are altered (i.e., single nucleotide polymorphisms [SNPs]) are associated with increased alcohol consumptions in male patients (Spanagel 2005*a*) and adolescent boys (Comasco et al. 2010). These observations suggest that clock genes are targets through which alcohol may alter circadian functions. However, in-depth molecular studies are necessary to elucidate the potential mechanisms by which alcohol directly or indirectly affects clock gene expression and cellular functions.

[4] POMC is a precursor molecule primarily produced in and secreted by the pituitary gland but also in the hypothalamus. POMC subsequently can be processed in other tissues into numerous different products, which in turn exert specific effects on the organism and play a role in a wide range of physiological processes. One of these products is adrenocorticotropic hormone (ACTH), which is produced in the pituitary gland and is part of the body's stress response system, the hypothalamic–pituitary–adrenal (HPA) axis.

Circadian Systems, the Stress Response, and Alcohol Consumption

The Stress Response System

The circadian system also may be involved in regulating alcohol-drinking behavior by interacting with a hormone system called the hypothalamic–pituitary–adrenal (HPA) axis, which plays a central role in the body's stress response as well as in reward mechanisms. Stress increases the production of a hormone called corticotrophin-releasing hormone (CRH) in certain cells in a region known as the paraventricular nucleus (PVN) in the hypothalamus. The CRH then is secreted into the blood vessels leading to the pituitary gland, where it interacts with a specific molecule, the CRH receptor1 (CRHR1), on specific cells in the anterior pituitary. In response, these cells begin the synthesis and release of adrenocorticotropic hormone (ACTH) into the circulation. ACTH, in turn, stimulates the release of glucocorticoids (i.e., corticosterone in rats and cortisol in humans) from the outer layer (i.e., cortex) of the adrenal glands that are located on top of the kidneys. The glucocorticoids then act on numerous tissues throughout the organism to coordinate the body's stress response. However, the CRH/CRHR1 system is found not only in the hypothalamus but also in other areas of the brain and helps mediate the actions of the brain's central stress response systems.

The CRH–HPA system is controlled by many brain-signaling molecules (i.e., neurotransmitters) and their receptors, including opioid peptides[5] (e.g., β-endorphin [β-EP]) and their receptors. For example, in rats, the bodies of CRF-producing cells are found in the same locations of the PNV as the fibers of β-EP–releasing cells. In another area of the hypothalamus called the median eminence, a certain type of opioid receptors (i.e., μ-opioid receptors [MOP-r]) is located on the ends of CRH-releasing cells. Agents that stimulate the activity of this receptor (i.e., MOP-r agonists) can inhibit neurotransmitter-stimulated CRF release from the hypothalamus in vitro. Likewise, studies in living organisms found that β-EP infusion decreased CRH release in the blood vessels linking the hypothalamus and the pituitary (Plotsky 1991), and morphine pretreatment prevented stress-induced HPA activation (Zhou et al. 1999). Finally, transplantation of β-EP–producing cells into the PVN suppressed HPA activation under different conditions and normalized stress hyperresponse in fetal alcohol-exposed rats (Boyadjieva et al. 2009). All of these data suggest that endogenous opioids (and, by extension, opiate drugs) have a counterregulatory effect on the stress response.

Alcohol and the Stress Response

In the central nervous system, β-EP long has been suspected of contributing to the positive reinforcement and motivational

[5] Opioid peptides are short sequences of amino acids (i.e., peptides) that are naturally produced by the body and have effects resembling those of opiate drugs. The three main classes of endogenous opioids are endorphins, enkephalins, and dynorphins. Endorphins also are derived from POMC, which also is the precursor for ACTH.

properties of several addictive substances. For example, microinjection of this peptide to several regions of the brain's reward system that involves the neurotransmitter dopamine (i.e., the mesolimbic dopamine system), such as the nucleus accumbens, produced place preference (Bals-Kubik et al. 1993). In addition, several studies have demonstrated that repeated administration of alcohol, cocaine, or heroin significantly attenuated β-EP expression in various limbic areas (Jarjour et al. 2009; Rasmussen et al. 2002; Sweep et al. 1988), supporting the notion that β-EP may contribute significantly in the development of alcohol abuse and dependence.

The stress response system also interacts with these reward pathways. For example, the CRH/CRHR1 system can activate mesolimbic dopaminergic pathways and increase dopamine-mediated signal transmission in various parts of the mesolimbic system, including the nucleus accumbens, amygdala, and medial prefrontal cortex. Furthermore, elevation of plasma corticosterone has been associated with increases in alcohol self-administration (Fahlke et al. 1995). Finally, evidence indicates that corticosterone directly stimulates activity of the mesolimbic dopamine system, subsequently increasing drug-seeking behavior (Piazza et al. 1996). Thus, stress, via activation of the CRH–HPA circuits and/or extrahypothalamic CRH circuits, increases mesolimbic dopamine that, in turn, increases drug seeking in drug-treated animals. The relationship between the stress response and the mesolimbic dopamine system is further supported by findings that an abnormality in POMC-mediated regulation of the HPA axis may lead to excess alcohol drinking under stressful conditions. Finally, consistent with animal studies demonstrating acute and chronic effects of alcohol on the HPA axis (Koob and Bloos 1998), studies in humans have documented HPA axis alterations in both actively drinking and recently abstinent alcoholics (Sinha 2007; Uhart and Wand 2009).

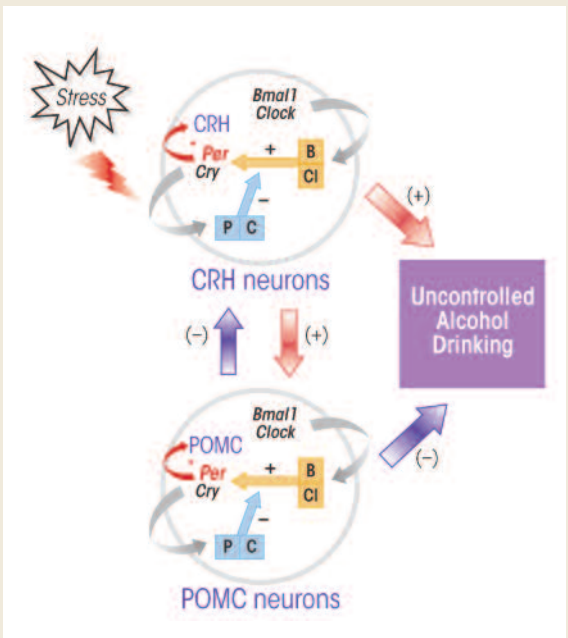


Figure Conceptual framework of how the circadian genes regulating stress-induced excess alcohol drinking. Clock genes (*Per* = P, *Cry* = C, *Bmal1* = B, and *Clock* = Cl) are key components of the circadian mechanism controlling the functions of nerve cells in the hypothalamus and pituitary that produce two molecules important in the body's stress response—corticotrophin-releasing hormone (CRH) and proopiomelanocortin (POMC). Of these clock genes, *Per* might be a potential target of alcohol (indicated by a * symbol) in CRH and POMC neurons and may control the stress-induced propensity to consume alcohol.

NOTE: (+) = stimulatory effect; (–) = inhibitory effect.

Circadian Genes, the Stress Response, and Alcohol

Several findings have suggested that interactions exist between the circadian system, the HPA axis, and alcohol-drinking behavior (see the figure). For example, in animal studies, forced-swimming and immobilization stress elevated expression of the murine *Per1* gene in CRH-positive cells of the PVN (Takahashi et al. 2001). On the other hand, stress-related (i.e., cortisol-induced) transcriptional activation of human *PER1* was reduced in a type of human blood cells (i.e., B-lymphoblastoid cells) that carried an altered form of the *PER1* gene (i.e., the rs3027172 genotype), which has been associated with an increased risk of alcoholism (Dong et al. 2011). Moreover, alcohol consumption can decrease *Per2* expression in POMC-producing neurons in the hypothalamus (Chen et al. 2004), and certain mutations in the murine *Per2* gene interfere with alcohol's stimulatory effect on POMC neurons (Agapito et al. 2010) and alter the rhythmic changes in corticosterone levels in the blood (Yang et al. 2009). Thus, it seems that the *Per1* and *Per2* genes may control functions of CRH- and POMC-producing neurons and that these interactions are affected by alcohol.

It is possible that alcohol-mediated modulation of *Per* genes may play a significant role in modulating HPA axis function, which in turn may lead to an increased propensity to drink alcohol following a stressful event. This view is supported by the recent findings by Dong and colleagues (2011) that the presence of certain *Per1* mutations increased psychosocial stress-induced alcohol drinking in mice, increased alcohol-drinking behavior in human adolescents following psychosocial adversity, and reduced cortisol-induced transcriptional activation of *Per1* in human B-lymphoblastoid cells. Other recent findings, although preliminary, showed that a certain *Per2* mutation increased basal levels of plasma corticosterone and alcohol drinking while preventing stress-induced increases in corticosterone levels and alcohol drinking in mice (Logan et al. 2011). In this context, it is interesting to note that mice carrying mutations in *Per2*, but not *Per1*, display ethanol reinforcement and alcohol-seeking behavior (Spanagel et al. 2005*a*; Zghoul et al. 2007).

Conclusions

The studies reviewed here suggest an intricate interaction between circadian genes, the body's stress response, and alcohol consumption. Thus, it seems that particularly the *Per1* and *Per2* genes, which have a distinct influence on the HPA axis, may control stress-induced propensity to alcohol drinking behavior. However, additional research is needed to address this novel concept involving clock genes, stress, and alcohol drinking. ■

Financial Disclosure

The author declares that he has no competing financial interests.

References

Agapito, M.; Mian, N.; Boyadjieva, N.I.; and Sarkar, D.K. Period 2 gene deletion abolishes ß-endorphin neuronal response to ethanol. *Alcoholism: Clinical and Experimental Research* 34(9):1613–1618, 2010. PMID: 20586752

Ando, H.; Ushijima, K.; Kumazaki, M.; et al. Associations of metabolic parameters and ethanol consumption with messenger RNA expression of clock genes in healthy men. *Chronobiology International* 27(1):194–203, 2010. PMID: 20205566

Arjona, A.; Boyadjieva, N.; Kuhn, P.; and Sarkar, D.K. Fetal ethanol exposure disrupts the daily rhythms of splenic granzyme B, IFN-gamma, and NK cell cytotoxicity in adulthood. *Alcoholism: Clinical and Experimental Research* 30(6):1039–1044, 2006. PMID: 16737463

Bals-Kubik, R.; Ableitner, A.; Herz, A.; and Shippenberg, T.S. Neuroanatomical sites mediating the motivational effects of opioids as mapped by the conditioned place preference paradigm in rats. *Journal of Pharmacology and Experimental Therapeutics* 264(1): 489–495, 1993. PMID: 8093731

Boyadjieva, N.I.; Ortigüela, M.; Arjona, A.; et al. ß-endorphin neuronal cell transplant reduces corticotropin releasing hormone hyperresponse to lipopolysaccharide and eliminates natural killer cell functional deficiencies in fetal alcohol exposed rats. *Alcoholism: Clinical and Experimental Research* 33(5):931–937, 2009. PMID: 19320628

Chen, C.P.; Kuhn, P.; Advis, J.P.; and Sarkar, D.K. Chronic ethanol consumption impairs the circadian rhythm of pro-opiomelanocortin and period genes mRNA expression in the hypothalamus of the male rat. *Journal of Neurochemistry* 88(6):1547–1554, 2004. PMID: 15009656

Chen, C.P.; Kuhn, P.; Advis, J.P.; and Sarkar, D.K. Prenatal ethanol exposure alters the expression of period genes governing the circadian function of beta-endorphin neurons in the hypothalamus. *Journal of Neurochemistry* 97(4):1026–1033, 2006. PMID: 16686691

Comasco, E.; Nordquist, N.; Göktürk, C.; et al. The clock gene PER2 and sleep problems: Association with alcohol consumption among Swedish adolescents. *Upsala Journal of Medical Sciences* 115(1):41–48, 2010. PMID: 20187847

Danel, T.; Cottencin, O.; Tisserand, L.; and Touitou, Y. Inversion of melatonin circadian rhythm in chronic alcoholic patients during withdrawal: Preliminary study on seven patients. *Alcohol and Alcoholism* 44(1):42–45, 2009. PMID: 19029096

Devaney, M.; Graham, D.; and Greeley, J. Circadian variation of the acute and delayed response to alcohol: Investigation of core body temperature variations in humans. *Pharmacology, Biochemistry, and Behavior* 75(4):881–887, 2003. PMID: 12957231

Dong, L.; Bilbao, A.; Laucht, M.; et al. Effects of the circadian rhythm gene period 1 (per1) on psychosocial stress-induced alcohol drinking. *American Journal of Psychiatry* 168(10):1090–1098, 2011. PMID: 21828288

Fahlke, C.; Härd, E.; Eriksson, C.J.; et al. Consequence of long-term exposure to corticosterone or dexamethasone on ethanol consumption in the adrenalectomized rat, and the effect of type I and type II corticosteroid receptor antagonists. *Psychopharmacology (Berl)* 117(2):216–224, 1995. PMID: 7753970

Farnell, Y.Z.; Allen, G.C.; Nahm, S.S.; et al. Neonatal alcohol exposure differentially alters clock gene oscillations within the suprachiasmatic nucleus, cerebellum, and liver of adult rats. *Alcoholism: Clinical and Experimental Research* 32(3):544–552, 2008. PMID: 18215209

Grant, B.F.; Dawson, D.A.; Stinson, F.S. et al. The 12-month prevalence and trends in DSM-IV alcohol abuse and dependence: United States, 1991-1992 and 2001-2002. *Drug and Alcohol Dependence* 74: 223–234, 2004. PMID: 15194200

Handa, R.J.; Zuloaga, D.G.; and McGivern, R.F. Prenatal ethanol exposure alters core body temperature and corticosterone rhythms in adult male rats. *Alcohol* 41(8):567–575, 2007. PMID: 18047910

Huang, M.C.; Ho, C.W.; Chen, C.H.; et al. Reduced expression of circadian clock genes in male alcoholic patients. *Alcoholism: Clinical and Experimental Research* 34(11):1899–1904, 2010. PMID: 20735373

Jarjour, S.; Bai, L.; and Gianoulakis, C. Effect of acute ethanol administration on the release of opioid peptides from the midbrain including the ventral tegmental area. *Alcoholism: Clinical and Experimental Research* 33(6): 1033–1043, 2009. PMID: 19302084

Ko, C.H., and Takahashi, J.S. Molecular components of the mammalian circadian clock. *Human Molecular Genetics* 15 (Spec. No. 2):R271–R277, 2006. PMID: 16987893

Koob, G., and Bloom, F.E. Cellular and molecular mechanisms of drug dependence. *Science* 242(4879):715–723, 1998. PMID: 2903550

Kovanen, L.; Saarikoski, S.T.; Haukka, J.; et al. Circadian clock gene polymorphisms in alcohol use disorders and alcohol consumption. *Alcohol and Alcoholism* 45(4):303–311, 2010. PMID: 20554694

Logan, R.W.; O' Connell, S.; Levitt, D.; et al. The involvement of clock gene Per2 in mediating stress-induced alcohol drinking behavior in fetal-alcohol exposed mice. *Alcoholism: Clinical and Experimental Research* 35:107, 2011.

Nakashita, M.; Ohkubo, T.; Hara, A.; et al. Influence of alcohol intake on circadian blood pressure variation in Japanese men: The Ohasama study. *American Journal of Hypertension* 22(11):1171–1176, 2009. PMID: 19713946

Perreau-Lenz, S.; Zghoul, T.; de Fonseca, F.R.; et al. Circadian regulation of central ethanol sensitivity by the mPer2 gene. *Addiction Biology* 14(3):253–259, 2009. PMID: 19523042

Piazza, P.V.; Barrot, M.; Rougé-Pont, F.; et al. Suppression of glucocorticoid secretion and antipsychotic drugs have similar effects on the mesolimbic dopaminergic transmission. *Proceedings of the National Academy of Sciences of the United States of America* 93(26):15445–15450, 1996. PMID: 8986831

Plotsky, P.M. Pathways to the secretion of adrenocorticotropin: A view from the portal. *Journal of Neuroendocrinology* 3(1):1–9, 1991. PMID: 19215439

Prosser, R.A.; Mangrum, C.A.; and Glass, J.D. Acute ethanol modulates glutamatergic and serotonergic phase shifts of the mouse circadian clock in vitro. *Neuroscience* 152(3):837–848, 2008. PMID: 18313227

Rasmussen, D.D.; Boldt, B.M.; Wilkinson, C.W.; and Mitton, D.R. Chronic daily ethanol and withdrawal: 3. Forebrain pro-opiomelanocortin gene expression and implications for dependence, relapse, and deprivation effect. *Alcoholism: Clinical and Experimental Research* 26(4):535–546, 2002. PMID: 11981131

Rosenwasser, A.M.; Clark, J.W.; Fixaris, M.C.; et al. Effects of repeated light-dark phase shifts on voluntary ethanol and water intake in male and female Fischer and Lewis rats. *Alcohol* 44(3):229–237, 2010. PMID: 20488643

Rosenwasser, A.M.; Fecteau, M.E.; Logan, R.W.; et al. Circadian activity rhythms in selectively bred ethanol-preferring and nonpreferring rats. *Alcohol* 36(2):69–81, 2005. PMID: 16396740

Ruby, C.L.; Brager, A.J.; DePaul, M.A.; et al. Chronic ethanol attenuates circadian photic phase resetting and alters nocturnal activity patterns in the hamster. *American Journal*

of Physiology: Regulatory, Integrative and Comparative Physiology 297(3):R729–R737, 2009. PMID: 19553498

Sakata-Haga, H.; Dominguez, H.D.; Sei, H.; et al. Alterations in circadian rhythm phase shifting ability in rats following ethanol exposure during the third trimester brain growth spurt. *Alcoholism: Clinical and Experimental Research* 30(5):899–907, 2006. PMID: 16634860

Seggio, J.A.; Fixaris, M.C.; Reed, J.D.; et al. Chronic ethanol intake alters circadian phase shifting and free-running period in mice. *Journal of Biological Rhythms* 24(4):304–312, 2009. PMID: 19625732

Sinha, R. The role of stress in addiction relapse. *Current Psychiatry Reports* 9(5):388–395, 2007. PMID: 17915078

Spanagel, R.; Pendyala, G.; Abarca, C.; et al. The clock gene Per2 influences the glutamatergic system and modulates alcohol consumption. *Nature Medicine* 11(1):35–42, 2005*a*. PMID: 15608650

Spanagel, R.; Rosenwasser, A.M.; Schumann, G.; and Sarkar, D.K. Alcohol consumption and the body's biological clock. *Alcoholism: Clinical and Experimental Research* 29(8): 1550–1557, 2005*b*. PMID: 16156052

Sweep, C.G.; Van Ree, J.M.; and Wiegant, V.M. Characterization of beta-endorphin-immunoreactivity in limbic brain structures of rats self-administering heroin or cocaine. *Neuropeptides* 12(4):229–236, 1988. PMID: 2976900

Takahashi, S.; Yokota, S.; Hara, R.; et al. Physical and inflammatory stressors elevate circadian clock gene mPer1 mRNA levels in the paraventricular nucleus of the mouse. *Endocrinology* 142(11):4910–4917, 2001. PMID: 11606459

Taylor, A.N.; Branch, B.J.; Cooley-Matthews, B.; and Poland, R.E. Effects of maternal ethanol consumption in rats on basal and rhythmic pituitary-adrenal function in neonatal offspring. *Psychoneuroendocrinology* 7(1):49–58, 1982. PMID: 7201653

Trinkoff, A.M., and Storr, C.L. Work schedule characteristics and substance use in nurses. *American Journal of Industrial Medicine* 34(3): 266–271, 1988. PMID: 9698996

Uhart, M., and Wand, G.S. Stress, alcohol and drug interaction: An update of human research. *Addiction Biology* 14(1):43–64, 2009. PMID: 18855803

Yang, S.; Liu, A.; Weidenhammer, A.; et al. The role of mPer2 clock gene in glucocorticoid and feeding rhythms. *Endocrinology* 150(5):2153–2160, 2009. PMID: 19179447

Zghoul, T.; Abarca, C.; Sanchis-Segura, C.; et al. Ethanol self-administration and reinstatement of ethanol-seeking behavior in Per1(Brdm1) mutant mice. *Psychopharmacology (Berl)* 190(1):13–19, 2007. PMID: 17051414

Zhou, Y.; Spangler, R.; Maggos, C.E.; et al. Hypothalamic-pituitary-adrenal activity and pro-opiomelanocortin mRNA levels in the hypothalamus and pituitary of the rat are differentially modulated by acute intermittent morphine with or without water restriction stress. *Journal of Endocrinology* 163(2):261–267, 1999. PMID: 10556776

Immune Function Genes, Genetics, and the Neurobiology of Addiction

Fulton T. Crews, Ph.D.

The neuroimmune system (i.e., the immune system and those components of the nervous system that help regulate immune responses), and in particular the innate immune system, play a role in the development of addictions, including alcoholism, particularly in the context of stressful situations. Certain cells of the neuroimmune system are activated both by stress and by environmental factors such as alcohol, resulting in the induction of genes involved in innate immunity. One of the molecules mediating this gene induction is a regulatory protein called nuclear factor-κB, which activates many innate immune genes. Innate immune gene induction in certain brain regions (e.g., the frontal cortex), in turn, can disrupt decision making, which is a characteristic of addiction to alcohol and other drugs. Likewise, altered neuroimmune signaling processes are linked to alcohol-induced negative affect and depression-like behaviors and also regulate alcohol-drinking behavior. Moreover, the expression of several genes and proteins involved in innate immunity is enhanced in addicted people. Finally, specific variants of multiple innate immune genes are associated with the genetic risk for alcoholism in humans, further strengthening the connection between increased brain innate immune gene expression and alcohol addiction. KEY WORDS: Other drug dependence; alcoholism; addiction; causes of alcohol and other drug use; genetic factors; environmental factors; neurobiology; neuroimmune system; immune system; innate immune system; innate immune genes; immune function genes; nuclear factor-κB; stress; decision making; depression

The nervous system and the immune system interact closely to regulate the body's immune responses, including inflammatory responses. Accordingly, the term "neuroimmune system" refers to the immune system and those components of the nervous system that help regulate immune responses and also encompasses the hormones and other signaling molecules that convey signals between the immune and nervous systems. Part of the neuroimmune system is the innate immune system—a network of cells and the signaling molecules they release that are present from birth and form the first line of the body's defense system, including such responses as inflammatory reactions. This article summarizes the role that the neuroimmune system and genes encoding components of the innate immune system play in the development of addiction, including alcoholism.

Neuroimmune Signaling, Drug Abuse, and Stress

Neuroimmune signaling influences the responses and functions of a variety of body systems, including the digestive (i.e., enteric) system, sensory pathways, and the hormonal axis known as the hypothalamic–pituitary–adrenal (HPA) axis, which is involved in the body's stress response and also plays a role in addiction to alcohol and other drugs (AODs).[1] Immune cells called monocytes and monocyte-like cells in the brain (e.g., microglia) are sensitive key cells involved in neuroimmune signaling. When the immune system is stimulated or tissue damage occurs, these cells go through multiple stages of activation, which at the molecular level are reflected by the activation of a cascade of innate immune genes (Graeber 2010). These responses of the monocytes and microglia involve the production and secretion of signaling molecules, including inflammation-promoting (i.e., proinflammatory) cytokines and chemokines, such as monocyte chemotactic protein (MCP)-1, tumor necrosis factor α (TNFα), and interleukin 1β (IL1β). In the brain, microglial activation contributes to the activation of another type of cell called astroglia, or astrocytes, which, like microglia, show multiple stages of neuroimmune activation. In the microglia, the different stages of activation are accompanied by morphological changes. Thus, these cells change from their resting state with multiple branches (i.e., the ramified form) to a less branched, bushy morphology after mild activation and a rounded morphology after strong activation (i.e., when major brain cell death occurs). Chronic alcohol treatment induces mild, bushy microglial activation as well as mild astrocyte activation (see figure 1).

Activated glia show increased production of a wide range of proteins. For example, they produce and secrete increased amounts of proteases as well as of proteins found in the space between cells (i.e., extracellular matrix proteins). In addition, they generate increased amounts of proteins called toll-like receptors (TLRs) that play a role in alcohol-induced depressed mood and negative emotions (see below) and

[1] Among the main molecules involved in the HPA system are the glucocorticoids (e.g., cortisol), and cycles of stress as well as AOD abuse lead to elevated basal glucocorticoid levels and promote addiction (Armario 2010).

Fulton T. Crews, Ph.D., *is a John Andrews Distinguished Professor, professor of pharmacology and psychiatry, and director of the Bowles Center for Alcohol Studies, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina.*

show enhanced activity of enzymes known as oxidases that promote inflammatory reactions (e.g., nicotinamide adenine dinucleotide phosphate [NADPH] oxidases [NOX], cyclooxygenase [COX], and inducible nitric oxide synthases [iNOS]).

Microglia easily can become activated, and the initial stages of activation are characterized by the secretion of signaling molecules, slight morphological changes, and increased production of molecules involved in immune responses (i.e., major histocompatibility complex [MHC]) as well as of TLRs (Graeber 2010). Activation of microglia and astrocytes also increases proinflammatory agents, including TNFα, that alter the transmission of nerve signals (i.e., neurotransmission), including signal transmission mediated by the excitatory neurotransmitter glutamate. Likewise, studies have suggested that alcoholism is related to excessive glutamate levels (i.e., a hyperglutamate state). In the outer layer of the brain (i.e., the cerebral cortex), chronic alcohol-induced neuroimmune activation leads to a hyperglutamate state that reduces cortical function (figure 2). One mechanism contributing to this hyperglutamate state involves TNFα, which acts to reduce the activity of glutamate transporters[2] in the astrocytes (Zou and Crews 2005). Similarly, beverage alcohol (i.e., ethanol) has been shown to inhibit glutamate transport (Zou and Crews 2006). This blockade of glutamate transporters increases glutamate levels outside the cells and particularly in the space between two neurons where nerve signals are transmitted (i.e., the synapse), resulting in excessive neuronal activity (i.e., hyperexcitability). TNFα also stimulates the production of certain proteins found on signal-receiving neurons that interact with glutamate (i.e., the AMPA glutamate receptors) (Beattie et al. 2010). Increases in synaptic glutamate receptors and glutamate concentrations cause hyperexcitability that disrupts the normal concentration of the brain's response to a specific area of the cortex (i.e., cortical focus), thereby reducing cortical function. Through these mechanisms, monocytes, microglia, and astrocytes progressively become activated by stress and environmental factors, including ethanol, resulting in the induction of genes that encode proteins involved in the innate immune response.

Stress and Drug Abuse Increase Transcription of Innate Immune Genes

Stress and AODs, as well as sensory and hormonal signals, activate a regulatory protein (i.e., transcription factor[3]) called nuclear factor κ–light-chain enhancer of activated B cells (NF-κB) that is produced in large amounts (i.e., is highly expressed) in monocytes and microglia. Although NF-κB is found in most cells, it is the key transcription factor involved in the induction of innate immune genes in microglia and other monocyte-like cells. A wide range of stimuli, such as stress, cytokines, oxidative free radicals, ultraviolet irradiation, bacterial or viral molecules, and many other signaling molecules, increase binding of NF-κB to specific sequences of the DNA. This binding increases the transcription of many genes, particularly those encoding signaling molecules (e.g., chemokines and cytokines) and enzymes (e.g., oxidases and proteases) (figure 3). Studies found that ethanol can increase the binding of NF-κB to its corresponding DNA sequences both in the brains of living organisms (Crews et al. 2006) and in cultured brain slices obtained from a brain area called the hippocampal–entorhinal

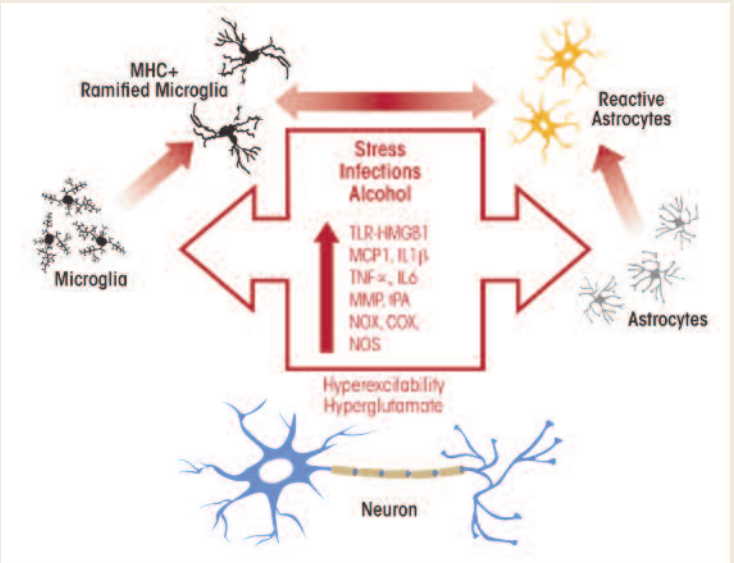


Figure 1 Activation of microglia and astrocytes by alcohol in the brain. Microglia and astrocytes undergo multiple stages of activation that include characteristic changes in morphology. Resting microglia become ramified microglia with that express molecules called major histocompatibility complex (MHC) on their surface. Similarly, astrocytes begin to show markers of reactive astrocytes. Alcohol-induced glial activation is associated with increased expression of innate immune genes, including increased expression of the chemokine monocyte chemoattractant protein-1 (MCP1); the cytokines tumor necrosis factor-α (TNFα), interleukin-1 β (IL-1β) , and interleukin-6 (IL-6); the proteases matrix metalloproteinase (MMP) and tissue plasminogen activator (TPA); and the oxidases nicotinamide adenine dinucleotide phosphate oxidase (NOX), cyclooxygenase (COX), and nitric oxide synthetase (NOS). The alcohol-induced activation of glial innate immune genes increases neuronal hyperexcitability (Crews et al. 2011).

[2] Glutamate transporters are proteins that shuttle glutamate released by nerve cells (i.e., neurons) into the space between cells back into the neuron; this is essential to terminate transmission of a nerve signal and thus ensure appropriate regulation of neuronal activity.

[3] Transcription factors are proteins that are necessary for a set of reactions called transcription, which is the first step of the process during which the genetic information encoded in the DNA is used as a template for the generation of functional proteins.

cortex (HEC) (Zou and Crews 2006). These and other studies also have indicated that ethanol increases transcription of NF-κB target genes, including the genes encoding the following:

- MCP-1;
- Certain proinflammatory cytokines, such as TNFα, IL-1β, and IL-6;
- Certain proinflammatory oxidases, such as iNOS (Zou and Crews 2010), COX-2 (Knapp and Crews 1999), and NOX (Qin et al. 2008); and
- Certain proteases, such as TNF–converting enzyme (TACE) and tissue plasminogen activator (Zou and Crews 2010).

Not only ethanol but also chronic stress increases brain NF-κB activation (Koo et al. 2010; Madrigal et al. 2002), as well as the levels of cytokines, prostaglandin,[4] and COX-2 (Madrigal et al. 2003), all of which have proinflammatory effects. Although acute stress–induced responses, such as elevated glucocorticoid levels, are anti-inflammatory by blocking NF-κB production, chronic elevation of glucocorticoid levels during cycles of stress and/or AOD abuse reverses these anti-inflammatory effects and indeed results in proinflammatory NF-κB activation in the frontal cortex (Munhoz et al. 2010). Thus, activation of NF-κB is a common molecular mechanism through which stress and AODs can induce innate immune genes.

Addiction and Neuroimmune Signaling

Alcoholism is a progressive disease related to repeated episodes of alcohol abuse that reduce the brain's behavioral control and decision-making ability; at the same time, increasing habitual

[4] Prostaglandins are lipid compounds that are produced by almost all cells in the body and have a variety of important physiological effects, including the regulation of inflammatory reactions.

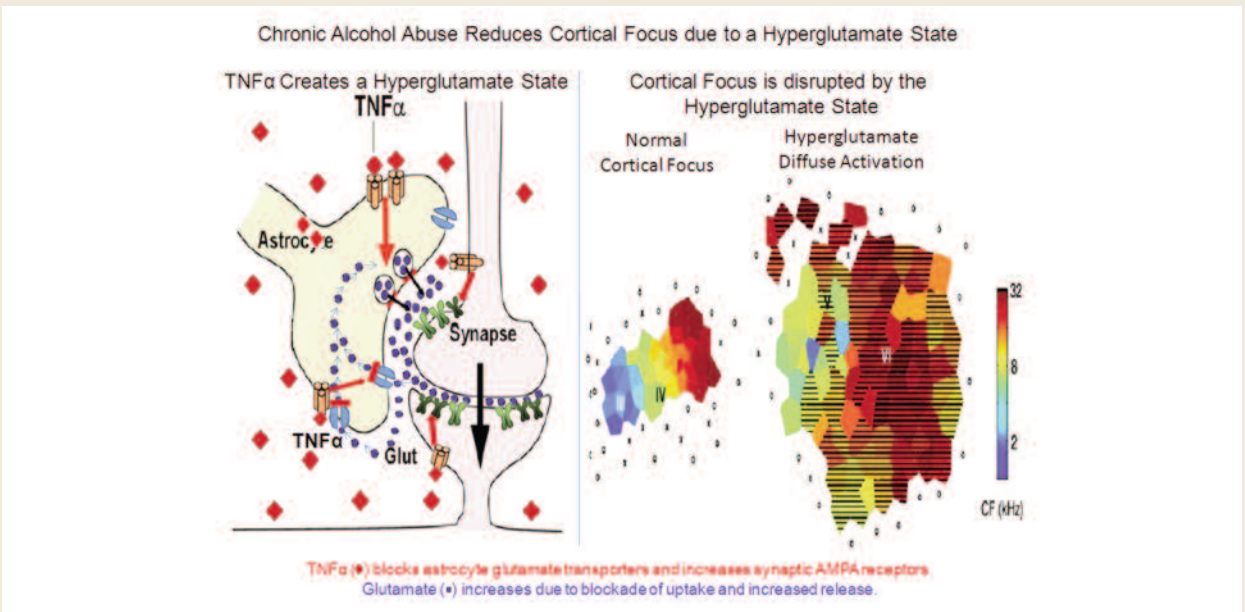


Figure 2 Mechanisms of alcohol-induced excessive glutamate activity in the cortex and loss of cortical focus. Ethanol-induced activation of microglia and astrocytes increases the levels of proinflammatory cytokines, including tumor necrosis factor-alpha (TNFα). (Left panel) TNFα creates a state characterized by excess activity of the neurotransmitter glutamate (i.e., a hyperglutamate state). Thus, TNFα reduces the levels of the primary glutamate transporters, GLT-1, in the astrocytes, in the cerebral cortex, and inhibits glutamate transport, possibly through induction of TNFα and other proinflammatory genes. As a result, glutamate levels outside the neurons, and particularly at the synapse, increase, resulting in a hyperglutamate state. In addition, TNFα increases the levels of certain molecules that interact with glutamate (i.e., AMPA receptors). All these processes causes excessive neuronal excitability. (Right panel) Hyperexcitability disrupts cortical focus. The left image shows the response of a normal adult auditory cortex to a series of tones with a frequence of 2-32 kHz colorized as blue to red. The response to a specific tone involves activation of a specific focal cortical region, which likely relates to the ability to distinguish specific tones of sounds. The right image shows the disrupted hyperglutamate-state–like response to sound that involves the entire auditory cortex without specific tonal areas of focus. The hyperglutatamate state increases cortical excitability, which in turn decreases function because it results in loss of focal activation and likely loss of tonal discrimination. In alcoholism, the hyperglutamate state most strongly affects the frontal cortex, which may disrupt decision making as well as attention and behavioral control mechanisms.

SOURCE: Image in right panel adapted from Chang and Merzenich (2003).

urges combined with increasing bad feelings (i.e., negative affect) promote continued drinking. Frontal cortical brain regions that designate attention and motivation, using information to predict the result of actions (Schoenbaum and Shaham 2008), play a role in addiction development. Frontal cortical dysfunction often is investigated using reversal-learning tasks. In reversal learning, the subject first learns to make one choice (e.g., responding to the black objects in a series of black and white objects) and then has to learn to reverse this choice (e.g., to respond to the white objects). Thus, the initially expected responses suddenly are considered wrong, requiring the subject to exhibit flexible behavior in response to outcomes that do not match those predicted by preceding cues (Stalnaker et al. 2009).

In behavioral studies, poor performance on such tasks is supposed to reflect the inability of drug-addicted individuals to learn new healthy behaviors and avoid the negative consequences of their drug consumption. Such learning and/or changes in behavior require signals from the frontal cortex to indicate the value of decisions. Studies found that binge drinking induces persistent deficits in reversal learning in rats (Obernier et al. 2002; Pascual et al. 2007) and in adult mice following a model of adolescent binge drinking (Coleman 2010). Other investigators similarly have demonstrated that cocaine use results in abnormally slow reversal learning, even though initial learning is normal (Calu et al. 2007; Schoenbaum et al. 2004). Specifically, human cocaine and alcohol addicts exhibit dysfunctional decision making in reversal-learning tasks that probe cognitive flexibility (Bechara et al. 2002). Lesions in the frontal cortex cause reversal-learning deficits comparable to those induced by chronic drug abuse (Schoenbaum et al. 2006). The persistence of addiction matches the persistent increases in innate immune gene activation (Qin et al. 2007, 2008) and loss of behavioral flexibility. Thus, it is thought that innate immune gene induction in the frontal cortex disrupts decision making consistent with addiction (Crews et al. 2011).

Addiction to alcohol, opiates, and stimulant drugs involves both changes in attention–decision making and increased temporal lobe anxiety–negative affect urgency. Addiction-induced negative affect and depression-like behaviors also are linked to neuroimmune signaling because neuroimmune signals can alter moods. For example, a compound called lipopolysaccharide (LPS) that can induce brain innate immune genes causes depression-like behavior that mimics components of addiction-like negative affect. LPS naturally binds with one of the TLRs (i.e., TLR4) and this interaction results in NF-κB activation, ultimately leading to the induction of innate immune genes. In humans, LPS infusions reduce reward responses and increase depressed mood (Eisenberger et al. 2010). Likewise, when patients with cancer or viral infections are treated with agents such as interferon and IL that influence innate immune genes, they may experience severe depression as a major adverse effect (Kelley and Dantzer 2011). Innate immune activators such as LPS, chemokines, and cytokines can mimic the amplification of depressed mood that occurs during repeated cycles of drug abuse or stress (Breese et al. 2008). All of these observations further support the link between neuroimmune signaling and mood as well as the role of neuroimmune signaling as a key component of addiction neurobiology. Of interest, chronic alcohol leads to withdrawal anxiety in normal mice

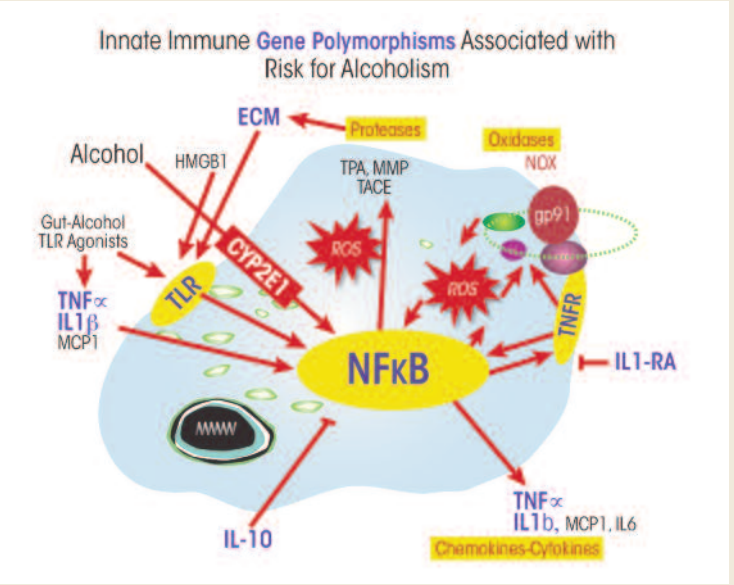


Figure 3 Innate immune gene polymorphisms associated with risk for alcoholism. The schematic shows a representative astrocyte or microglial cell. Genes associated with genetic risk for alcoholism are in light blue. Nuclear factor κ-lightchain–enhancer of activated B cells (NF-κB) is a key transcription factor involved in induction of innate immune genes that is sensitive to reactive oxygen species (ROS). These ROS are generated by the enzyme CYP2E1 during alcohol metabolism, and certain DNA sequences (i.e., polymorphisms) in the CYP2E1 gene are associated with alcoholism. CYP2E1 is highly expressed in monocyte-like cells, which are activated when CYP2E1 metabolizes alcohol. The ROS formed during this process activate proinflammatory NF-κB responses. Chronic ethanol treatment increases CYP2E1 expression in the brain, particularly in astrocytes.The resulting elevated ROS levels activate NF-κB–mediated transcription of innate immune genes, and this response may be amplified in the presence of certain NF-κB polymorphisms (i.e., NF-κB1). Certain variants of other genes also are associated with alcoholism, including polymorphisms of T NFα, interleukin-10 (IL-10), interleukin-1 receptor antagonist (IL-1RA), and other components of the IL-1 gene complex, as well as of certain proteins in the space surrounding the cells (i.e., extracellular matrix proteins [ECM]).

tent with the hypothesis that innate immune activation drives negative affect and associated anxiety responses. Thus, the anxiety–depression negative affect that contributes to addiction occurs with increased brain neuroimmune signaling.

Neuroimmune signaling also regulates alcohol drinking behavior. Genetic comparisons among different strains of rats and mice found that addiction-like drinking behavior was associated with increased levels or activity of NF-κB, its regulatory proteins, and multiple innate immune genes (Mulligan et al. 2006). Furthermore, induction of innate immune genes resulted in increased ethanol consumption, whereas inactivation of such genes reduced drinking behavior (Blednov et al. 2005, 2011*b*). Thus, across genetically divergent strains of mice, innate immune responses to LPS corresponded to increases in ethanol consumption (Blednov et al. 2005, 2011*b*). In fact, even a single injection of LPS was able to produce a long-lasting increase in ethanol consumption (Blednov et al. 2011*a*) that corresponded to sustained increases in brain innate immune gene expression (Qin et al. 2007). These studies identified several innate immune molecules (e.g., β2-microglobulin, cathepsins, and CD14, a key innate immune signaling protein) as important for regulating drinking behavior. Thus, innate immune gene induction may underlie the progressive loss of behavioral flexibility, increasing negative affect, and increased alcohol drinking associated with repeat episodes of alcohol abuse and alcoholism.

Activity of Innate Immune Genes Is Increased in the Addicted Brain

Direct analyses of changes in the activity or levels of various proteins in the brains of alcoholics and other drug addicts also can provide insight into the neurobiology of addiction. Such studies found the following:

- Postmortem studies of the brains of human alcoholics indicate that the innate immune chemokine MCP-1 is increased severalfold in multiple brain regions (Breese et al. 2008). Consistent with this, chronic alcohol treatment of mice (Qin et al. 2008) or of cultured brain slices from the rat hippocampus (Zou and Crews 2010) also increases expression of MCP-1 and other innate immune genes.

- Proteins that serve as markers of microglial activation are increased across the alcoholic brain (He and Crews 2008).

- Consistent with alcoholism being related to neuroimmune signaling, postmortem studies of gene expression in the brains of human alcoholics found increased levels of a subunit of NF-κB; moreover, 479 genes targeted by NF-κB showed increased expression in the frontal cortex of alcoholics (Okvist et al. 2007).

- Postmortem analyses of alcoholic human brain gene expression found innate immune activation of cell adhesion and extracellular membrane components of innate immune gene signaling (Liu et al. 2006).

Thus, the findings of several studies of gene or protein expression are consistent with increased neuroimmune signaling in the brains of addicted individuals.

Polymorphisms of Innate Immune Genes and Genetic Risk of Addiction

Genetic factors account for approximately 50 percent of the risk of alcohol dependence (Schuckit 2009). Multiple genes linked to innate immune function also have been linked to the risk for alcoholism (see figure 3). DNA variations (i.e., polymorphisms) at specific locations on the chromosomes result in gene variants (i.e., alleles) that differ in their function or activity and thereby may increase or reduce the risk of alcoholism. For example, polymorphisms in the gene encoding an enzyme called CYP2E1, which is involved in ethanol metabolism, have been associated with the risk for alcoholism (Webb et al. 2010). In the body, CYP2E1 is highly expressed in monocyte-like cells; ethanol metabolism by CYP2E1 leads to the activation of these cells. Specifically, CYP2E1-mediated ethanol metabolism causes an increased production of highly reactive molecules called reactive oxygen species (ROS) within the monocytes that activate proinflammatory NF-κB responses (Cao et al. 2005) (see figure 3). In the brain, ethanol exposure leads to increased CYP2E1 expression, particularly in astrocytes (Montoliu et al. 1994, 1995), which likely contributes to astrocyte activation of NF-κB transcription during chronic alcohol exposure.

Human genetic association studies also have directly linked certain polymorphisms of the genes encoding NF-κB to alcohol dependence (Edenberg et al. 2008; Flatscher-Bader et al. 2005; Okvist et al. 2007). For example, polymorphisms in a precursor gene called *NF-kB1* that encodes one of the subunits of the transcription factor (i.e., the NF-κB p50 subunit) and which is important for activation of transcription have been associated with the risk for alcoholism (Edenberg et al. 2008). Likewise, alleles of the proinflammatory cytokine TNFα that result in increased TNFα expression have been linked to alcoholism and alcoholic liver disease (Pastor et al. 2000, 2005; Powell et al. 2000). Another genetic linkage exists between certain alleles of the anti-inflammatory, NF-κB–inhibiting cytokine IL-10 and alcoholism (Marcos et al. 2008). Additional genetic evidence regarding innate immune genes and the risk for alcoholism comes from polymorphisms of the gene encoding a molecule called the IL-1 receptor antagonist as well as from multiple other alleles of the IL-1 gene complex (Saiz et al. 2009).

In general, gene polymorphisms associated with increased risk of alcoholism tend to increase proinflammatory responses. For example, alcohol exposure may increase the expression of proinflammatory cytokines or individuals at risk of alcohol dependence may carry alleles associated with decreased anti-

inflammatory cytokine secretion. Thus, multiple innate immune gene polymorphisms are associated with genetic risk for alcoholism in humans, consistent with the assumption that increased brain innate immune gene expression contributes to the neurobiology of alcohol addiction.

Summary

The findings summarized in this article link innate immune gene induction to addiction and alcoholism. Monocytes, microglia, and astrocytes are sensitive to AODs and stress, with repeated AOD use causing progressive innate immune gene induction that parallels changes in decision making, mood, and alcohol consumption. Stress and AODs activate NF-κB transcription in the brain, which in turn enhances expression of proinflammatory NF-κB target genes. As a result, molecules related to the innate immune response, such as the chemokine MCP-1, the proinflammatory cytokines TNFα, IL-1β, and IL-6; the proinflammatory oxidases iNOS, COX, and NOX (Qin et al. 2008); and proinflammatory proteases are found following chronic ethanol treatment. Postmortem analyses of human alcoholic brain also have demonstrated increased expression of innate immune genes, which can disrupt cognition, mood, and drug consumption and is consistent with addition-like behavior. Finally, polymorphisms of genes involved in the innate immune responses influence the risk for alcoholism. These studies suggest that innate immune genes contribute to alcoholism and may be involved in the genetic risk for alcoholism. ■

Acknowledgements

The author acknowledges support from the Bowles Center for Alcohol Studies, School of Medicine, University of North Carolina, and the National Institutes of Health, National Institute on Alcohol Abuse and Alcoholism (grants AA–020023, AA–020024, AA–020022, AA–019767, AA–11605, and AA–007573).

Financial Disclosure

The author declares that he has no competing financial interests.

References

Armario, A. Activation of the hypothalamic-pituitary-adrenal axis by addictive drugs: Different pathways, common outcome. *Trends in Pharmacological Sciences* 31(7): 318–325, 2010. PMID: 20537734

Beattie, M.S.; Ferguson, A.R.; and Bresnahan, J.C. AMPA-receptor trafficking and injury-induced cell death. *European Journal of Neuroscience* 32(2):290– 297, 2010. PMID: 20646045

Bechara, A.; Dolan, S.; and Hindes, A. Decision-making and addiction (part II): Myopia for the future or hypersensitivity to reward? *Neuropsychologia* 40(10):1690–1705, 2002. PMID : 11992657

Blednov, Y.A.; Benavidez, J.M.; Gell, C.; et al. Activation of inflammatory signaling by lipopolysaccharide produces a prolonged increase of voluntary alcohol intake in mice. *Brain, Behavior, and Immunity* 25(Suppl 1): S92–S105, 2011*a*. PMID: 21266194

Blednov, Y.A.; Bergeson, S.E.; Walker, D.; et al. Perturbation of chemokine networks by gene deletion alters the reinforcing actions of ethanol. *Behavioural Brain Research* 165(1):110–125, 2005. PMID: 16105698

Blednov, Y.A.; Ponomarev, I.; Geil, C.; et al. Neuroimmune regulation of alcohol consumption: Behavioral validation of genes obtained from genomic studies. *Addiction Biology* doi: 10.1111/j.1369-1600.2010.00284.x, 2011*b*. PMID: 21309947

Breese, G.R.; Knapp, D.J.; Overstreet, D.H.; et al. Repeated lipopolysaccharide (LPS) or cytokine treatments sensitize ethanol withdrawal-induced anxiety-like behavior. *Neuropsychopharmacology* 33(4):867–876, 2008. PMID: 17551540

Calu, D.J.; Roesch, M.R.; Stalnaker, T.A.; and Schoenbaum, G. Associative encoding in posterior piriform cortex during odor discrimination and reversal learning. *Cerebral Cortex* 17(6):1342–1349, 2007. PMID: 16882682

Cao, Q.; Mak, K.M.; and Lieber, C.S. Cytochrome P4502E1 primes macrophages to increase TNF-alpha production in response to lipopolysaccharide. *American Journal of Physiology. Gastrointestinal and Liver Physiology* 289(1):G95– G107, 2005. PMID: 15961886

Chang, E.F., and Merzenich M.M. Environmental noise retards auditory cortical development. *Science* 300(5618):498–504, 2003. PMID: 12702879

Coleman, L.G. Jr.; He, J.; Lee, J.; et al. Adolescent binge drinking alters adult brain neurotransmitter gene expression, behavior, brain regional volumes, and neurochemistry in mice. *Alcoholism: Clinical and Experimental Research* 35(4):671–688, 2011. PMID: 21223304

Crews, F.T.; Bechara, R.; Brown, L.A.; et al. Cytokines and alcohol. *Alcoholism: Clinical and Experimental Research* 30(4):720–730, 2006. PMID: 16573591

Crews, F.; Nixon, K.; Kim, D.; et al. BHT blocks NF-kappaB activation and ethanol-induced brain damage. *Alcoholism: Clinical and Experimental Research* 30(11):1938–1949, 2006. PMID: 17067360

Crews, F.T.; Zou, J.; and Qin, L. Induction of innate immune genes in brain create the neurobiology of addiction. *Brain, Behavior, and Immunity* 25(Suppl. 1):S4–S12, 2011. PMID: 21402143

Edenberg, H.J.; Xuei, X.; Wetherill, L.F.; et al. Association of NFKB1, which encodes a subunit of the transcription factor NF-kappaB, with alcohol dependence. *Human Molecular Genetics* 17(7):963–970, 2008. PMID: 18079108

Eisenberger, N.I.; Berkman, E.T.; Inagaki, T.K.; et al. Inflammation-induced anhedonia: Endotoxin reduces ventral striatum responses to reward. *Biological Psychiatry* 68(8):748–754, 2010. PMID: 20719303

Flatscher-Bader, T.; van der Brug, M.; Hwang, J.W.; et al. Alcohol-responsive genes in the frontal cortex and nucleus accumbens of human alcoholics. *Journal of Neurochemistry* 93(2):359–370, 2005. PMID: 15816859

Ghosh, S., and Hayden, M.S. New regulators of NF-kappaB in inflammation. *Nature Reviews Immunology* 8(11):837–848, 2008. PMID: 18927578

Graeber, M. B. Changing face of microglia. *Science* 330(6005):783–788, 2010. PMID: 21051630

He, J., and Crews, F.T. Increased MCP-1 and microglia in various regions of the human alcoholic brain. *Experimental Neurology* 210(2):349–358, 2008. PMID: 18190912

Kelley, K.W., and Dantzer, R. Alcoholism and inflammation: Neuroimmunology of behavioral and mood disorders. *Brain, Behavior, and Immunity* 25(Suppl. 1):S13–S20, 2011. PMID: 21193024

Knapp, D.J., and Crews, F.T. Induction of cyclooxygenase-2 in brain during acute and chronic ethanol treatment and ethanol withdrawal. *Alcoholism: Clinical and Experimental Research* 23(6):633–643, 1999. PMID: 10235299

Koo, J.W.; Russo, S.J.; Ferguson, D.; et al. Nuclear factor-kappaB is a critical mediator of stress-impaired neurogenesis and depressive behavior. *Proceedings of the National*

Academy of Sciences of the United States of America 107(6):2669–2674, 2010. PMID: 20133768

Liu, J.; Lewohl, J.M.; Harris, R.A.; et al. Patterns of gene expression in the frontal cortex discriminate alcoholic from nonalcoholic individuals. *Neuropsychopharmacology* 31(7):1574–1582, 2006. PMID: 16292326

Madrigal, J.L.; Garcia-Bueno, B.; Moro, M.A.; et al. Relationship between cyclooxygenase-2 and nitric oxide synthase-2 in rat cortex after stress. *European Journal of Neuroscience* 18(6):1701–1705, 2003. PMID: 14511348

Madrigal, J.L.; Moro, M.A.; Lizasoain, I.; et al. Stress-induced increase in extracellular sucrose space in rats is mediated by nitric oxide. *Brain Research* 938(1–2):87–91, 2002. PMID: 12031539

Marcos, M.; Pastor, I.; Gonzalez-Sarmiento, R.; and Laso, F.J. Interleukin-10 gene polymorphism is associated with alcoholism but not with liver disease. *Alcohol and Alcoholism* 43(5):523–528, 2008. PMID: 18436572

Montoliu, C.; Sancho-Tello, M.; Azorin, I.; et al. Ethanol increases cytochrome P4502E1 and induces oxidative stress in astrocytes. *Journal of Neurochemistry* 65(6):2561–2570, 1995. PMID: 7595552

Montoliu. C.; Valles, S.; Renau-Piqueras, J.; and Guerri, C. Ethanol-induced oxygen radical formation and lipid peroxidation in rat brain: Effect of chronic alcohol consumption. *Journal of Neurochemistry* 63(5):1855–1862, 1994. PMID: 79311342

Mulligan, M.K.; Ponomarev, I.; Hitzemann, R.J.; et al. Toward understanding the genetics of alcohol drinking through transcriptome metaanalysis. *Proceedings of the National Academy of Sciences of the United States of America* 103(16):6368–6373, 2006. PMID: 16618939

Munhoz, C.D.; Sorrells, S.F.; Caso, J.R.; et al. Glucocorticoids exacerbate lipopolysaccharide-induced signaling in the frontal cortex and hippocampus in a dose-dependent manner. *Journal of Neuroscience* 30(41):13690– 13698, 2010. PMID: 20943909

Obernier, J.A.; White, A.M.; Swartzwelder, H.S.; and Crews, F.T. Cognitive deficits and CNS damage after a 4-day binge ethanol exposure in rats. *Pharmacology, Biochemistry, and Behavior* 72(3):521–532, 2002. PMID: 12175448

Okvist, A.; Johansson, S.; Kuzmin, A.; et al. Neuroadaptations in human chronic alcoholics: Dysregulation of the NF-kappaB system. *PLoS One* 2(9):e930, 2007. PMID: 17895871

Pascual, M.; Balino, P.; Alfonso-Loeches, S.; et al. Impact of TLR4 on behavioral and cognitive dysfunctions associated with alcohol-induced neuroinflammatory damage. *Brain, Behavior, and Immunity* 25(Suppl 1):S80–S91, 2011. PMID: 21352907

Pascual, M.; Blanco, A.M.; Cauli, O.; et al. Intermittent ethanol exposure induces inflammatory brain damage and causes long-term behavioural alterations in adolescent rats. *European Journal of Neuroscience* 25(2):541–550, 2007. PMID: 17284196

Pastor, I.J.; Laso, F.J.; Avila, J.J.; et al. Polymorphism in the interleukin-1 receptor antagonist gene is associated with alcoholism in Spanish men. *Alcoholism: Clinical and Experimental Research* 24(10):1479–1482, 2000. PMID: 11045853

Pastor, I.J.; Laso, F.J.; Romero, A.; and Gonzalez-Sarmiento R. -238 G>A polymorphism of tumor necrosis factor alpha gene (TNFA) is associated with alcoholic liver cirrhosis in alcoholic Spanish men. *Alcoholism: Clinical and Experimental Research* 29(11):1928–1931, 2005. PMID: 16340448

Powell, E.E.; Edwards-Smith, C.J.; Hay, J.L.; et al. Host genetic factors influence disease progression in chronic hepatitis C. *Hepatology* 31(4):828– 833, 2000. PMID: 10733535

Qin, L.; He, J.; Hanes, R.N.; et al. Increased systemic and brain cytokine production and neuroinflammation by endotoxin following ethanol treatment. *Journal of Neuroinflammation* 5:10, 2008. PMID: 18348728

Qin, L.; Wu, X.; Block, M.L.; et al. Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia* 55(5):453–462, 2007. PMID: 17203472

Saiz, P.A.; Garcia-Portilla, M.P.; Florez, G.; et al. Polymorphisms of the IL-1 gene complex are associated with alcohol dependence in Spanish Caucasians: Data from an association study. *Alcoholism: Clinical and Experimental Research* 33(12):2147–2153, 2009. PMID: 19764937

Schoenbaum, G., and Shaham, Y. The role of orbitofrontal cortex in drug addiction: A review of preclinical studies. *Biological Psychiatry* 63(3):256–262, 2008. PMID: 17719014

Schoenbaum, G.; Roesch, M.R.; and Stalnaker, T.A. Orbitofrontal cortex, decision-making and drug addiction. *Trends in Neurosciences* 29(2):116–124, 2006. PMID: 16406092

Schoenbaum, G.; Saddoris, M.P.; Ramus, S.J.; et al. Cocaine-experienced rats exhibit learning deficits in a task sensitive to orbitofrontal cortex lesions. *European Journal of Neuroscience* 19(7):1997–2002, 2004. PMID: 15078575

Schuckit, M.A. An overview of genetic influences in alcoholism. *Journal of Substance Abuse Treatment* 36(1):S5–S14, 2009. PMID: 19062348

Stalnaker, T.A.; Takahashi, Y.; Roesch, M.R.; and Schoenbaum, G. Neural substrates of cognitive inflexibility after chronic cocaine exposure. *Neuropharmacology* 56(Suppl 1):63–72, 2009. PMID: 18692512

Webb, A.; Lind, P.A.; Kalmijn, J.; et al. The investigation into CYP2E1 in relation to the level of response to alcohol through a combination of linkage and association analysis. *Alcoholism: Clinical and Experimental Research* 35(1):10–18, 2011. PMID: 20958328

Zou, J.Y., and Crews, F.T. TNF alpha potentiates glutamate neurotoxicity by inhibiting glutamate uptake in organotypic brain slice cultures: Neuroprotection by NF kappa B inhibition. *Brain Research* 1034(1–2):11–24, 2005. PMID: 15713255

Zou, J., and Crews, F. CREB and NF-kappaB transcription factors regulate sensitivity to excitotoxic and oxidative stress induced neuronal cell death. *Cellular and Molecular Neurobiology* 26(4–6):385–405, 2006. PMID: 16633891

Zou, J., and Crews, F. Induction of innate immune gene expression cascades in brain slice cultures by ethanol: Key role of NF-kappaB and proinflammatory cytokines. *Alcoholism: Clinical and Experimental Research* 34(5):777–789, 2010. PMID: 20201932

Alcohol Dependence and Genes Encoding α2 and γ1 $GABA_A$ Receptor Subunits

Insights from Humans and Mice

Cecilia M. Borghese, Ph.D., and R. Adron Harris, Ph.D.

One approach to identifying the causes of alcoholism, particularly without crossing ethical boundaries in human subjects, is to look at the person's genome (and particularly at the variations that naturally arise in the DNA) to identify those variations that seem to be found more commonly in people with the disease. Some of these analyses have focused on the genes that encode subunits of the receptor for the brain chemical (i.e., neurotransmitter) γ-aminobutyric acid (GABA). Different epidemiological genetic studies have provided evidence that variations in certain $GABA_A$ receptor ($GABA_A$-R) subunits, particularly subunits α2 and γ1, are correlated with alcohol dependence. Manipulations of these genes and their expression in mice and rats also are offering clues as to the role of specific $GABA_A$-Rs in the molecular mechanisms underlying alcoholism and suggest possibilities for new therapeutic approaches. KEY WORDS: Alcohol dependence; alcoholism; genetic factors; DNA; genetic theory of alcohol and other drug use (AODU); genetic vulnerability to AODU; genetic variants; γ-aminobutyric acid (GABA); $GABA_A$ receptor ($GABA_A$-R) subunits; GABRA2; GABRG1; single nucleotide polymorphisms (SNPs); ion channels; neurotransmitters; gene association studies; human studies; animal studies; mice; rats

Even though the consequences of alcohol dependence (AD) clearly are devastating and obvious to observers, the molecular mechanisms involved in the development of the disease are far from clear and understood. The search for these mechanisms is made even more difficult by the vast number of genes, proteins, and pathways in the human body that potentially could be involved, and by the obvious limitations of conducting research with human subjects without crossing ethical boundaries. Yet despite these complexities, various approaches already have allowed researchers to gather much knowledge in recent years, and the essential players in alcohol's mechanisms of action and in the development of AD already may have been identified. Thus, research has found that the primary targets of alcohol seem to be proteins prominently involved in neuronal communication, including:

- Ion channels in the neuronal membrane that are activated by signaling molecules (i.e., neurotransmitters) such as γ-aminobutyric acid (GABA) (i.e., $GABA_A$ receptors), glycine (i.e., glycine receptors), glutamate (i.e., *N*-methyl-D-aspartate receptors [NMDA-Rs]), acetylcholine (i.e., nicotinic receptors), and serotonin (i.e., $5\text{-}HT_3$ receptors);
- Ion channels regulated by changes in the electric potential across the neuronal membrane (i.e., voltage-gated channels), such as voltage-gated calcium channels; and
- Ion channels regulated by a type of regulatory molecules called G-proteins, such as G-protein–coupled inwardly rectifying potassium channels (GIRKs).

Alcohol's actions on these primary targets trigger the involvement of other systems that ultimately culminate in the development of dependence (Vengeliene et al. 2008).

Many techniques have yielded insight into alcohol's effects on the organism, but perhaps the most challenging field, given the logical ethical constrains, is the study of the neuronal structures and mechanisms that are affected by alcohol and/or which play a role in the development of AD in living humans. One way of circumventing these limitations is by studying how the natural variations (i.e., polymorphisms) between individuals in the genomic DNA relate to AD—that is, whether any specific variants are found more or less commonly than would be expected by chance in people with the disorder. This analysis can provide a glimpse of which genes or gene variants contribute to and shape the development of the disorder.

These natural differences in the genomic DNA between individuals arise from spontaneous mutations of single DNA building blocks (i.e., nucleotides) and are called single nucleotide polymorphisms (SNPs). (For more information on SNPs and their analysis, see the sidebar). In the past 10 years, different genetic association studies in alcohol-dependent subjects have identified several genes linked to this condition. Some examples of proteins that are encoded by genes in which the AD-linked SNPs are located include the following:

- The μ-opioid receptor (encoded by the OPRM1 gene) (Bart et al. 2005; Kim et al. 2004; Nishizawa et al. 2006; Ray and Hutchison 2004; Rommelspacher et al. 2001; Zhang et al. 2006);
- The κ-opioid receptor (OPRK1) (Edenberg et al. 2008; Xuei et al. 2006; Zhang et al. 2008);

Cecilia M. Borghese, Ph.D., *is a research associate and* **R. Adron Harris, Ph.D.,** *is M. June and J. Virgil Waggoner Chair in Molecular Biology and director of the Waggoner Center for Alcohol and Addiction Research, both at the University of Texas at Austin, Austin, Texas.*

- Neuropeptide Y (NPY) (Ilveskoski et al. 2001; Lappalainen et al. 2002; Mottagui-Tabar et al. 2005);
- The muscarinic acetylcholine receptor M2 (CHRM2) (Dick et al. 2007; Luo et al. 2005; Wang et al. 2004); and
- The corticotropin-releasing hormone receptor 1 (CRHR1) (Chen et al. 2010).

Another group of genes related to alcohol dependence encode the $GABA_A$ receptors ($GABA_A$-Rs). This article will summarize what is known about the role of these receptors in the development of alcohol dependence.

$GABA_A$ Receptors

The $GABA_A$-Rs are proteins that span the membrane encasing the nerve cells (i.e., neurons) and which are composed of five subunits arranged around a central pore. There are several classes of subunits, including alpha (α), beta (β), gamma (γ), delta (δ), epsilon (ε), pi (π), theta (θ), and rho (ρ) subunits.

Single-Nucleotide Polymorphisms and Their Analysis

What Are Single-Nucleotide Polymorphisms?

Single-nucleotide polymorphisms (SNPs, pronounced "snips") are spontaneous mutations of single building blocks (i.e., nucleotides) in the genomic DNA. They can occur randomly, in any region of the DNA, including within those regions of the gene that actually encode parts of the resulting protein (i.e., coding sequences), within "silent" regions of a gene that ultimately do not encode parts of the resulting protein (i.e., non-coding regions), or in the regions between genes (i.e., intergenic regions). When a SNP occurs within a coding sequence, it may or may not change the amino acid sequence of the encoded protein. Each amino acid is represented by a three-nucleotide block of DNA (i.e., a codon). Because there are four different nucleotides (represented as A, C, G, and T), 64 possible codons exist; however, these encode only 20 amino acids. As a result, the genetic code is degenerate—that is, several codons may encode the same amino acid (e.g., both ACT and ACC encode threonine). A SNP in which both the original codon and the mutant codon produce the same protein sequence is called a synonymous polymorphism or silent mutation. If a different polypeptide sequence is produced, it is called a replacement polymorphism. This can result either in the introduction of a different amino acid, which is called a missense mutation, or in a premature stop of the protein, which is called a nonsense mutation (see the figure). Even if the SNP occurs in a noncoding region of the gene, it still may affect regulatory processes that could result, for instance, in altered protein levels.

When aligning DNA sequences from different individuals and comparing them at the same positions (i.e., loci) in the DNA, the occurrence of a SNP results in different "versions" of DNA called alleles. For example, in the figure, the two alleles for the SNP rs279868 are "A" and "G". Alleles frequently are transmitted from one generation to the next in a larger DNA block, usually from 5,000 to 100,000 nucleotides long. These blocks, which can contain numerous SNPs, are known as haplotypes. Thus, a haplotype specifies markers on one member of a pair of homologous chromosomes (i.e., either the chromosome inherited from the mother or the one inherited from the father).

Haplotypes are not always transmitted from one generation to the next, however, because of a process called recombination that randomly occurs during the formation of germ cells. As a result of recombination, new haplotypes should be formed based on the frequencies of the different alleles involved in the general population. Sometimes, however, certain combinations of alleles occur more or less frequently in a given population than would be expected from random formation of haplotypes. This nonrandom association of alleles at two or more loci is referred to as linkage disequilibrium. Identification of alleles that are in linkage disequilibrium can be useful for determining genes that are involved in conditions such as alcohol dependence.

How Can SNPs Be Analyzed?

Some genetic studies try to determine whether a certain allele (or haplotype) is present more or less frequently in people who suffer from a medical condition (e.g., alcoholism) than those without the disease; these are called genetic-association studies. One type of genetic-association studies are case–control studies, which include both individuals affected by the condition and disease-free control individuals from the same population. The frequency of alleles then is determined in both case and control subjects. Differences in the frequency of an allele between the two groups suggest that an association exists between the involved gene and the medical condition, with a certain allele conferring an increased or decreased risk for the condition. Identification of the precise nature of this association then requires additional studies.

units. The most commonly found $GABA_A$-Rs consist of two α, two β, and one γ or δ subunit. For some classes of subunits, several variants exist that are encoded by different genes, including six for the α subunit, three for the β subunit, and three for the γ subunit, allowing for numerous different subunit combinations. When GABA binds to the $GABA_A$-R, it activates the receptor and the central channel opens, allowing the entrance of negatively charged ions (i.e., anions), specifically Cl^-, to enter the neuron. This results in an increase in the difference in electrical charge between the inside and outside of the neuron (i.e., hyperpolarization), which in turn makes it more difficult for the neuron to transmit a nerve impulse, thus ultimately leading to inhibition of neuronal activity. Accordingly, GABA is considered an inhibitory neurotransmitter.

Considerable evidence points to the $GABA_A$-R as one of the main targets of alcohol (Kumar et al. 2009). The most abundant subunit combination in the brain, α1β2γ2, has been the most studied. Recently, the δ subunit–containing $GABA_A$-Rs also have been scrutinized in relationship to alcohol (Lobo and Harris 2008).

Single-Nucleotide Polymorphisms and Their Analysis *continued*

For example, the allele may alter the sequence, the splicing, or the levels of expression of the protein encoded or it may be in linkage disequilibrium with another allele that constitutes the genetic basis for the difference.

A caveat of these studies is that the frequencies of alleles/haplotypes can vary with ethnicity and geography; this is known as population stratification. One way to avoid this problem is to use family-based association designs. In this situation, unaffected family members (e.g., parents or siblings) are used as control subjects for the affected individuals. If an allele increases the risk of having the disease, then that allele would appear more frequently in the affected family members than in the unaffected members.

Other studies, such as twin and adoption studies, focus on the interaction between genes and environment. Twin studies compare the similarity of identical (i.e., monozygotic) and fraternal (i.e., dizygotic) twins. Identical twins generally are more similar than fraternal twins, because they are not only exposed to the same environment but also share a higher genetic similarity. In adoption studies, the adopted individuals are compared with control individuals (i.e., non-adopted individuals either from the adoptive family or the general population or adopted but unrelated children in the adoptive family). By comparing large numbers of twin pairs or adoptees and control subjects (e.g., with respect to the frequency of certain SNPs as well as the disease of interest), it is possible to better understand the role of genes and environment in the characteristics of a person.

Another approach to using SNPs to identify genes involved in a certain disease is to conduct genome-wide association studies (GWASs). With this strategy, a genetic association with the disease is investigated using many SNPs that cover the entire genome, instead of just a few genes as in the study approaches described above. This allows researchers to identify associations with genes that previously had not been expected to play a role in the disease under investigation. The downside of the GWASs is that they require a much larger number of subjects than do the other studies.

Segment of a single strand of DNA representing a fragment of the coding region from the *GABRA2* gene from two different people. There are two SNPs in this gene region—one in which both variants of the DNA encode the same amino acid (i.e., a silent mutation) and one in which the two variants of the DNA encode different amino acids (i.e., a missense mutation).

The genes encoding the $GABA_A$-R subunits are located in clusters on different chromosomes, including one cluster on chromosome 4 that carries genes called *GABRB1*, *GABRA4*, *GABRA2*, and *GABRG1*, which encode the β1, α4, α2, and γ1 subunits, respectively (see the figure). Previous human genetic studies have linked genetic polymorphisms in two regions of the *GABRA2* gene (i.e., in the middle and at the 3′ end of the gene) and in the region between the *GABRA2* and *GABRG1* genes (i.e., the *GABRA2* to *GABRG1* intergenic region) with AD (Agrawal et al. 2006; Covault et al. 2004; Edenberg et al. 2004; Enoch et al. 2006; Fehr et al. 2006; Lappalainen et al. 2005; Soyka et al. 2008). However, the evidence is not unequivocal, because other studies found no association between AD and the SNPs in this area (Covault et al. 2008; Drgon et al. 2006; Matthews et al. 2007). Even among the studies that did find a correlation, some inconsistencies existed. For instance, the first study of the association between *GABRA2* and AD came from the Collaborative Study on the Genetics of Alcoholism (COGA), a vast family-based association study (Edenberg et al. 2004). When Agrawal and colleagues (2006) extended the study of the COGA sample to include illicit drug dependence and comorbid dependence on alcohol and other drugs, the association was found only in subjects with AD and co-occurring drug dependence. On the other hand, Covault and colleagues (2004) found that the association was stronger when alcoholics with comorbid drug dependence were removed from the sample. Despite these inconsistencies, however, most of the clinical and genetic evidence points to *GABRA2* as a major genetic player in AD (see Enoch 2008).

All the SNPs that have been studied in the *GABRA2* and *GABRG1* genes to date are nonfunctional polymorphisms—that is, they do not alter the amino acid sequence of the encoded proteins. An alternative explanation for their role in the development of AD would be that the SNPs may alter the amount of protein that is produced. To address this possibility, researchers have analyzed the levels of an intermediate molecule called messenger RNA (mRNA) that is generated when the information encoded in the DNA is used for the production of a functional protein (i.e., during gene expression). An analysis of α2 mRNA levels in the prefrontal cortex of AD and control subjects found an association between α2 mRNA levels and the different variants (i.e., alleles) in the SNP rs279858,[1] although mRNA levels did not differ between control subjects and alcoholics (Haughey et al. 2008). Other recent data suggest that the apparent correlation between AD and *GABRA2* may result from a linkage disequilibrium with a not-yet-detected functional variant in the neighboring *GABRG1* gene. These findings on *GABRA2* and *GABRG1* and their association with AD are reviewed in the following sections, focusing on human studies and correlates in genetically modified rodents.

Analyses of the *GABRA2* and *GABRG1* Genes

Genetic-Association Studies

Several gene-association studies have examined the relationship of the *GABRA2* and *GABRG1* genes with AD, with varying results. Two studies—a large twin sample of the Australian population that also investigated the association with smoking and illicit drug use (Lind et al. 2008), and a small case–control study in an Italian sample (Onori et al. 2010)—reported no association between *GABRA2* and AD. Another case–control and family-association study (Sakai et al. 2010) sought to analyze the correlation between *GABRA2* genotype and substance abuse and behavioral problems in adolescents. The investigators only analyzed a single SNP in *GABRA2*, which was found not to be associated with conduct disorder or AD in adolescents.

Enoch and colleagues (2010) conducted a case–control study in African-American men with single and comorbid diagnoses of alcohol, cocaine, and heroin dependence, assessing the *GABRA2* genotype as well as childhood trauma. The exposure to childhood trauma predicted substance dependence.

[1] SNP rs279858 encodes a silent mutation (i.e., a mutation that does not result in an altered amino acid sequence) in the coding region of GABRA2.

[2] A haplotype is a set of closely linked genetic markers (e.g., SNPs) present on one chromosome that tends to be inherited together.

Figure Schematic representation of the cluster of $GABA_A$ receptor genes on chromosome 4. Arrows indicate gene position, size, and direction of transcription. The subunits and the names of the corresponding genes are α2 *(GABRA2)*, α4 *(GABRA4)*, β1 *(GABRB1)*, and γ1 *(GABRG1)*.

common in control subjects and seemed to confer resilience to addiction after exposure to severe childhood trauma. These findings suggest that in African-American men, childhood trauma, *GABRA2* SNPs, and their interaction determine (at least in part) the risk of or resilience to substance dependence. However, the data did not show a direct association between *GABRA2* and AD.

In a case–control adoption study of substance abuse (Philibert et al. 2009), the researchers determined the participants' genotypes for SNPs encompassing the *GABRA2* gene and analyzed them with respect to their history of alcohol, nicotine, and/or cannabis dependence. Both *GABRA2* genotype and haplotype were significantly related to vulnerability to all three types of substance dependence, particularly nicotine, and this association was more pronounced in female than in male subjects.

A small study using Japanese subjects (mostly social drinkers) examined the association between genetic variation in *GABRA2* (as assessed via seven SNPs) and subjective responses to alcohol as well as stimulant and sedative effects of alcohol (Roh et al. 2010). Three of these seven SNPs, all of which were located in the middle of the *GABRA2* gene, showed significant associations with subjective effects of alcohol. Specifically, individuals carrying one or two copies of the more common *GABRA2* allele (which is not associated with AD) showed greater subjective responses to alcohol than did individuals carrying two copies of the allele associated with AD. These results are, to some extent, in agreement with previous studies (Haughey et al. 2008; Pierucci-Lagha et al. 2005).

Another study (Kareken et al. 2010) examined the association between *GABRA2* SNPs and the brain's reward system. The participants, which included social drinkers, heavy drinkers, and alcohol-dependent individuals, first were assessed with respect to the brain's responses to alcohol cues (i.e., exposure to the odor of their preferred alcoholic beverage or a control odor) under both alcohol intoxication and control conditions using an imaging technique called functional MRI (fMRI). Then, the subjects were stratified according to their genotype at a SNP in *GABRA2* that previously had been shown to be associated with AD (Edenberg et al. 2004). All participants carried at least one copy of the high-risk allele of the SNP. Under both alcohol intoxication and control conditions, participants with two copies of this allele (i.e., homozygous subjects) exhibited a larger response to alcoholic odors than to control odors in one brain region (i.e., the medial frontal cortical areas), whereas participants with only one copy of this allele (i.e., heterozygous subjects) exhibited a larger response in another brain area (i.e., the ventral tegmental area). Thus, *GABRA2* variants seem to modify the activation of reward-related areas after exposure to alcohol-associated cues. Another study (Villafuerte et al. 2011) used fMRI to analyze the relationship between two *GABRA2* SNPs, the personality trait of impulsivity, and activation of a brain region called the insula cortex during anticipation of reward or loss in a family sample with high numbers of alcohol-dependent individuals. The investigators detected an association of all three variables, suggesting that *GABRA2* genotype influences insula responses and therefore impulsivity.

Another type of study called linkage disequilibrium analyzes whether certain alleles located close to each other on the same chromosome are inherited together more or less frequently than would be expected by chance alone.[3] Such studies in different populations have focused on *GABRA2* and either *GABRG1* (Ittiwut et al. 2008) or the intergenic region between the two genes (Philibert et al. 2009). The findings of these studies led to the conclusion that associations observed between *GABRA2* and the condition under investigation could be attributable to functional genetic variation at the *GABRG1* locus or that disease-related variants may exist at both loci.

Some new studies have focused on the *GABRG1* gene. Ray and Hutchinson (2009) examined associations between two SNPs of the *GABRG1* gene and alcohol use in hazardous drinkers. The data indicated that variation in one of the SNPs was associated with level of response to alcohol, drinking behavior, and alcohol problems.

Additional evidence of a significant *GABRG1* association with AD was found in a study involving Finnish Caucasian and Plains American Indians that examined both the *GABRA2* and *GABRG1* genes (Enoch et al. 2009). In both populations, there were significant haplotype and SNP associations of *GABRG1,* but not *GABRA2,* with AD. However, in the Finnish study population, the association of three less common haplotypes with AD was determined by *GABRA2*. Taken together, the findings of all of these studies suggest that independent contributions from both *GABRG1* and *GABRA2* likely contribute to the risk of AD.

Genome-Wide Association Studies

In contrast to the approaches used in the studies described above, genome-wide association studies (GWASs) investigate the genetic association with a disease using many SNPs that cover the entire genome instead of just a few genes. GWASs therefore also may discover associations with genes not previously suspected to be involved in the disease. One GWASs (Bierut et al. 2010) identified 15 SNPs that showed a significant association with AD. Moreover, when the investigators performed an independent evaluation for *GABRA2*, they found that five SNPs at that gene showed a modest association with AD.

Another GWASs was carried out in a case–control sample drawn from the families in the COGA, using individuals with AD (56 percent of whom also were dependent on illicit drugs) and individuals who used alcohol but were not dependent on alcohol or illicit drugs (Edenberg et al. 2010). The study identified no single SNP that met genome-wide criteria for significance; however, several clusters of SNPs provided mutual support for an association with the disease. An analysis of SNPs in genes encoding $GABA_A$-R subunits

[3] Even genes located close to each other on a chromosome may not always be inherited together, because of a process called genetic recombination that occurs with a certain probability during the generation of the germ cells.

in this sample found that a SNP in a gene called *GABRR2,* which encodes the $GABA_A$-R α2 subunit, was highly correlated with AD in this GWASs. This supported previous results, even though the level of significance was not high enough for genome-wide significance (Xuei et al. 2009). Likewise, SNPs in *GABRG1* were associated with AD, consistent with previous studies (Covault et al. 2008; Enoch et al. 2009). However, there was no evidence that the neighboring *GABRA2* gene was associated with AD. Finally, SNPs in other genes encoding $GABA_A$-R subunits (i.e., *GABRG3,* which encodes the γ3 subunit; *GABRA1,* which encodes the α1 subunit; and *GABRG2,* which encodes the γ2 subunit) also were associated with AD, again confirming findings of other investigators (Dick et al. 2004, 2006).

It is important to note that there are some inconsistencies in the findings of these genetic studies, which can be attributed to the inherent differences among the study types, the variability (i.e., heterogeneity) of the disease, and the genetic differences among the populations studied. In general, however, evidence continues to accumulate supporting an association between variations in genes encoding $GABA_A$-R subunits, particularly α2 and γ1 subunits, and AD.

Studies in Genetically Engineered Rodents

The studies in humans discussed above have been supplemented with studies of alcohol's effects on behavior in genetically engineered mice carrying mutations in GABA-related genes (Crabbe et al. 2006). For example, Boehm and colleagues (2004) found that mice in which the $GABA_A$-R α2 subunit had been deleted (i.e., α2 knockout mice) differed from control mice both in behavioral tests conducted without alcohol exposure (e.g., showed decreased spontaneous locomotion when tested for locomotor response to novelty) and in some behavioral responses to alcohol. Thus, the α2 knockout mice showed a shorter duration of alcohol-induced loss of righting reflex (LORR), which is a measure of alcohol's hypnotic effects. However, the sensitivity of the mice to acute ethanol withdrawal seemed unchanged, as did alcohol's anxiety-reducing (i.e., anxiolytic) effects. This lack of an effect of α2 deletion on alcohol's anxiolytic effects was unexpected, because mice genetically modified to possess a benzodiazepine-insensitive α2 subunit called α2(H101R) (i.e., knockin mice) no longer exhibited anxiolytic behavior when they were treated with the benzodiazepine diazepam (Low et al. 2000). Both alcohol and benzodiazepines are anxiolytic, and both increase $GABA_A$-R function. If both benzodiazepines and alcohol acted through α2-containing $GABA_A$-Rs to produce anxiolytic effects, deletion of the α2 subunit in the knockout mice should reduce alcohol's anxiolytic effects, and that did not happen. Furthermore, accurate assessment of alcohol's anxiolytic effects in the α2 knockout mice was complicated by the altered locomotor responses in these animals. Finally, female α2 knockout mice, but not males, preferred and consumed less alcohol than did the controls. However, interpretation of these findings is complicated by the female mice's greater aversion to bitter-tasting substances.

Another study was conducted in knockin mice carrying α1, α2, α3, or α5 $GABA_A$-R subunits that are insensitive to benzodiazepines through a mutation in a single amino acid (Tauber et al. 2003). The investigators administered diazepam and alcohol in combination to these mice and then determined the mice's LORR. All animals except for the α2 (H101R) mice showed similar sensitivity (i.e., increased LORR and reduced locomotor activity) to the combined drugs. Furthermore, the α2 (H101R) mice exhibited normal responses to alcohol alone (i.e., normal LORR and locomotor activity) and to a combination of low-dose alcohol and diazepam (i.e., normal locomotor activity). Thus, the benzodiazepine-induced increase in the alcohol-mediated hypnosis depends on the α2 $GABA_A$-R subunit.

Although null mutant mice that completely lack a certain $GABA_A$-R subunit provide important contributions to our knowledge of alcohol's targets, the deletion of a receptor in the brain, particularly an important one, is likely to trigger compensatory changes. For example, other receptor subunits could become more abundant and take on the functions of the missing subunit. An alternative approach is to design specific mutations that will render that receptor insensitive to alcohol but normal in every other aspect. One study compared the activities of $GABA_A$-Rs containing wild-type and mutated α2 subunits expressed in frog egg cells (i.e., *Xenopus* oocytes) (Blednov et al. 2011). With the normal $GABA_A$-Rs containing the wild-type subunit α2 (SL),[4] submaximal GABA responses were enhanced (i.e., potentiated) by alcohol. However, this potentiation was absent in the mutant α2 (HA)-containing $GABA_A$-Rs; there even was a small inhibition of the receptor's activity. In contrast, the mutation did not affect the receptor's sensitivity to GABA or the modulation by zinc, the benzodiazepine flunitrazepam, or the anesthetic etomidate (Werner et al. 2011).

On the basis of these findings, researchers developed and studied two corresponding mouse lines, the α2 SL/SL (i.e., wild-type) mice and the α2 HA/HA (i.e., knockin) mice. The responses to alcohol in these animals then were studied using a variety of tests (Blednov et al. 2011). The analyses found that some typical effects of alcohol (e.g., conditioned taste aversion and motor stimulation) were absent in the knockin mice. Moreover, the knockin animals showed changes in alcohol intake and preference in multiple tests as well as increased alcohol-induced hypnosis. In contrast, the knockin animals exhibited no changes in alcohol's anxiolytic and motor incoordination effects. These altered behavioral responses to alcohol in mutant (i.e., both knockout and knockin) mice may be related to altered subjective effects of alcohol in humans with different α2-associated SNPs (Kareken et al. 2010; Roh et al. 2010). In summary, the

[4] The wild-type α2 (SL) subunit carries the amino acid serine at position 270 and the amino acid leucine at position 277. In contrast, the mutant α2 (HA) subunit carried the amino acids histidine at position 270 and alanine at position 277.

study suggests that α2-containing $GABA_A$-Rs may be responsible for specific alcohol-induced effects. A subsequent study of the changes in mRNA levels induced by these mutations in the α2 subunit in the outer layer of the brain (i.e., the cerebral cortex) underlines the advantages of using knockin over knockout mice. Of almost 11,000 probes tested, the expression of only three genes was significantly modified in the knockin mice, and the behavioral responses to the sedative agents pentobarbital and flurazepam were unchanged (Harris et al. 2011). This confirms that the introduction of these mutations has minimal impact on the knockin animals compared with controls, minimizing the risk that effects unrelated to the behavior being investigated confound the results.

Another study in mice focused on the role of the $GABA_A$-R α2 subunit in changes in behavior produced by adaptation to chronic cocaine's effects (i.e., cocaine behavioral plasticity), such as locomotor sensitization, as well as in addiction (Dixon et al. 2010). In $GABA_A$-R α2 null mutant mice, cocaine did not induce a greater effect after repeated administration (i.e., did not produce behavioral sensitization) as it did in wild-type mice. Conversely, in mice carrying the benzodiazepine-insensitive $GABA_A$-R α2 (H101R) subunit, an agent called Ro 15–4513 that can increase the receptor responses in this mutant α2 subunit could stimulate locomotor activity if it was delivered into a brain region called the nucleus accumbens and induced behavioral sensitization to this effect after repeated administration. These results suggest that activation of α2-containing $GABA_A$-Rs in the nucleus accumbens is sufficient and necessary for behavioral sensitization. Furthermore, the investigators conducted a genetic case–control study in a diverse population (mainly Caucasian) that demonstrated an association of *GABRA2* with cocaine addiction in humans, emphasizing the relevance of α2-containing $GABA_A$-Rs in drug dependence.

Finally, researchers used an established animal model of human alcohol abuse, the selectively bred alcohol-preferring (P) rats, to look at the role of $GABA_A$-R subunits in alcohol's effects (Liu et al. 2011). The levels of $GABA_A$-R α1 subunits are elevated in a brain region called the ventral pallidum of these rats, and both α1 and α2 levels are increased in another region called the central nucleus of the amygdala (CeA). The study used molecules known as small-interfering RNAs (siRNA), which can interfere with gene expression, to specifically prevent production of α1 and α2 subunits. When siRNA targeted to α2 was infused into the CeA of P rats, both α2 expression and $GABA_A$-R density were reduced, and this was associated with inhibition of binge drinking. In contrast, siRNA targeted to α1 did not cause any of these changes when introduced in the CeA but did reduce α1 expression and binge drinking when administered into the ventral pallidum. These results highlight that not only the kind of $GABA_A$-R subunit but also the brain region in which it is located are relevant for alcohol consumption.

Implications of Genetic Findings for Therapeutic Approaches

Extending a previous study (Bauer et al. 2007), Das and colleagues (2010) analyzed the association between a SNP in the *GABRA2* gene and the efficacy of three psychotherapies for alcoholism (i.e., motivational enhancement therapy, cognitive–behavioral therapy, or 12-step facilitation) in preventing extreme drinking in AD patients. The study found that men with a high-risk *GABRA2* allele had a significantly higher probability of extreme drinking than did men without that allele. However, both men and women carrying at least one high-risk allele responded better to the therapy than did those who were homozygous for the low-risk allele. Among the female participants, the most effective therapy was cognitive–behavioral therapy, whereas among male subjects motivational enhancement therapy was most effective.

Tailoring pharmacotherapy to alcohol-dependent patients on the basis of genetic indicators also may be within reach. Two medications, naltrexone and acamprosate, currently are used for the treatment of alcoholism, but often with limited success. In an effort to identify potential associations between genotype and treatment outcome, Ooteman and colleagues (2009) determined SNPs in genes encoding different receptors involved in AD processes (i.e., opioid, dopamine, glutamate, and $GABA_A$ receptors) in alcohol-dependent individuals randomly assigned to acamprosate or naltrexone treatment. The investigators also quantified treatment effectiveness using tests administered the day before treatment initiation and on the last day the medication was administered. The tests included a cue exposure (i.e., participants were exposed to the sight and smell of their favorite alcoholic beverage while listening to a mood-induction script), followed by an assessment of self-reported cue-induced craving and physiological cue reactivity (i.e., heart rate). Significant association effects were found for several SNPs, suggesting that this may be the first step in matching patients to pharmacotherapy based on $GABA_A$-Rs and other genotypic markers.

Summary

Studies of genetically modified mice and rats have demonstrated that manipulation of α2 $GABA_A$-R subunits produces changes in alcohol-related phenotypes. The findings were more equivocal in human genetic studies, although strong evidence suggests that several $GABA_A$-R subunits, particularly α2 and γ1, have a role in AD in humans. Future studies hopefully will elucidate what exact mechanism creates these variations in the genetic code that affect AD, and how such variations can be used to provide a path to individualized therapy for patients with AD. ■

Acknowledgements

The preparation of this manuscript was supported by National Institutes of Health (NIH) grant AA–06399.

Financial Disclosure

The authors declare that they have no competing financial interests.

References

Agrawal, A.; Edenberg, H.J.; Foroud, T., et al. Association of GABRA2 with drug dependence in the collaborative study of the genetics of alcoholism sample. *Behavior Genetics* 36(5):640–650, 2006. 16622805

Bart, G.; Kreek, M.J.; Ott, J.; et al. Increased attributable risk related to a functional mu-opioid receptor gene polymorphism in association with alcohol dependence in central Sweden. *Neuropsychopharmacology* 30(2):417– 422, 2005. PMID: 15525999

Bauer, L.O.; Covault, J.; Harel, O.; et al. Variation in GABRA2 predicts drinking behavior in project MATCH subjects. *Alcoholism: Clinical and Experimental Research* 31(11): 1780–1787, 2007. PMID: 17949392

Bierut, L.J.; Agrawal, A.; Bucholz, K.K.; et al. A genome-wide association study of alcohol dependence. *Proceedings of the National Academy of Sciences of the United States of America* 107(11):5082–5087, 2010. PMID: 20202923

Blednov, Y.A.; Borghese, C.M.; McCracken, M.L.; et al. Loss of ethanol conditioned taste aversion and motor stimulation in knockin mice with ethanol-insensitive alpha2-containing GABA(A) receptors. *Journal of Pharmacology and Experimental Therapeutics* 336(1):145–154, 2011. PMID: 20876231

Boehm, S.L., 2nd; Ponomarev, I.; Jennings, A.W.; et al. gamma-Aminobutyric acid A receptor subunit mutant mice: New perspectives on alcohol actions. *Biochemical Pharmacology* 68(8):1581–1602, 2004. PMID: 15451402

Chen, A.C.; Manz, N.; Tang, Y.; et al. Single-nucleotide polymorphisms in corticotropin releasing hormone receptor 1 gene (CRHR1) are associated with quantitative trait of event-related potential and alcohol dependence. *Alcoholism: Clinical and Experimental Research* 34(6):988–996, 2010. PMID: 20374216

Covault, J.; Gelernter, J.; Hesselbrock, V.; et al. Allelic and haplotypic association of GABRA2 with alcohol dependence. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics* 129B(1):104–109, 2004. PMID: 15274050

Covault, J.; Gelernter, J.; Jensen, K.; et al. Markers in the 5'-region of GABRG1 associate to alcohol dependence and are in linkage disequilibrium with markers in the adjacent GABRA2 gene. *Neuropsychopharmacology* 33(4): 837–848, 2008. PMID: 17507911

Crabbe, J.C.; Phillips, T.J.; Harris, R.A.; et al. Alcohol-related genes: Contributions from studies with genetically engineered mice. *Addiction Biology* 11(3–4): 195–269, 2006. PMID: 16961758

Das, S.; Harel, O.; Dey, D.K.; et al. Analysis of extreme drinking in patients with alcohol dependence using Pareto regression. *Statistics in Medicine* 29(11):1250–1258, 2010. PMID: 20225194

Dick, D.M.; Agrawal, A.; Wang, J.C.; et al. Alcohol dependence with comorbid drug dependence: Genetic and phenotypic associations suggest a more severe form of the disorder with stronger genetic contribution to risk. *Addiction* 102(7):1131–1139, 2007. PMID: 17567401

Dick, D.M.; Edenberg, H.J.; Xuei, X.; et al. Association of GABRG3 with alcohol dependence. *Alcoholism: Clinical and Experimental Research* 28(1):4–9, 2004. PMID: 14745296

Dick, D.M.; Plunkett, J.; Wetherill, L.F.; et al. Association between GABRA1 and drinking behaviors in the collaborative study on the genetics of alcoholism sample. *Alcoholism: Clinical and Experimental Research* 30(7):1101–1110, 2006. PMID: 16792556

Dixon, C.I.; Morris, H.V.; Breen, G.; et al. Cocaine effects on mouse incentive-learning and human addiction are linked to alpha2 subunit-containing GABAA receptors. *Proceedings of the National Academy of Sciences of the United States of America* 107(5):2289–2294, 2010. PMID: 20133874

Drgon, T.; D'Addario, C.; and Uhl, G.R. Linkage disequilibrium, haplotype and association studies of a chromosome 4 GABA receptor gene cluster: Candidate gene variants for addictions. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics* 141B(8):854–860, 2006. PMID: 16894595

Edenberg, H.J.; Dick, D.M.; Xuei, X.; et al. Variations in GABRA2, encoding the alpha 2 subunit of the GABA(A) receptor, are associated with alcohol dependence and with brain oscillations. *American Journal of Human Genetics* 74(4):705–714, 2004. PMID: 15024690

Edenberg, H.J.; Koller, D.L.; Xuei, X.; et al. Genome-wide association study of alcohol dependence implicates a region on chromosome 11. *Alcoholism: Clinical and Experimental Research* 34(5):840–852, 2010. PMID: 20201924

Edenberg, H.J.; Wang, J.; Tian, H.; et al. A regulatory variation in OPRK1, the gene encoding the kappa-opioid receptor, is associated with alcohol dependence. *Human Molecular Genetics* 17(12):1783–1789, 2008. PMID: 18319328

Enoch, M.A. The role of GABA(A) receptors in the development of alcoholism. *Pharmacology, Biochemistry, and Behavior* 90(1):95–104, 2008. PMID: 18440057

Enoch, M.A.; Hodgkinson, C.A.; Yuan, Q.; et al. GABRG1 and GABRA2 as independent predictors for alcoholism in two populations. *Neuropsychopharmacology* 34(5):1245–1254, 2009. PMID : 18818659

Enoch, M.A.; Hodgkinson, C.A.; Yuan, Q.; et al. The influence of GABRA2, childhood trauma, and their interaction on alcohol, heroin, and cocaine dependence. *Biological Psychiatry* 67(1):20–27, 2010. PMID: 19833324

Enoch, M.A.; Schwartz, L.; Albaugh, B.; et al. Dimensional anxiety mediates linkage of GABRA2 haplotypes with alcoholism. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics* 141B(6):599–607, 2006. PMID: 16874763

Fehr, C.; Sander, T.; Tadic, A.; et al. Confirmation of association of the GABRA2 gene with alcohol dependence by subtype-specific analysis. *Psychiatric Genetics* 16(1):9–17, 2006. PMID: 16395124

Harris, R.A.; Osterndorff-Kahanek, E.; Ponomarev, I.; et al. Testing the silence of mutations: Transcriptomic and behavioral studies of GABA(A) receptor alpha1 and alpha2 subunit knock-in mice. *Neuroscience Letters* 488(1): 31–35, 2011. PMID: 21056629

Haughey, H.M.; Ray, L.A.; Finan, P.; et al. Human gamma-aminobutyric acid A receptor alpha2 gene moderates the acute effects of alcohol and brain mRNA expression. *Genes, Brain, and Behavior* 7(4):447–454, 2008. PMID: 18005236

Ilveskoski, E.; Kajander, O.A.; Lehtimaki, T.; et al. Association of neuropeptide y polymorphism with the occurrence of type 1 and type 2 alcoholism. *Alcoholism: Clinical and Experimental Research* 25(10):1420–1422, 2011. PMID: 11696660

Ittiwut, C.; Listman, J.; Mutirangura, A.; et al. Interpopulation linkage disequilibrium patterns of GABRA2 and GABRG1 genes at the GABA cluster locus on human chromosome 4. *Genomics* 91(1):61–69, 2008. PMID: 17976953

Kareken, D.A.; Liang, T.; Wetherill, L.; et al. A polymorphism in GABRA2 is associated with the medial frontal response to alcohol cues in an fMRI study. *Alcoholism: Clinical and Experimental Research* 34(12):2169–2178, 2010. PMID: 20698837

Kim, S.A.; Kim, J.W.; Song, J.Y.; et al. Association of polymorphisms in nicotinic acetylcholine receptor alpha 4 subunit gene (CHRNA4), mu-opioid receptor gene (OPRM1), and ethanol-metabolizing enzyme genes with alcoholism in Korean patients. *Alcohol* 34(2–3):115–120, 2004. PMID : 15902904

Kumar, S.; Porcu, P.; Werner, D.F.; et al. The role of GABA(A) receptors in the acute and chronic effects of ethanol: A decade of progress. *Psychopharmacology (Berlin)* 205(4): 529–564, 2009. PMID: 19455309

Lappalainen, J.; Kranzler, H.R.; Malison, R.; et al. A functional neuropeptide Y Leu7Pro polymorphism associated with alcohol dependence in a large population sample from the United States. *Archives of General Psychiatry* 59(9):825–831, 2002. PMID: 12215082

Lappalainen, J.; Krupitsky, E.; Remizov, M.; et al. Association between alcoholism and gamma-amino butyric acid alpha2 receptor subtype in a Russian population. *Alcoholism: Clinical and Experimental Research* 29(4):493–498, 2005. PMID: 15834213

Lind, P.A.; MacGregor, S.; Montgomery, G.W.; et al. Effects of GABRA2 variation on physiological, psychomotor and subjective responses in the Alcohol Challenge Twin Study. *Twin Research and Human Genetics* 11(2):174–182, 2008. PMID: 18361719

Liu, J.; Yang, A.R.; Kelly, T.; et al. Binge alcohol drinking is associated with GABAA alpha2-regulated Toll-like receptor 4 (TLR4) expression in the central amygdala. *Proceedings of the National Academy of Sciences of the United States of America* 108(11):4465–4470, 2011. PMID: 21368176

Lobo, I.A., and Harris, R.A. GABA(A) receptors and alcohol. *Pharmacology, Biochemistry, and Behavior* 90(1):90–94, 2008. PMID: 18423561

Low, K.; Crestani, F.; Keist, R.; et al. Molecular and neuronal substrate for the selective attenuation of anxiety. *Science* 290(5489):131–134, 2000. PMID: 11021797

Luo, X.; Kranzler, H.R.; Zuo, L.; et al. CHRM2 gene predisposes to alcohol dependence, drug dependence and affective disorders: Results from an extended case-control structured association study. *Human Molecular Genetics* 14(16):2421–2434, 2005. PMID: 16000316

Matthews, A.G.; Hoffman, E.K.; Zezza, N.; et al. The role of the GABRA2 polymorphism in multiplex alcohol dependence families with minimal comorbidity: Within-family association and linkage analyses. *Journal of Studies on Alcohol and Drugs* 68(5):625–633, 2007. PMID: 17690794

Mottagui-Tabar, S.; Prince, J.A.; Wahlestedt, C.; et al. A novel single nucleotide polymorphism of the neuropeptide Y (NPY) gene associated with alcohol dependence. *Alcoholism: Clinical and Experimental Research* 29(5):702– 707, 2005. PMID: 15897713

Nishizawa, D.; Han, W.; Hasegawa, J.; et al. Association of mu-opioid receptor gene polymorphism A118G with alcohol dependence in a Japanese population. *Neuropsychobiology* 53(3):137–141, 2006. PMID: 16679777

Onori, N.; Turchi, C.; Solito, G.; et al. GABRA2 and alcohol use disorders: No evidence of an association in an Italian case-control study. *Alcoholism: Clinical and Experimental Research* 34(4):659–668, 2010. PMID: 20102561

Ooteman, W.; Naassila, M.; Koeter, M.W.; et al. Predicting the effect of naltrexone and acamprosate in alcohol-dependent patients using genetic indicators. *Addiction Biology* 14(3):328–337, 2009. PMID: 19523047

Philibert, R.A.; Gunter, T.D.; Beach, S.R.; et al. Role of GABRA2 on risk for alcohol, nicotine, and cannabis dependence in the Iowa Adoption Studies. *Psychiatric Genetics* 19(2):91–98, 2009. PMID : 19672139

Pierucci-Lagha, A.; Covault, J.; Feinn, R.; et al. GABRA2 alleles moderate the subjective effects of alcohol, which are attenuated by finasteride. *Neuropsychopharmacology* 30(6):1193–1203, 2005. PMID: 15702134

Ray, L.A., and Hutchison, K.E. A polymorphism of the mu-opioid receptor gene (OPRM1) and sensitivity to the effects of alcohol in humans. *Alcoholism: Clinical and Experimental Research* 28(12):1789–1795, 2004. PMID: 15608594

Ray, L.A., and Hutchison, K.E. Associations among GABRG1, level of response to alcohol, and drinking behaviors. *Alcoholism: Clinical and Experimental Research* 33(8):1382–1390, 2009. PMID: 19426171

Roh, S.; Matsushita, S.; Hara, S.; et al. Role of GABRA2 in moderating subjective responses to alcohol. *Alcoholism: Clinical and Experimental Research,* 35(3):400–407, 2011. PMID: 21118274

Rommelspacher, H.; Smolka, M.; Schmidt, L.G.; et al. Genetic analysis of the mu-opioid receptor in alcohol-dependent individuals. *Alcohol* 24(2):129– 135, 2001. PMID: 11522434

Sakai, J.T.; Stallings, M.C.; Crowley, T.J.; et al. Test of association between GABRA2 (SNP rs279871) and adolescent conduct/alcohol use disorders utilizing a sample of clinic referred youth with serious substance and conduct problems, controls and available first degree relatives. *Drug and Alcohol Dependence* 106(2–3):199–203, 2010. PMID: 19783384

Soyka, M.; Preuss, U.W.; Hesselbrock, V.; et al. GABA-A2 receptor subunit gene (GABRA2) polymorphisms and risk for alcohol dependence. *Journal of Psychiatric Research* 42(3):184–191, 2008. PMID: 17207817

Tauber, M.; Calame-Droz, E.; Prut, L.; et al. alpha2-gamma-Aminobutyric acid (GABA)A receptors are the molecular substrates mediating precipitation of narcosis but not of sedation by the combined use of diazepam and alcohol in vivo. *European Journal of Neuroscience* 18(9):2599–2604, 2003. PMID: 14622161

Vengeliene, V.; Bilbao, A.; Molander, A.; and Spanagel, R. Neuropharmacology of alcohol addiction. *British Journal of Pharmacology* 154(2):299–315, 2008. PMID: 18311194

Villafuerte, S.; Heitzeg, M.M.; Foley, S.; et al. Impulsiveness and insula activation during reward anticipation are associated with genetic variants in GABRA2 in a family sample enriched for alcoholism. *Molecular Psychiatry,* Epub ahead of print, 2011. PMID: 21483437

Wang, J.C.; Hinrichs, A.L.; Stock, H.; et al. Evidence of common and specific genetic effects: Association of the muscarinic acetylcholine receptor M2 (CHRM2) gene with alcohol dependence and major depressive syndrome. *Human Molecular Genetics* 13(17):1903–1911, 2004. PMID: 15229186

Werner, D.F.; Swihart, A.; Rau, V.; et al. Inhaled anesthetic responses of recombinant receptors and knockin mice harboring alpha2 (S270H/L277A) GABA(A) receptor subunits that are resistant to isoflurane. *Journal of Pharmacology and Experimental Therapeutics* 336(1):134–144, 2011. PMID: 20807777

Xuei, X.; Dick, D.; Flury-Wetherill, L.; et al. Association of the kappa-opioid system with alcohol dependence. *Molecular Psychiatry* 11(11):1016–1024, 2006. PMID: 16924269

Xuei, X.; Flury-Wetherill, L.; Dick, D.; et al. GABRR1 and GABRR2, encoding the GABA-A receptor subunits rho1 and rho2, are associated with alcohol dependence. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics* 153B(2):418–427, 2010. PMID: 19536785

Zhang, H.; Kranzler, H.R.; Yang, B.Z.; et al. The OPRD1 and OPRK1 loci in alcohol or drug dependence: OPRD1 variation modulates substance dependence risk. *Molecular Psychiatry* 13(5):531–543, 2008. PMID: 17622222

Zhang, H.; Luo, X.; Kranzler, H.R.; et al. Association between two mu-opioid receptor gene (OPRM1) haplotype blocks and drug or alcohol dependence. *Human Molecular Genetics* 15(6):807–819, 2006. PMID: 16476706

Genes Encoding Enzymes Involved in Ethanol Metabolism

Thomas D. Hurley, Ph.D., and Howard J. Edenberg, Ph.D.

The effects of beverage alcohol (ethanol) on the body are determined largely by the rate at which it and its main breakdown product, acetaldehyde, are metabolized after consumption. The main metabolic pathway for ethanol involves the enzymes alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). Seven different ADHs and three different ALDHs that metabolize ethanol have been identified. The genes encoding these enzymes exist in different variants (i.e., alleles), many of which differ by a single DNA building block (i.e., single nucleotide polymorphisms [SNPs]). Some of these SNPs result in enzymes with altered kinetic properties. For example, certain ADH1B and ADH1C variants that are commonly found in East Asian populations lead to more rapid ethanol breakdown and acetaldehyde accumulation in the body. Because acetaldehyde has harmful effects on the body, people carrying these alleles are less likely to drink and have a lower risk of alcohol dependence. Likewise, an ALDH2 variant with reduced activity results in acetaldehyde buildup and also has a protective effect against alcoholism. In addition to affecting drinking behaviors and risk for alcoholism, ADH and ALDH alleles impact the risk for esophageal cancer. KEY WORDS: Alcohol consumption; alcohol dependence; alcoholism; ethanol metabolism; genetic factors; protective factors; risk factors; DNA; genetics; genetic variance; enzymes; acetaldehyde; alcohol dehydrogenase (ADH); aldehyde dehydrogenase (ALDH); single nucleotide polymorphisms (SNPs); esophageal cancer

The duration and extent of the body's exposure to beverage alcohol (i.e., ethanol) is the primary determinant of ethanol's pleiotropic effects on human health (Edenberg 2007). The time course of its concentration and the concentration of its byproducts in the tissues and the circulation, and, consequently, its effects, are determined mainly by the rate of ethanol's processing (i.e., metabolism) in the body. Ethanol can be metabolized in several reactions, but this review focuses on the primary pathway through which it is eliminated from the systemic circulation. In humans, this primary pathway of ethanol metabolism involves oxidation to acetaldehyde by the enzyme alcohol dehydrogenase (ADH). The acetaldehyde then is further oxidized by the enzyme aldehyde dehydrogenase (ALDH) to acetate, which is either excreted in the urine or reincorporated into intermediary metabolism as acetyl-CoA. The hydrogen atoms that are released during these reactions are used to generate a compound called reduced nicotinamide dinucleotide (NADH), with two NADH molecules produced per molecule of acetate generated. The resulting NADH and acetate are thought to provide both the excess reducing equivalents and excess acetyl-CoA that are needed as starting material for fatty acid synthesis, which results in the development of fatty liver disease if high amounts of alcohol are ingested over time.

Both ADH and ALDH exist in different variants with different levels of activity, therefore resulting in different rates of ethanol metabolism. This article discusses how these differences influence a person's sensitivity to ethanol's effects and his or her risk of alcohol dependence.

ADH Variants

Humans have seven ADHs that can carry out the first step in alcohol metabolism. The genes encoding these enzymes all are localized on chromosome 4 in a head-to-tail array about 370 kb long. The enzymes produced from these genes all differ slightly in their activities (see table 1):

- The *ADH1A*, *ADH1B*, and *ADH1C* genes[1] produce closely related proteins that function as homo- and heterodimers (Hurley et al. 2002); their kinetic properties, tissue localization, and developmental expression all support major roles in oxidative ethanol metabolism in the liver.
- The *ADH4* gene is expressed almost exclusively in the liver (Hurley et al. 2002), where it contributes significantly to ethanol oxidation at higher levels of consumption.
- The product of the ubiquitously expressed *ADH5* gene is the glutathione-dependent formaldehyde dehydrogenase (also known as nitrosoglutathione reductase [GSNOR]). The physiological substrates for ADH5 (α-ADH) are compounds (i.e., adducts) formed during the reaction between glutathione and formaldehyde and between glutathione and nitric oxide (Que et al. 2005; Sanghani et al.

[1] By convention, the names of genes are written in italics, whereas the names of the corresponding proteins are written in normal font.

Thomas D. Hurley, Ph.D., *is a Chancellor's Professor in the Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana.*

Howard J. Edenberg, Ph.D., *is a Distinguished Professor and Chancellor's Professor in the Department of Biochemistry and Molecular Biology and the Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, Indiana.*

2000). The main functions of this enzyme are to oxidize formaldehyde to formic acid and to terminate nitric oxide signaling. The human ADH5 enzyme is nonsaturable with ethanol as a substrate, unless medium-chain fatty acids are present in the assay (Engeland et al. 1993), and was originally thought to contribute little to ethanol oxidation. However, its relatively high maximal velocity, coupled with its ubiquitous expression pattern and the high concentrations of ethanol found in gastric tissues, has led some researchers to suggest that it plays a significant role in first-pass metabolism (Lee et al. 2003).

- Although the *ADH6* gene has been identified, there are as yet no physiological data on the functions of the ADH6 enzyme.

- The *ADH7* gene has a limited expression pattern and mainly is found in endothelial cells, such as those lining the esophageal and stomach tissues, as well as during embryonic development when it may contribute to the metabolism of retinol, a form of vitamin A (Hurley et al. 2002). In adults, ADH7 has been implicated in the first-pass metabolism of ethanol taking place in the gastroesophageal tissues, before the ethanol is delivered to the liver via the portal vein (Hurley et al. 2002).

The *ADH* gene cluster contains many single-nucleotide polymorphisms (SNPs)—that is, sites in which the DNA sequence differs by a single building block (i.e., nucleotide) from the reference sequence. Some of these variations result in an altered amino acid sequence of the encoded enzyme and therefore are considered functional or coding SNPs (cSNPs). Detailed functional studies are lacking for all these cSNPs except those that give rise to the *ADH1B* and *ADH1C* gene variants (i.e., alleles).

The ADH1B Alleles

The three most studied alleles of *ADH1B* usually are referred to as *ADH1B*1* (the reference allele, which encodes the β_1 form of the enzyme and carries the amino acid arginine [Arg] at positions 48 and 370 in the amino acid chain), *ADH1B*2* (encoding β_2 and carrying histidine [His] at position 48: ADH1B-His48Arg370; rs1229984), and *ADH1B*3* (encoding β_3 and carrying cysteine [Cys] at position 370: ADH1B-Arg48Cys370; rs2066702). The encoded enzyme variants differ significantly in the ethanol concentrations they require for maximal function and in how fast they metabolize the ethanol (i.e., in their kinetic properties), with both *ADH1B*2* and *ADH1B*3* encoding enzymes with faster turnover (i.e., higher V_{max}) than the reference allele (see table 1). One study of Japanese alcoholics who checked into a hospital 1 day after heavy drinking showed that those who carried two copies of (i.e., were homozygous for) the *ADH1B*1* allele still had significant blood ethanol concentrations (BECs), whereas those who carried at least one *ADH1B*2* allele had very low BECs, consistent with a more rapid ethanol metabolism by *ADH1B*2* (Yokoyama et al. 2007). The current model posits that more rapid oxidation of ethanol at least transiently elevates acetaldehyde levels in one or more tissues. Because acetaldehyde has toxic or at least unpleasant effects on the body, leading to a flushing response after alcohol consumption, this acetaldehyde accumulation is thought to produce aversion that tends to limit heavy alcohol consumption by people who carry at least one *ADH1B*2* allele.

The *ADH1B*2* allele is very common in East Asian population, where it is the major allele with a frequency of 75 percent among Japanese and Chinese individuals (Eng et al. 2007; Li et al. 2007). It also is relatively common in the Middle East (frequency 20 percent) but is uncommon elsewhere in Europe or Africa. In general, its allele frequency is less than 4 percent in populations of European descent, and it was not found in any of the 90 individuals of European descent that were studied in a large genotyping project (i.e., HapMap). Likewise, it is absent from most African populations. As a result, the allele is not included in most of the genotyping arrays used in genome-wide association studies (GWASs).

In the Asian populations where *ADH1B*2* is common, there is very strong evidence that it is protective against alcohol dependence (Chen et al. 1999; Edenberg 2007; Li et al. 2007; Thomasson et al. 1991; Whitfield 2002). An analysis among Han Chinese in Taiwan showed that the relative risk of alcohol dependence was reduced to 0.2 if a person carried a single *ADH1B*2* allele and to 0.12 for homozygotes (Chen et al. 1999). A more recent meta-analysis similarly showed a very strong protective effect for the *ADH1B*2* allele in Asians (odds ratio ~ 0.44; $P < 10^{-36}$) (Li et al. 2007). It has been more difficult to detect the effect of *ADH1B*2* in Europeans; the meta-analysis by Li and colleagues (2007) showed an odds ratio of 0.65 and $P = 0.04$ in alcohol-depen-

Table 1 Kinetic Constants for Ethanol Oxidation by Human Alcohol Dehydrogenases[1]

Gene (Enzyme)	K_M (mM)	V_{max} (min^{-1})	% liver contribution[2] at 22 mM ethanol[3]
ADH1A ($\alpha\alpha$)	4	20	8.1
ADH1B*1 (ADH1B-Arg48Arg370; $\beta1\beta1$)	0.05	4	21.8
ADH1B*2 (ADH1B-His48Arg370; $\beta2\beta2$)	0.9	350	-2
ADH1B*3 (ADH1B-Arg48Cys370; $\beta3\beta3$)	40	300	-2
ADH1C*1 (ADH1C-Arg272, Ile350; $\gamma1\gamma1$)	1	90	41.5
ADH1C*2 (ADH1C-Gln272, Val350; $\gamma2\gamma2$)	0.6	40	-2
ADH4 ($\pi\pi$)	30	20	28.6
ADH5 ($\chi\chi$)	>1,000	100	<1
ADH7 ($\sigma\sigma$)	30	1800	<1

[1] Data from Hurley, Edenberg and Li, 2002.
[2] Calculated for an individual homozygous for both ADH1B*1 and ADH1C*1; expression data for polymorphic ADH variants are uncertain.
[3] 22.7 mM corresponds to a blood alcohol concentration of 100 mg/dL

dent people without secondary disease. A study in European Americans found that each *ADH1B*2* allele lowered the number of symptoms of alcoholism as specified in the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* (DSM–IV) and also reduced the maximum number of drinks consumed in one sitting (Sherva et al. 2009). Another analysis across three large, well-characterized samples of European Americans demonstrated a strong effect of *ADH1B*2*, close to what is seen in Asian populations (odds ratio <0.4; $P < 10^{-8}$) (Bierut et al. 2012). The *ADH1B*2* allele also was associated with hypersensitivity to alcohol in a Scandinavian population (Linneberg et al. 2010), as well as with significantly less drinking before and during pregnancy in European women (Zuccolo et al. 2009). Finally, both maternal and fetal *ADH1B*2* reduced the risk for fetal alcohol spectrum disorders in a mixed population from South Africa (Warren and Li 2005).

The *ADH1B*3* allele is relatively common in Eastern African populations, with frequencies of 27 percent among Yoruba, 14 percent among Luhya, and 24 percent among people of African ancestry in the Southwestern United States but only 4 percent among Maasai in Kenya. In contrast, the allele is rare elsewhere and is not found in the European or Asian HapMap samples. Fewer studies of alcohol dependence have been conducted among people of African ancestry, but *ADH1B*3* has been shown to be protective against alcohol dependence (Edenberg 2007; Edenberg et al. 2010). Moreover, the presence of *ADH1B*3* in pregnant women leads to less drinking at conception and fewer adverse effects in the children born to these women (Jacobson et al. 2006).

The ADH1C Alleles

ADH1C also has cSNPs, of which *ADH1C*1* and *ADH1C*2* are the most studied. These two alleles differ at two sites, resulting in two amino acid changes: the enzyme encoded by *ADH1C*1* (γ1-ADH) has Arg at position 272 and isoleucine (Ile) at position 350, whereas that encoded by *ADH1C*2* (γ2-ADH) has glutamine (Gln) at position 272 and valine (Val) at position 350 (Osier et al. 2002).[2] The kinetic differences between γ1-ADH and γ2-ADH are smaller than those between the ADH1B isozymes (table 1). Studies in Asian populations have shown an association between *ADH1C* alleles and alcoholism, but the protective effect of *ADH1C*1* in that population may be explained in large part by its coinheritance with the *ADH1B*2* allele. A SNP in the *ADH1C* gene that is always inherited together with (i.e., is in complete linkage disequilibrium) with one of the amino acid changes at this locus was associated with alcoholism in two GWASs candidate gene substudies on people of European descent, but the difference did not reach statistical significance for genome-wide analyses (Kendler et al. 2011; Treutlein et al. 2009). The *ADH1C*1* allele has been associated with increased risk for alcohol-related cancer, particularly in people who consume alcohol (Seitz and Meier 2007).

Many other variations in and around the seven *ADH* genes have been associated with risk for alcohol dependence or alcohol-related traits. Among these, variations in and near *ADH4* are among the most widely replicated associations with alcohol dependence, in several populations. Moreover, noncoding SNPs in the region of *ADH1A*, *ADH1B*, and *ADH1C* have been associated with alcoholism and drinking phenotypes, as have SNPs in *ADH7* and *ADH5*. Some noncoding SNPs have been shown to affect gene expression in cultured cells and tissues (i.e., in vitro) (Chen et al 2005; Pochareddy and Edenberg 2010, 2011), and it is likely that many different variations in this region also affect the level of expression of the different ADH enzymes in the intact organism (i.e., in vivo), thereby influencing ethanol metabolism (in some cases possibly only in specific tissues), its physiological effects, and, ultimately, drinking behavior and risk for alcoholism. Without molecular studies, however, detailed analyses of which SNPs might be functional are difficult because many of the *ADH* variations are inherited together with nearby SNPs as haplotypes.

ALDH Variants

The acetaldehyde produced by the action of one or more ADH enzymes must be oxidized efficiently by one or more ALDH enzymes in order for the cell/tissue to maintain nontoxic levels of this reactive molecule. Even transient elevation of acetaldehyde can provoke aversive reactions in people whose ALDH activity is reduced either genetically or pharmacologically. Unlike the human *ADH* genes, the *ALDH* genes are not localized to a single chromosome. Humans have 18 genes encoding for members of the ALDH enzyme superfamily (Jackson et al. 2011). Three of these—*ALDH1A1*, *ALDH1B1*, and *ALDH2*—are most relevant to acetaldehyde oxidation (table 2). The three ALDH enzymes encoded by these genes share more than 68 percent amino acid sequence identity; all three enzymes function in the cell as homotetramers. The ALDH1A1 enzyme is found in the cytosol, whereas both ALDH1B1 and ALDH2 are produced in the nucleus but have leader sequences that direct them to cell components called mitochondria, where they exert their functions in the mitochondrial interior (i.e., the matrix) (Jackson et al. 2011). Of the three isoenzymes, ALDH2 seems to carry out most of the oxidation of ethanol-derived acetaldehyde, as demonstrated by the effects of its inhibition by activated forms of the medication disulfiram (Antabuse®) and by the effects of a functional polymorphism commonly found in East Asian populations (*ALDH2*2*), in which a critical glutamate is substituted by a lysine residue at position 504 of the precursor protein (487 of the mature protein) (ALDH2-Lys504; rs671) (Hurley et al. 2002). With both disulfiram and the ALDH2*2 enzyme, ALDH2 activity is severely compromised, resulting in increased levels of

[2] Another allele, *ADH1C*3*, which carries threonine instead of proline at position 352 on the background of *ADH1C*2* has been reported in Native Americans (Osier et al. 2002).

acetaldehyde, which enters the systemic circulation and initiates the commonly observed facial flushing syndrome. In vitro kinetic analyses also are consistent with the key role of ALDH2, demonstrating that the ALDH2 isoenzyme has the highest catalytic efficiency for acetaldehyde oxidation (Hurley et al. 2002). The *ALDH2*2* allele is relatively common in East Asia (frequencies of 12 to 41 percent [Li et al. 2009]), where it has a very strong effect on risk for alcoholism. Thus, people who carry one copy of the inactive allele are strongly protected against alcoholism (odds ratio from 0.5 to 0.12 [Chen et al. 1999; Thomasson et al. 1991]), and homozygotes are almost completely protected.

It is likely that the ALDH1A1 and ALDH1B1 enzymes significantly contribute to acetaldehyde metabolism only in situations where ALDH2 is inactivated, either pharmacologically or because of the presence of the *ALDH2*2* allele. The K_M values[3] of the ALDH1A1 and ALDH1B1 enzymes exceed those of the ALDH2 enzyme by at least 100-fold, and, thus, are not likely to be operating at full capacity when acetaldehyde levels are kept at the usual physiological state (i.e., below 5 µmol/L) (Klyosov et al. 1996; Stagos et al. 2010). Numerous polymorphisms have been identified for the *ALDH1A1* gene, and linkage to alcohol-related phenotypes has been found in both European populations (rs8187974 [Lind et al. 2008; Sherva et al. 2009]) and people of Indo-Trinidadian background (*ALDH1A1*2,* rs67952887 [Moore et al. 2007]). In addition, a functional polymorphism (i.e., rs2228093) in the *ALDH1B1* gene found in northern European populations seems to correlate with alcohol-aversive reactions (Husemoen et al. 2008; Linneberg et al. 2010), suggesting that the ALDH1A1 and ALDH1B1 isoenzymes contribute to ethanol metabolism under typical ethanol loads even in populations where the *ALDH2*2* allele virtually is nonexistent.

Cytochrome P450 Isoenzymes

Although the ADH/ALDH system is the primary pathway of ethanol metabolism in the body, another system called the microsomal ethanol oxidizing system (MEOS) also contributes to ethanol metabolism, particularly in alcoholics in whom chronic ethanol exposure induces higher expression levels of the enzymes involved. The primary component of the MEOS is cytochrome P450 2E1 (CYP2E1). Increased production of acetaldehyde through this pathway is associated with increased risk for liver damage (Lu and Cederbaum 2008), presumably because of the propensity of the P450 enzymes to produce reactive oxygen species as a byproduct of the catalytic activation of molecular oxygen. Until recently, most studies did not find a significant correlation between CYP2E1 polymorphisms and alcohol elimination rates or alcohol-induced liver injury. Recent work in India, however, has found a significant association between the *CYP2E1*B5* allele (rs2031920) and alcoholic liver cirrhosis (Khan et al. 2009, 2010). These studies raise the possibility that additional associations exist between *CYP2E1* polymorphisms and alcohol-induced liver disease, warranting more detailed study in other populations.

Table 2 Kinetic Constants for Acetaldehyde Oxidation by Human Aldehyde Dehydrogenases

Enzyme	K_M (µM)	V_{max} (min^{-1})	V_{max} ($min^{-1}µM^{-1}$)
ALDH1A1	180	380	2.1
ALDH1B1	55	40	0.7
ALDH2*1	0.2	280	1400
ALDH2*2	1.4	20	14

[1]Data for ALDH1A1 and ALDH2*1 from Klyosov, 1996; data for ALDH2*2 oxidation of propionaldehyde from Farrés et al., 1994 and data for ALDH1B1 from Stagos et al., 2010.

Alcohol Metabolism and Cancer

In addition to affecting drinking behaviors and risk for alcoholism, *ADH* and *ALDH* alleles affect the risk for esophageal cancer. In a meta-analysis of studies (primarily of East Asian populations), the presence of *ADH1B*1* was associated with a higher risk for esophageal cancer even in men who drank little or rarely and had a greater effect in heavier drinkers (Yang et al. 2010). Moreover, although the presence of *ALDH2*2* alleles reduces drinking, the risk for esophageal cancer is elevated among those who drink despite carrying these alleles. Finally, a large study of European subjects showed that *ADH1B*2* and an allele in *ADH7* (the minor allele of rs1573496) independently were protective against upper aerodigestive cancers, particularly among heavier drinkers (Hashibe et al. 2008).

Conclusions

The onset of the genomics era has initiated a rapid increase in researchers' ability to find and analyze polymorphisms within the enzymes responsible for ethanol metabolism. In fact, the rate of discovery of polymorphisms in and near these genes far outpaces the ability to functionally characterize them. Future studies of the expression and kinetic properties of the variant enzymes are important. In particular, methodologies for rapidly and accurately determining protein expression levels of specific forms are needed. For example, a commonly used approach to identify individual problems—that is, the use of specific antibodies—has not yet worked with the ADH1A, ADH1B, and ADH1C proteins because these proteins are highly conserved, with their sequence at the protein level more than 93 percent identical; moreover,

[3] K_M is the concentration of an enzyme's substrate—in this case ethanol—at which the rate of the enzyme reaction is half the maximum rate. The lower the K_M, the more efficient the enzyme is toward that substrate.

many of the sequence changes are located within functional sites and not optimally situated for antibody recognition. Knowledge of expression level changes, however, will be critical to develop models for predicting the metabolic consequences of both the currently characterized functional polymorphisms and those that are yet to be discovered. ■

Acknowledgments

Related work in the authors' laboratories is supported by National Institute on Alcohol Abuse and Alcoholism grants AA–018123, AA–019746, AA–006460, AA–008401, AA–016660, AA–017941, AA–007611, and AA–020892.

Financial Disclosure

The authors declare that they have no competing financial interests.

References

Bierut, L.J.; Goate, A.M.; Breslau, N.; et al. ADH1B is associated with alcohol dependence and alcohol consumption in populations of European and African ancestry. *Molecular Psychiatry,* 17(4):445–450, 2012. PMID: 21968928

Chen, H.-J.; Tian, H.; and Edenberg, H.J. Natural haplotypes in the regulatory sequences affect human alcohol dehydrogenase 1C (*ADH1C*) gene expression. *Human Mutation* 25(2):150–155, 2005. PMID: 15643610

Chen, C-C.; Lu, R-B.; Chen, Y-C.; et al. Interaction between the functional polymorphisms of the alcohol-metabolism genes in protection against alcoholism. *American Journal of Human Genetics* 65(3):795–807, 1999. PMID: 10441588

Ding, J.H.; Li, S.P.; Cao, H.X.; et al. Alcohol dehydrogenase-2 and aldehyde dehydrogenase-2 genotypes, alcohol drinking and the risk for esophageal cancer in a Chinese population. *Journal of Human Genetics* 55(2):97–102, 2010. PMID: 20010786

Edenberg, H.J. The genetics of alcohol metabolism: Role of alcohol dehydrogenase and aldehyde dehydrogenase variants. *Alcohol Research & Health* 30(1):5–13, 2007. PMID: 17718394

Edenberg, H.J.; Koller, D.L.; Xuei, X.; et al. Genome-wide association study of alcohol dependence implicates a region on chromosome 11. *Alcoholism: Clinical and Experimental Research* 34(5):840–852, 2010. PMID : 20201924

Eng, M.Y.; Luczak, S.E.; and Wall, T.L. ALDH2, ADH1B, and ADH1C genotypes in Asians: A literature review. *Alcohol Research & Health* 30(1):22–27, 2007. PMID: 17718397

Engeland, K.; Höög, J.O.; Holmquist, B.; et al. Mutation of Arg-115 of human class III alcohol dehydrogenase: A binding site required for formaldehyde dehydrogenase activity and fatty acid activation. *Proceedings of the National Academy of Sciences of the United States of America* 90(6):2491–2494, 1993. PMID: 8460164

Farrés, J.; Wang, X.; Takahashi, K.; et al. Effects of changing glutamate 487 to lysine in rat and human liver mitochondrial aldehyde dehydrogenase: A model to study human (Oriental type) class 2 aldehyde dehydrogenase. *Journal of Biological Chemistry* 269(19):13854–13860, 1994. PMID: 7910607

Hashibe, M.; McKay, J.D.; Curado, M.P.; et al. Multiple ADH genes are associated with upper aerodigestive cancers. *Nature Genetics* 40(6):707–709, 2008. PMID: 18500343

Higuchi, S.; Matsushita, S.; Imazeki, H.; et al. Aldehyde dehydrogenase genotypes in Japanese alcoholics. *Lancet* 343(8899):741–742, 1994. PMID: 7907720

Hurley, T.D.; Edenberg, H.J.; and Li, T.-K. The pharmacogenomics of alcoholism. In: Licinio, J., and Wong, M.-L., Eds. *Pharmacogenomics: The Search for Individualized Therapeutics*. Weinheim, Germany: Wiley-VCH, 2002, pp. 417–441.

Husemoen, L.L., Fenger, M., Friedrich, N.; et al. The association of ADH and ALDH gene variants with alcohol drinking habits and cardiovascular disease risk factors. *Alcoholism: Clinical and Experimental Research* 32(11):1984–1991, 2008. PMID: 18782342

Jackson, B.; Brocker, C.; Thompson, D.C.; et al. Update on the aldehyde dehydrogenase gene (ALDH) superfamily. *Human Genomics* 5(4):283–303, 2011. PMID: 21712190

Jacobson, S.W.; Carr, L.G.; Croxford, J.; et al. Protective effects of the alcohol dehydrogenase-ADH1B allele in children exposed to alcohol during pregnancy. *Journal of Pediatrics* 148(1):30–37, 2006. PMID: 16423594

Kendler, K.S.; Kalsi, G.; Holmans, P.A.; et al. Genomewide association analysis of symptoms of alcohol dependence in the molecular genetics of schizophrenia (MGS2) control sample. *Alcoholism: Clinical and Experimental Research* 35(5):963–975, 2011. PMID: 21314694

Khan, A.J.; Ruwali, M.; Choudhuri, G.; et al. Polymorphism in cytochrome P450 2E1 and interaction with other genetic risk factors and susceptibility to alcoholic liver cirrhosis. *Mutation Research* 664(1–2):55–63, 2009. PMID: 19428381

Khan, A.J.; Husain, Q.; Choudhuri, G.; and Parmar, D. Association of polymorphism in alcohol dehydrogenase and interaction with other genetic risk factors with alcoholic liver cirrhosis. *Drug and Alcohol Dependence* 109(1–3): 190–197, 2010. PMID: 20171022

Klyosov, A.A.; Rashkovetsky, L.G.; Tahir, M.K.; and Keung, W.M. Possible role of liver cytosolic and mitochondrial aldehyde dehydrogenases in acetaldehyde metabolism. *Biochemistry* 35(14):4445–4456, 1996. PMID: 8605194

Lee, S.L.; Wang, M.F.; Lee, A.I.; and Yin, S.J. The metabolic role of human ADH3 functioning as ethanol dehydrogenase. *FEBS Letters* 544(1–3):143– 147, 2003. PMID: 12782305

Li, D.; Zhao, H.; and Gelernter, J. Strong association of the alcohol dehydrogenase 1B gene (ADH1B) with alcohol dependence and alcohol-induced medical diseases. *Biological Psychiatry* 70(6):504–512, 2011. PMID: 21497796

Li, H.; Borinskaya, S.; Yoshimura, K.; et al. Refined geographic distribution of the oriental ALDH2*504Lys (nee 487Lys) variant. *Annals of Human Genetics* 73(Pt. 3):335–345, 2009. PMID: 19456322

Li, H.; Mukherjee, N.; Soundararajan, U.; et al. Geographically separate increases in the frequency of the derived ADH1B*47His allele in eastern and western Asia. *American Journal of Human Genetics* 81(4):842–846, 2007. PMID: 17847010

Lind, P.A.; Eriksson, C.J.; and Wilhelmsen, K.C. The role of aldehyde dehydrogenase-1 (ALDH1A1) polymorphisms in harmful alcohol consumption in a Finnish population. *Human Genomics* 3(1):24–35, 2008. PMID: 19129088

Linneberg, A.; Gonzalez-Quintela, A.; Vidal, C.; et al. Genetic determinants of both ethanol and acetaldehyde metabolism influence alcohol hypersensitivity and drinking behaviour among Scandinavians. *Clinical and Experimental Allergy* 40(1):123–130, 2010. PMID: 20205700

Lu, Y., and Cederbaum, A.I. CYP2E1 and oxidative liver injury by alcohol. *Free Radical Biology & Medicine* 44(5):723–738, 2008. PMID: 18078827

Macgregor, S.; Lind, P.A.; Bucholz, K.K.; et al. Associations of ADH and ALDH2 gene variation with self report alcohol reactions, consumption and dependence: An integrated analysis. *Human Molecular Genetics* 18(3):580–593, 2009. PMID: 18996923

Maly, I.P.; Toranelli, M.; and Sasse, D. Distribution of alcohol dehydrogenase isoenzymes in the human liver acinus. *Histochemistry and Cell Biology* 111(5):391–397, 1999. PMID: 10403118

McKay, J.D.; Truong, T.; Gaborieau, V.; et al. A genome-wide association study of upper aerodigestive tract cancers conducted within the INHANCE Consortium. *PLoS Genetics* 7(3):e1001333, 2011. PMID: 21437268

Moore, S.; Montane-Jaime, K.; Shafe, S.; et al. Association of ALDH1 promoter polymorphisms with alcohol-related phenotypes in Trinidad and Tobago. *Journal of Studies on Alcohol and Drugs* 68(2):192–196, 2007. PMID: 17286337

Osier, M.V.; Pakstis, A.J.; Goldman, D.; et al. A proline-threonine substitution in codon 351 of ADH1C is common in Native Americans. *Alcoholism: Clinical and Experimental Research* 26(12):1759–1763, 2002. PMID: 12500098

Pochareddy, S., and Edenberg, H.J. Identification of a FOXA-dependent enhancer of human alcohol dehydrogenase 4 (*ADH4*). *Gene* 460(1–2):1–7, 2010. PMID: 20363298

Pochareddy, S, and Edenberg, H.J. Variation in the *ADH1B* proximal promoter affects expression. *Chemico-Biological Interactions* 191(1–3):38–41, 2011. PMID: 21168396

Que, L.G.; Liu, L.; Yan, Y.; et al. Protection from experimental asthma by an endogenous bronchodilator. *Science* 308(5728):1618–1621, 2005. PMID: 15919956

Sanghani, P.; Stone, C.L.; Ray, B.D.; et al. Kinetic mechanism of human glutathione-dependent formaldehyde dehydrogenase. *Biochemistry* 39(35): 10720–10729, 2000. PMID: 10978156

Seitz, H.K., and Meier, P. The role of acetaldehyde in upper digestive tract cancer in alcoholics. *Translational Research* 149(6):293–297, 2007. PMID: 17543846

Sherva, R.; Rice, J.P.; Neuman, R.J.; et al. Associations and interactions between SNPs in the alcohol metabolizing genes and alcoholism phenotypes in European Americans. *Alcoholism: Clinical and Experimental Research* 33(5):848–857, 2009. PMID: 19298322

Stagos, D.; Chen, Y.; Brocker, C.; et al. Aldehyde dehydrogenase 1B1: Molecular cloning and characterization of a novel mitochondrial acetaldehyde-metabolizing enzyme. *Drug Metabolism and Disposition* 38(10):1679– 1687, 2010. PMID: 20616185

Thomasson, H.R.; Edenberg, H.J.; Crabb, D.W.; et al. Alcohol and aldehyde dehydrogenase genotypes and alcoholism in Chinese men. *American Journal of Human Genetics* 48(4):677–681, 1991. PMID: 2014795

Treutlein, J.; Cichon, S.; Ridinger, M.; et al. Genome-wide association study of alcohol dependence. *Archives of General Psychiatry* 66(7):773–784, 2009. PMID: 19581569

Warren, K.R., and Li, T.K. Genetic polymorphisms: Impact on the risk of fetal alcohol spectrum disorders. *Birth Defects Research, Part A: Clinical and Molecular Teratology* 73(4):195–203, 2005. PMID: 15786496

Whitfield, J.B. Alcohol dehydrogenase and alcohol dependence: Variation in genotype-associated risk between populations. *American Journal of Human Genetics* 71(5):1247–1250, 2002. PMID: 12452180

Yang, S.J.; Yokoyama, A.; Yokoyama, T.; et al. Relationship between genetic polymorphisms of ALDH2 and ADH1B and esophageal cancer risk: A meta-analysis. *World Journal of Gastroenterology* 16(33):4210–4220, 2010. PMID: 20806441

Yokoyama, A.; Tsutsumi, E.; Imazeki, H.; et al. Contribution of the alcohol dehydrogenase-1B genotype and oral microorganisms to high salivary acetaldehyde concentrations in Japanese alcoholic men. *International Journal of Cancer* 121(5):1047–1054, 2007. PMID: 17471563

Zhang, G.H.; Mai, R.Q.; and Huang, B. Meta-analysis of ADH1B and ALDH2 polymorphisms and esophageal cancer risk in China. *World Journal of Gastroenterology* 16(47):6020–6025, 2010. PMID: 21157980

Zuccolo, L.; Fitz-Simon, N.; Gray, R.; et al. A non-synonymous variant in ADH1B is strongly associated with prenatal alcohol use in a European sample of pregnant women. *Human Molecular Genetics* 18(22):4457–4466, 2009. PMID: 19687126

Genes Contributing to the Development of Alcoholism

An Overview

Howard J. Edenberg, Ph.D.

Genetic factors (i.e., variations in specific genes) account for a substantial portion of the risk for alcoholism. However, identifying those genes and the specific variations involved is challenging. Researchers have used both case–control and family studies to identify genes related to alcoholism risk. In addition, different strategies such as candidate gene analyses and genome-wide association studies have been used. The strongest effects have been found for specific variants of genes that encode two enzymes involved in alcohol metabolism—alcohol dehydrogenase and aldehyde dehydrogenase. Accumulating evidence indicates that variations in numerous other genes have smaller but measurable effects. **KEY WORDS: Alcoholism; alcohol metabolism; genetic basis of alcoholism; genetics; genetic factors; human studies; case–control studies; family studies; candidate gene analyses; genome-wide association studies; alcohol dehydrogenase; aldehyde dehydirogenase**

A major goal of genetic research into alcoholism and related traits is to better understand the biology underlying this disease by identifying specific genes in which variations contribute to a person's risk of developing the disease and then examining the pathways through which these genes and their variants affect the disease. Researchers hope to use this knowledge to develop new, more effective, and more targeted treatment and prevention strategies. For complex diseases such as alcoholism, however, this is a very difficult endeavour. There is no one gene (or several) whose particular variants "cause" the disease. Instead, variations in many, and perhaps hundreds, of genes likely have a small but measurable influence on disease risk that ultimately adds up to a substantial impact. Moreover, the impact of any one gene variation depends both on the individual's genetic background (i.e., other genetic variations the person carries) and on the environment. These factors further complicate the identification and confirmation of the role of any one gene. This overview briefly summarizes some of the strategies that can be used to identify specific gene variants that influence the risk of alcoholism and reviews some of the findings obtained to date, setting the stage for the following articles in this Special Section.

Strategies for Identifying Genes Associated With Alcoholism Risk

Several study designs—including case–control studies, population studies, and family studies—have been used to test whether a specific gene or gene variant affects risk for a disease (for more information, see the article by Foroud and Phillips, pp. 266–272). There are advantages and disadvantages to each approach. For example, it is much easier to collect individual cases (i.e., people with alcoholism) and control subjects (i.e., nonalcoholic people) or samples of the general population than it is to recruit family samples. Moreover, family studies require more effort to determine the participants' genetic makeup (i.e., genotype), because even with the simplest type of family study, genotypes must be determined for sets of three people (e.g., two parents and an affected child) rather than just for individual case and control subjects. On the other hand, family studies avoid the problem of incomplete ethnic/population matching[1] that can confound case–control studies. Furthermore, family studies can be more powerful than case–control studies if different variants (i.e., alleles) of the same gene affect a given trait in different families, because multiple families can show an effect of that gene despite not sharing the same alleles. In addition, broad regions of the genome generally are inherited within a family, increasing the sensitivity of the approach to detect an effect; however, the tradeoff is that for the same reason, family studies have less resolution to identify the specific allele(s) involved. When both types of studies point to the same genes, however, it provides additional evidence for the involvement of these genes.

There also are two strategies for deciding which genetic variations to test. The first involves focusing the testing on specific genes that are selected on the basis of their physiological roles or their reported involvement in related traits. These so-called candidate gene studies have been fruitful in alcohol research. For example, they led to strong evidence that genes that encode the two main enzymes involved in alcohol metabolism—alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH)—affect risk, which will be discussed in the next section. Some of these studies, particularly the earlier ones, only have assessed a single allele of a candidate gene, whereas in other studies a set of alleles was chosen to provide information on most of the common variations in the gene.

[1] This means that the samples of case and control subjects may not be sufficiently matched with respect to such factors as ethnicity or other population characteristics, which influence the prevalence of many gene variants or other factors that also may influence alcoholism risk.

Howard J. Edenberg, Ph.D., *is a Distinguished Professor and Chancellor's Professor in the Department of Biochemistry and Molecular Biology and the Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, Indiana.*

The other approach is the genome-wide association study (GWASs), which examines a large set of variations in many genes distributed acrosss the entire genome. Each of these variations involves only a single DNA building block (i.e., nucleotide), and they therefore are known as single nucleotide polymorphisms (SNPs). Because such studies may test 1 million SNPs (although not all of these are independent of each other), this can involve 1 million separate tests for association, and therefore an increased chance of false positives if the *P* value is not adjusted. In fact, to guard against false-positive associations between a SNP and a given trait, the current view is that a *P* value smaller than 5×10^{-8} is required. However, some think that this requirement may be too stringent, because many genes and interactions are expected to play a role in a complex disorder such as alcoholism. In practice, the GWASs approach requires very large samples or the aggregation of many studies, and most genome-wide studies on alcoholism do not have sufficient statistical power to detect the small effects expected for individual genes. In contrast, the statistical penalty for multiple testing is reduced greatly with candidate gene studies because these studies test specific hypotheses, and most of the specific genes thus far associated with alcoholism have been identified in candidate gene studies.

Genes Implicated in Alcoholism Risk

Genes Encoding Enzymes Involved in Alcohol Metabolism

The genes most strongly implicated and best characterized are those encoding the key enzymes of alcohol metabolism, ADHs and ALDHs. A variation in the gene encoding mitochondrial ALDH2 (i.e., the *ALDH2*2* allele)[2] renders the resulting enzyme nearly inactive so that the levels of acetaldehyde circulating in the body increase substantially when alcohol is consumed. This acetaldehyde accumulation underlies the strongly aversive flushing reaction (for more information, see the article by Hurley and Edenberg, pp. 339–344). An abundance of physiological and molecular data has demonstrated how this allele affects alcohol metabolism. People carrying a single copy of the *ALDH2*2* allele in their genome are highly protected against alcoholism. Yet this strong effect still can be modified by the environment, as clearly shown by Higuchi and colleagues (1994), who found that the level of protection afforded by *ALDH2*2* in the Japanese population dropped significantly with time as the social pressures for drinking increased. People carrying two copies of the *ALDH2*2* allele, however, become so ill after consuming alcohol that their risk of becoming alcohol dependent is near zero.

[2] By convention, gene names in animals are written in uppercase and lowercase and italicized. Gene names in humans are written in all caps and are italicized, whereas the acronyms for the encoded proteins are all caps but not italicized.

Another well-studied gene variant concerns the gene encoding the ADH1B enzyme. This *ADH1B*2* allele, which encodes an enzyme with higher activity, also is highly protective against alcoholism. Detailed molecular studies of this allele have been carried out, and although physiological studies did not detect the same dramatic rise in circulating acetaldehyde as with the *ALDH2*2* allele, the *ADH1B*2* allele has a similar effect on risk (see the article by Hurley and Edenberg, pp. 339–344).

Gene variants related to alcoholism risk that are present in a population at low frequency are difficult to detect because the number of people who need to be genotyped increases dramatically. For example, the *ALDH2*2* allele that has such a strong effect is essentially absent in many areas of the world and therefore is not detected in studies of most populations. However, the effect of *ALDH2*2* on risk for alcoholism is easy to detect even in relatively small studies of populations in which it is common, for example in China and Japan. Likewise, the *ADH1B*2* allele, which is very common in East Asia and relatively common in the Middle East, is relatively uncommon (i.e., generally has allele frequencies of less than 4 percent) in most other places. Thus, it is easy to detect the effect of *ADH1B*2* even in small studies of Asian populations but much more difficult in other populations (Li et al. 2011). However, a recent study in which the *ADH1B*2* allele was genotyped in several thousand people of European ancestry showed a highly significant protective effect, comparable in magnitude to that in Asians (Bierut et al. 2012). Therefore, the exact population studied, the size of the study, and the exact trait studied all are important to consider when comparing results.

To date, no other gene has been identified that harbors variations with effects on alcohol dependence as strong as those of the *ALDH2*2* or *ADH1B*2* alleles. Other alleles of the *ADH* and *ALDH* genes also have been reported to affect risk; however, these effects are much smaller and are not detected in all studies. For example, several studies found other variations in and near the *ADH1B* gene, as well as in or near the *ADH4*, *ADH1C*, *ADH5*, *ADH6*, and *ADH7* genes that affect risk for alcoholism or the level of alcohol consumption (see the article by Hurley and Edenberg, pp. 339–344). Importantly, many of these other alleles do not affect the structure of the encoded protein but probably act by altering the level of gene expression. Therefore, it is important to also study the effects of various alleles on gene regulation. Another complication is that many *ADH* and *ALDH* genes are located on the chromosomes in clusters, and many nearby variations therefore are inherited together (i.e., in haplotype blocks). As a result, researchers cannot always determine which allele in such a block has the observed effect or whether several alleles might be involved. Studies in populations with different genetic histories can help disentangle the roles of individual alleles, although environmental differences between these populations might complicate the analyses.

Genes Encoding Other Proteins

Other gene variants also have been associated with the risk for alcoholism, including genes encoding many of the subunits of a receptor for the brain signaling molecule (i.e., neurotransmitter) γ-aminobutyric acid (GABA). This $GABA_A$ receptor consists of five subunits, and studies have found that certain alleles of several subunit genes can influence the risk for alcoholism and other addictions, as well as of conduct disorder symptomology and can modify electrophysiological traits related to these disorders. For example, many (although not all) studies have implicated alleles of the *GABRA2* gene, which encodes the α2 subunit of the $GABA_A$ receptor, in alcoholism risk (for more information, see the article by Borghese and Harris, pp. 345–353). Other $GABA_A$ receptor subunit genes also have been implicated, including *GABRG1*, *GABRA1*, *GABRG3*, *GABRR1*, *GABRR2*, and *GABRB3*. Both physiological and molecular evidence indicates that $GABA_A$ receptors are affected by alcohol and participate in many processes relevant to addiction, and studies in rodents have provided further evidence of this involvement. Overall, however, the effects even of the best studied of these genes, *GABRA2*, seem to be small. Although some GWASs have shown nominally significant support, genes encoding $GABA_A$ receptor subunits generally have not been among the top genes identified by GWASs.

Variations in many other genes also have been implicated in contributing to alcoholism risk. Among the genes and pathways highlighted in this brief section are genes involved in the immune system, including nuclear factor-κB–related genes (see the article by Crews, pp. 355–361), the circadian system (see the article by Sarkar, pp. 362–366), and genes whose function is not yet clear (see the article by Buck and colleagues, pp. 367–374). Other genes that also have been identified encode components of the neurotransmitter systems using dopamine, endogenous opioids, serotonin, and acetylcholine; nicotinic receptors; and a hormonal system known as the hypothalamic–pituitary axis. This list continues to grow as more GWASs are completed and analyzed.

Conclusions and Outlook

Although studies in recent years have identified a plethora of genes that may play a role in determining risk of alcoholism, much work remains to be done. For example, many genes have been reported in only one study. Therefore, it will be critical to confirm these associations in additional studies. A failure to replicate the initial findings may not always disprove the association but may result from differences in the genetic background of the study participants, the environment, or the study design (e.g., differences in the definition of alcohol dependence). Beyond replication, the exploration of which specific aspects of the alcoholism phenotype each involved gene affects and which other diseases or traits may be influenced by it is essential. Moreover, it will be equally important to determine the potential underlying mechanisms through functional studies, including the use of animal models, particularly those in which candidate genes or alleles are introduced into the organism (i.e., knocked-in). Although much work remains to be done, researchers already have made substantial progress. New technological developments that allow for faster and more complete genotyping and sequencing will accelerate progress, as will technical developments allowing targeted overproduction or inactivation of genes in animal models. ■

Acknowledgments

Related work in the author's laboratory is supported by National Institute on Alcohol Abuse and Alcoholism grants AA–006460, AA–008401, AA–016660, AA–017941, AA–007611, and AA–020892.

Financial Disclosure

The author declares that he has no competing financial interests.

References

Bierut, L.J.; Goate, A.M.; Breslau, N.; et al. ADH1B is associated with alcohol dependence and alcohol consumption in populations of European and African ancestry. *Molecular Psychiatry,* 17(4):445–450, 2012. PMID: 21968928.

Higuchi, S.; Matsushita, S.; Imazeki, H.; et al. Aldehyde dehydrogenase genotypes in Japanese alcoholics. *Lancet* 343(8899):741–742, 1994. PMID: 7907720

Li, D.; Zhao, H.; and Gelernter, J. Strong association of the alcohol dehydrogenase 1B gene (ADH1B) with alcohol dependence and alcohol-induced medical diseases. *Biological Psychiatry* 70(6):504–512, 2011. PMID: 21497796

Bridging Animal and Human Models

Translating From (and to) Animal Genetics

Amanda M. Barkley-Levenson and John C. Crabbe, Ph.D.

Amanda M. Barkley-Levenson *is a graduate student and* **John C. Crabbe, Ph.D.,** *is a professor, both at the Portland Alcohol Research Center, Department of Behavioral Neuroscience, Oregon Health and Science University, and VA Medical Center, Portland, Oregon.*

Genetics play an important role in the development and course of alcohol abuse, and understanding genetic contributions to this disorder may lead to improved preventative and therapeutic strategies in the future. Studies both in humans and in animal models are necessary to fully understand the neurobiology of alcoholism from the molecular to the cognitive level. By dissecting the complex facets of alcoholism into discrete, well-defined phenotypes that are measurable in both human populations and animal models of the disease, researchers will be better able to translate findings across species and integrate the knowledge obtained from various disciplines. Some of the key areas of alcoholism research where consilience between human and animal studies is possible are alcohol withdrawal severity, sensitivity to rewards, impulsivity, and dysregulated alcohol consumption. KEY WORDS: Alcoholism; alcohol dependence; alcohol use disorders (AUDs); alcohol research; genetic basis of alcoholism; genetics; genetic factors; phenotypes; human studies; animal models; consilience; alcohol withdrawal; alcohol sensitivity; impulsivity; dysregulated alcohol consumption

Alcoholism is a complex disorder arising from a combination of genetic and environmental factors. The *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* (DSM–IV) (American Psychiatric Association 1994) requires that three of seven criteria be present during a 12-month period for a diagnosis of alcohol dependence. These criteria are tolerance, withdrawal symptoms, loss of control of drinking, desire to quit, preoccupation with drinking, curtailing of other activities because of drinking, and persistence of drinking in the face of negative consequences. The use of animal models, such as rodents, nonhuman primates, and even invertebrates, allows for a degree of genetic and environmental control that would not be possible in human studies. By using these species to recapitulate discrete aspects of alcohol use disorders (AUDs) as they appear in human populations, researchers are able to target the specific biological underpinnings of the disease.

Achieving consilience between animal models and human disease is one important goal of translational research. Several years ago, a group of researchers staged a multidisciplinary meeting with the goal of identifying specific areas of alcoholism research with good potential for translation between human and animal studies (Crabbe 2010). This effort, known as the consilience project, sought to highlight both better animal models for these areas, as well as better-defined and more specific human phenotypes to target. The group focused on genetic studies because of the obvious direct translation possible across the genomes of species. Currently, animal models clearly are able to address the diagnostic criteria of tolerance and withdrawal but are less obviously capable of capturing complex emotional constructs, such as desire and preoccupation. However, behaviors such as excessive alcohol intake undoubtedly are related to AUDs, despite the fact that they do not directly lead to a diagnosis. By using various animal species to model these other behaviors and risk factors, it is possible to begin to dissect the complexities of alcoholism. After several meetings, members of the consilience project identified seven major areas for focusing translational attention (for the complete report of the consilience group, please see *Addiction Biology*, 2010, vol. 15, issue 2, entire issue). This article focuses on five of these areas, which encompass specific behavioral domains related to alcohol abuse: withdrawal, reward sensitivity, impulsivity, dysregulated alcohol consumption, and low level of response to alcohol. This article will discuss major findings from both the human and animal literature, as well as some strategies for achieving even better consilience across species in the future.

Genetic Animal Models

Before examining the consilience of animal models and human research, it is important to briefly mention the behavioral genetic strategies used in these types of studies. Although numerous animal species are used in alcohol research, this article will focus primarily on rodent models. However, many of the approaches described here can successfully be applied to other species as well. There are three broad types of genetic methods used in rodent studies of alcohol: testing of inbred strains, selective breeding, and the creation and testing of animals with targeted genetic manipulations.

Inbred mouse and rat strains have been developed over repeated generations through brother–sister matings so that all animals within a strain are assumed to be genetically identical. As a result, these animals provide an excellent means of examining environmental contributions to alcohol-related traits because genetic variation is held constant across subjects. On the other hand, testing animals of multiple strains under standardized environmental conditions can provide evidence for the dependence of a given behavioral phenotype on genetic factors if it is found to differ across strains. Studying differences in brain morphology and neurochemistry between strains with innate differences for alcohol-related traits allows for greater insight into biological factors promoting AUDs. For example, the C57BL/6J (B6) and DBA/2J (D2) inbred mouse strains represent opposite ends of the spectrum with regard to voluntary oral consumption of alcohol, with B6 mice readily drinking large quantities and D2 mice consuming very little (e.g., Lê et al. 1994). Many studies that might explain this difference have been conducted, comparing these strains and a large panel of recombinant inbred strains derived from them for both biological and behavioral factors. Given the presumed complexity of genetic contributions to alcoholism, it is preferable to use a large number of inbred strains in order to include more genetic variation and to provide a greater ability to detect a statistical genetic correlation between traits.

Selective breeding is another method of studying genetic contributions to alcoholism. Beginning from a genetically diverse population, animals are tested for a trait of interest and are bred on the basis of their level of response. In bidirectional selection, two divergent lines are produced by breeding high responders with high responders and low responders with low responders until animals from the two lines differ significantly for the selected measure. Selective breeding is useful both for demonstrating the heritability of a trait as well as for identifying the genetic relatedness of multiple traits that might select together (known as a correlated response to selection). That is, if two lines bred for divergence on a given trait (such as alcohol preference) also differ on another measure, it can be inferred that both the selected and the correlated response share some underlying genetic contribution. For example, the numerous pairs of alcohol preferring/nonpreferring and high-alcohol–drinking/low-alcohol–drinking selected rat lines have been shown to differ on such traits as locomotor stimulation in response to alcohol and an alcohol-conditioned taste aversion, as well as on biological factors such as endogenous neurotransmitter levels (Stewart and Li 1997).

Researchers also study specific genes of interest in animal models (particularly invertebrates and mice) by targeted manipulation of the gene. This can include knockout or knockdown studies, in which the gene is removed or its expression is minimized, respectively. In another technique, transgenic experiments use increased gene expression or the insertion of a particular polymorphism or mutated version of the gene to determine the effect on the phenotype of study. Although this article will not discuss them in depth, human gene-expression and linkage studies can provide a useful method for identifying candidate genes for transgenic and knockout studies in animals (for a recent review of some human and animal gene expression techniques, see Foroud et al. 2010). In brief, gene expression profiles can be determined from samples of a variety of tissue types, including brain and blood. Although brain tissue is advantageous in demonstrating that the gene expression is likely to be behaviorally relevant, the utility of these studies is limited because they need to be conducted postmortem. Peripheral blood samples, on the other hand, are readily obtained and can be measured repeatedly in the same individuals, although the generalization of expression determined from blood samples to the brain has to be inferred. In a recent example of a translational genetic approach, researchers produced transgenic mice that expressed an ethanol-insensitive mutant form of the α2 subunit of a receptor for the neurotransmitter γ-aminobutyric acid ($GABA_A$), the gene for which has been identified as a candidate gene for alcohol dependence through human studies (e.g., Reich et al. 1998). These mice showed less sensitivity to alcohol's aversive and motor-stimulant effects than controls (Blednov et al. 2011), providing a possible behavioral mechanism for the genetic linkage of this subunit with alcohol abuse. Studies with knockout animal models must be interpreted with care, however, because the absence of genes can have profound effects on development and may result in unanticipated compensations by other systems. Increasingly, techniques are available that allow for a greater degree of spatial and temporal control of genetic manipulations (e.g., inducible knockout systems, short-interfering RNA). As technology continues to improve, these methods may provide a way to bypass the limitations of conventional knockout strategies.

Withdrawal

Of the alcohol-related traits discussed in this article, withdrawal is the only one that also is among the DSM–IV criteria for diagnosis of an AUD. In human alcoholics, withdrawal can

include both physiological and mood symptoms, with the majority of physical symptoms occurring during acute withdrawal (48 to 72 hours) (first described by Victor and Adams 1953), and emotional and mood symptoms arising in early abstinence (3 to 6 weeks) and continuing indefinitely (for review, see Heilig et al. 2010). Physical symptoms include excessive autonomic nervous system activity, central nervous system hyperexcitability, and increased seizures and convulsions, whereas mood symptoms generally consist of increased anxiety, dysphoria, and anhedonia. Historically, many theories of addiction have stemmed from a "self-medication" hypothesis, wherein continued substance abuse occurs as an attempt to prevent or relieve the experience of these negative withdrawal symptoms (Markou et al. 1998). Although current theories tend away from offering this as the only explanation, withdrawal still is considered to be a likely contributor to continued alcohol abuse and relapse.

The highly parallel nature and time course of withdrawal symptoms across species make this a key area for assessing consilience. Withdrawal severity also seems to have a genetic component, and it has been shown that alcohol withdrawal is a significant genetic factor in explaining AUD diagnoses in twin pairs (Ystrom et al. 2011). Gene polymorphisms associated with multiple neurotransmitter systems, including the dopaminergic, serotonergic, GABAergic, and opiate systems, have been explored in relation to alcohol withdrawal (Schmidt and Sander 2000). However, findings that implicate a role for certain genetic variants in withdrawal often are not replicated across studies, and there is little that can conclusively be said about the genetics underlying this trait. Difficulty in replicating results across studies likely is a result of factors such as gene-by-environment interactions and the genetic heterogeneity of the subjects and serves to highlight the complexities inherent in conducting behavioral genetic research.

A significant portion of the evidence for a genetic contribution to withdrawal has come from research using animal models. Selective breeding has produced mouse lines showing robust differences in handling-induced convulsion severity after the induction of alcohol dependence via a 72-hour vapor chamber exposure (Kosobud and Crabbe 1986). Inbred strains also exhibit differences in withdrawal severity (Metten and Crabbe 2005), and fine-mapping genetic techniques using a specialized set of B6-D2–derived strains called recombinant inbred strains helped lead to the identification of *Mpdz*[1], a quantitative trait gene for withdrawal seizure severity (Fehr et al. 2002). This represents a significant achievement in relating human and mouse genetics because the human ortholog of this gene (*MPDZ*) has been shown to potentially contribute to alcoholism risk (Milner and Buck 2010). The behavioral significance of the seizure phenotype, however, is less clear cut because human studies thus far have failed to show a specific association between *MPDZ* and withdrawal (Karpyak et al. 2009). Tremors and seizures are observed during acute withdrawal in humans, but these physiological symptoms dissipate in later stages of withdrawal, and it currently is unknown how they may relate to the affective and other changes that occur during continued abstinence.

In recent years, the focus of withdrawal research has shifted somewhat to the mood-related symptoms of later withdrawal, such as anhedonia, dysphoria, and anxiety. Modeling emotional states in rodents proves to be more challenging than modeling seizures or central nervous system excitability. A variety of tasks exist to assess anxiety-like behavior in rodents during withdrawal, mostly based on the idea that an anxious rat or mouse will be more avoidant of situations, such as open or brightly lit areas. However, these tasks are not all influenced by the same constellation of genes in rodents (Milner and Crabbe 2008), and interpretation of data from some tasks is confounded by variations in locomotor activity (Kliethermes 2005). Increased stress reactivity during withdrawal allows researchers to study measurable physiological outcomes across species, which may be related to the negative affective symptoms of withdrawal (for review, see Breese et al. 2011). For example, blocking corticotropin-releasing hormone receptors can attenuate withdrawal-associated anxiety in rats (Gehlert et al. 2007), suggesting that the stress system might be involved in mediating anxiety that develops during abstinence. Chronic stress prior to alcohol dependence also can potentiate the anxiety-like response seen during withdrawal (Wills et al. 2010). Research in humans also has demonstrated enhanced response to negative stimuli during withdrawal (Gilman and Hommer 2008), but again the genetics remain largely unexplored.

Finally, it is important to consider that the pattern of alcohol exposure and withdrawal may be a critical factor for influencing behavioral outcomes. Studies in both humans and rodents have shown that experiencing withdrawal repeatedly can lead to a "kindling" or potentiation of both physiological and psychological withdrawal symptoms (e.g., Becker 1998; Breese et al. 2011). In addition to potentiating withdrawal symptoms, repeated cycles of induced ethanol dependence (via vapor inhalation chambers) and subsequent withdrawal seem to enhance voluntary alcohol consumption in some strains of rats and mice (e.g., Becker and Lopez 2004; Gilpin et al. 2008). This behavior generally is known as dependence- or withdrawal-induced drinking and represents an area of interest for continued consilience efforts because the genetics of this behavior have not been well explored. Some evidence suggests that genetically predisposed high-drinking animals may show greater withdrawal-associated drinking than lower-drinking animals because B6 mice show a robust effect

[1]By convention, gene names in animals are written in uppercase and lowercase and italicized. Gene names in humans are written in all caps and are italicized, whereas the acronyms for the encoded proteins are all caps but not italicized.

and male mice of the high-alcohol–preferring (HAP) line show modest enhancement of drinking relative to their low-alcohol–preferring (LAP) counterpart selected line (Lopez et al. 2011). Consequently, taking into account both previous withdrawal experience and the time course of withdrawal (i.e., acute versus later withdrawal) may be a useful tool for future attempts to relate animal findings to human data.

In summary, it is possible to model specific human withdrawal symptoms in rodents rather directly, but a better understanding of which human withdrawal symptoms reflect a genetic predisposition to AUDs will help guide further success with achieving consilience in this area.

Alcohol and Sensitivity to Rewards

Several of the current models of alcoholism include the idea of dysregulation of reward processes as a factor in the onset and maintenance of the disorder (Stephens et al. 2010). Some models propose a specific deficiency in reward sensitivity, wherein a lowered sensitivity to alcohol's rewarding effects is thought to drive an increase in use in order to achieve the desired hedonic levels (e.g., Bowirrat and Oscar-Berman 2005). Other theories suggest more generally a dysregulation of reward processing and a hijacking of other brain systems (e.g., stress system), especially with repeated alcohol use (Koob and Le Moal 2001). In humans, the rewarding effects of alcohol most commonly are measured with self-reports. Although this is certainly an advantage of studies using human subjects (i.e., because researchers cannot directly ask a mouse how much it likes alcohol), there always is some risk of unreliability. Consequently, using both self-reports and tasks with measurable behavioral outcomes, such as willingness to work to obtain alcohol or preference for alcohol over a placebo, provides a more objective and complete measure of reward. Such assessments can be achieved through laboratory studies of self-administration and through the incorporation of behavioral economic analyses, both of which have well-developed analogues in rodent models. Biological markers of reward also are possible. One recent electrophysiological study showed evidence for altered reward processing in high-drinking (but nonalcoholic) individuals, with high-frequency drinking participants showing a greater reward-associated brain response (i.e., event-related potential) to stimuli that predicted the unexpected absence of a reward in a passive gambling task than did low-frequency drinkers (Franken et al. 2010). When considering questions of sensitivity to reward, however, it should be kept in mind that "reward" actually may represent a multifaceted sensation. That is, someone experiencing euphoria could have the same subjective sense of pleasure as someone experiencing the alleviation of anxiety, but these two outcomes may represent different actions of alcohol at the level of the brain. A single behavior that can represent two different underlying genetic substrates sometimes is called a "phenocopy;" identifying genes associated with specific behaviors can be confusing in both animal and human studies.

Despite the inference of altered reward sensitivity in AUDs, research examining the genetic contributions to this trait is relatively underdeveloped in human subjects. The dopamine neurotransmitter system is heavily implicated in regulating reward and, consequently, has been widely studied in relation to alcohol. Many studies have looked at specific alleles of the dopamine receptors that may alter reward sensitivity and subsequently either lessen or intensify the risk of developing an AUD (for review, see Le Foll et al. 2009). One of the first studies in this area suggested that the presence of one allelic form of the D2 dopamine receptor correctly predicted alcoholic status a majority of the time (Blum et al. 1990). Subsequent results have been mixed, however, with some studies failing to find any association (e.g., Gelernter and Kranzler 1999). Animal studies have provided some evidence for the role of D2 receptors: mice lacking these receptors show lower operant responding for alcohol, decreased preference for drinking alcohol, and a diminished alcohol-conditioned place preference (Cunningham and Phillips 2003). Expression of the gene encoding D2 receptors also has been shown to correlate positively with alcohol-conditioned place preference in B6-D2–derived recombinant inbred strains of mice (Hitzemann et al. 2003). In contrast, overexpression of D2 receptors in certain brain areas has been shown to decrease alcohol consumption relative to wild-type animals (Thanos et al. 2005). This highlights the difficulty of drawing conclusions about reward from behavioral measures such as alcohol intake (see the next paragraph). Consequently, although the dopamine system and its role in reward processing seem to be related to AUDs, the contributions of specific genetic variants warrant additional study.

In general, animal behavioral tasks are better developed than human ones with regard to examining alcohol reward and its possible genetic determinants. Self-administration studies are perhaps the most widely used method in this area. Mice and rats can be trained to respond operantly at a fixed ratio for infusions of ethanol or for access to a drinkable ethanol solution. In this way, it is possible to determine the ability of alcohol to maintain responding at different concentrations/doses or under different response requirements. Operant self-administration studies also can use a progressive ratio, wherein the response requirement for subsequent access to alcohol continually increases until an animal will no longer respond. The response requirement at which responding ceases to be maintained is known as the break point, and shifts in the break point are taken as an indication of differences in the rewarding effects of alcohol. As alluded to earlier, however, unambiguous assessments of the reward value of alcohol are very

difficult to make using operant self-administration or home cage-drinking paradigms. An increase or decrease in responding presumably indicates a change in perceived reward, but it cannot be determined whether that change results from an increase or decrease in reward value. For example, were a given experimental manipulation to halve the operant responding for alcohol, this could indicate either that the animal now finds the alcohol to be half as rewarding as before, or that it finds it twice as rewarding and therefore only needs to administer half as much for the same perceived effect.

One widely used method for assessing reward sensitivity in animals that avoids this particular ambiguity, conditioned place preference (CPP), is based on ideas of Pavlovian conditioning. In brief, two distinct sensory cues (e.g., floor texture) are paired with either an ethanol or vehicle injection over repeated training trials. During the test, both cues are presented and the animal is allowed to choose between the two cued locations without any drug on board. A greater amount of time spent in proximity to the previously drug-paired cue suggests a drug-seeking behavior presumably resulting from rewarding effects. Alcohol-induced CPP has been shown to differ across inbred mouse strains and also between rodent lines selected for other alcohol-related traits. Mice bred for high alcohol consumption also showed greater CPP than their low-drinking counterparts (Phillips et al. 2005), and a similar relationship was found between severity of alcohol withdrawal and CPP (Chester et al. 1998). Meta-analysis of a large number of studies suggests that sensitivity to alcohol-induced CPP seems to be modestly correlated with voluntary drinking (Green and Grahame 2008). However, this relationship is not always observed. A previous study by Grahame and colleagues (2001) failed to show line differences in the ability of alcohol to condition a place preference in the HAP and LAP selected mouse lines at lower doses, whereas LAP mice showed greater preference than HAP mice at a higher dose. Furthermore, it is hard to know exactly how CPP expression relates to measures of reward in humans. Human implicit learning tests, such as the conditioned pattern-preference task, may be analogous to CPP (Johnsrude et al. 1999). This task pairs a neutral visual stimulus (e.g., monochrome pattern) with a food reward across multiple trials, while masking the pairing with a distractor memory task so that the subject is not aware of the conditioning. The subject then is presented with the paired stimulus, along with unpaired and novel stimuli, and asked to identify his or her favorite. As with CPP, a greater preference for the paired stimulus is believed to be indicative of greater sensitivity to the reward. A recent study found that self-reports of hazardous drinking were significantly correlated with stronger food-conditioned pattern preference, suggesting that repeated alcohol use might relate to increased sensitivity to nondrug reward (Balodis et al. 2010). Human conditioning tasks of this nature have not been widely implemented in the addiction field and may prove to be a promising avenue of research for relating human and animal studies of reward.

It should be noted that both CPP and self-administration paradigms also can be used to assess reward more indirectly through reinstatement procedures that aim to model drug-seeking behaviors and relapse in human alcoholics. The drug-free test session in CPP itself can arguably be seen as a measure of drug seeking, but it also is possible to test for reinstatement of place preference following extinction trials (i.e., confinement in the previously drug-paired location without a drug pairing). Place preference then can be reinstated using various manipulations (e.g., drug prime, stress, drug cues). Reinstatement of drug-paired lever pressing in operant models after extinction of the behavior also can be produced using similar methods. One key difference between animal reinstatement models and human relapse, however, is that a relapsing animal will not actually obtain any alcohol because responding on the previously drug-paired lever during reinstatement testing does not result in the delivery of alcohol (nor does the expression of a place preference result in alcohol administration). Nevertheless, drug-seeking and relapse obviously are highly relevant to the clinical treatment of AUDs, and these procedures provide a means to assess experimental manipulations or genetic factors that may prevent relapse-like behaviors following abstinence.

Despite the wide assortment of available tasks, the rewarding effects of alcohol in animal studies still must be inferred from behavioral outcomes, whereas they can be more directly reported by humans. In animals, it is not yet possible to definitively isolate "reward" as a construct separate from various contributing factors, such as subjective experience, reinforcing value, and other more nebulous inputs (Stephens et al. 2010). Nonetheless, the use of multiple approaches with animals should allow investigators to achieve convergent results. A greater focus on achieving homologous tasks between humans and animals may allow for an increased understanding of alcohol's actions as a reinforcer, even if reward sensitivity remains somewhat elusive.

Impulsivity

Much like alcoholism, impulsivity itself is a heterogeneous trait comprising multiple components. This can make relating the two traits even more complicated, but given the evidence for links between impulsivity and AUDs, it is worth the attempt. Impulsivity as a personality trait frequently is assessed via a variety of questionnaires, but tasks exist as well for measuring impulsive behavior (i.e., the "state" of impulsivity). Behavioral tasks seem to roughly dissociate five aspects of impulsive behavior: the inability to inhibit behavioral responses, susceptibility to distractor interference, susceptibility to proactive interference, preference for smaller immediate rewards over larger

rewards after a delay, and deficits in judging elapsed time (Dick et al. 2010). However, self-reported measures of impulsivity do not always correlate well with performance on behavioral tasks (Reynolds et al. 2006). The relationship between impulsivity and alcohol use is thought to be twofold: first, a propensity toward impulsive behaviors (impulsivity as a "trait") might coincide with a propensity toward alcohol abuse; and second, impulsive behaviors can be increased when alcohol is ingested (impulsivity as a "state"). Assessment of impulsive behavior is aided by the relatively good face validity of the tests used in both rodents and humans because many of the behavioral assays are very similar. For example, the Go/No-Go test measures behavioral inhibition and is widely used in mice, rats, and humans. This task consists of distinct cues that signal "go" trials and "no-go" trials, and a behavioral response (e.g., button push, lever press, etc.) must be made in response to the go cues and inhibited in response to the no-go cues on a series of repeated trials. Impulsive responding is characterized by responses on no-go trials. Delay-discounting procedures, which measure aversion to delayed reward, also have both human and rodent variations. These tasks offer the choice between an immediate small reward and a larger reward after a delay. By altering either the relative sizes of the rewards or the time delay to the large reward, it is possible to determine the indifference point at which the delayed and immediate rewards are valued equally. Impulsivity is associated with steeper discounting of delayed rewards (i.e., the perceived value of a delayed reward is smaller).

Despite the strong concordance in tasks across species, the genetic contributions to impulsivity remain elusive. Questionnaire-based longitudinal studies have shown that measures of impulsivity in childhood and adolescence are predictive of the development of problems with alcohol abuse later in life (Nigg et al. 2006), reinforcing the idea of similar underlying genetic risk factors. Twin studies also provide evidence for the genetic relatedness of impulsive behavior and alcohol abuse (Kendler et al. 2003). One issue in human studies that makes it difficult to tease apart the contribution of genetics to impulsivity and alcohol abuse is that many studies are conducted in people with previous drug or alcohol abuse experience. For example, it has been shown that alcohol-dependent individuals discount delayed rewards more steeply than do nondependent comparison subjects (for review, see Bickel et al. 2007), but these results can be difficult to interpret from a genetic standpoint because of the concurrent or past experience with substance use. This issue can be countered somewhat by using nonabusing subjects with a family history of AUDs and assessing them for impulsivity-related traits. One such study (Andrews et al., in press) used functional magnetic resonance imaging and found differences in the activation of brain reward circuitry during a monetary incentive delay task in individuals who had a family history of alcoholism (but who did not have a diagnosis of AUD themselves) relative to control subjects with no family history. Ideally, however, this type of study should be conducted using drug- and alcohol-naïve individuals, which can be very difficult to achieve with adult subjects. Consequently, studies often are conducted in children and adolescents at familial risk for alcoholism. Parental diagnosis with a substance use disorder has been shown to both predict behavioral disinhibition in childhood and to relate to a predisposition toward substance abuse in young adulthood (Tarter et al. 2004). Specific to alcohol abuse, a study of children of alcoholics showed greater impulsivity measures in this group relative to children with no family history of alcoholism (Dawes et al. 1997). However, despite a mean participant age of 11 years, there still were children in this study who had past experience with alcohol, tobacco, and/or other drugs, which serves to highlight how difficult it can be to conduct studies using drug- and alcohol- naïve human subjects.

A significant portion of the evidence for a genetic contribution to withdrawal has come from research using animal models.

An advantage of animal studies in this area, therefore, is the ability to assess impulsivity in alcohol-naïve animals of lines selected for alcohol consumption (for example) while also eliminating the complex environmental contributions that can confound human studies. This strategy has demonstrated differences in impulsivity among a variety of rodent lines differing in their level of alcohol preference. Rats of the alcohol-preferring (P) and high–alcohol-drinking (HAD) selected lines have shown a greater degree of impulsive behavior on delay discounting and behavioral inhibition tasks than their corresponding low-drinking counterparts (Steinmetz et al. 2000; Wilhelm and Mitchell 2008). A recent study in the HAP and LAP selected mouse lines found that the HAP mice showed more impulsive responding on a delay-discounting task than did the LAP mice or the progenitor strain (Oberlin and Grahame 2009). Although few attempts have been made to selectively breed for impulsivity-related phenotypes, inbred strains have been shown to differ in their impulsive behaviors, suggesting a degree of genetic control (e.g., Gubner et al. 2010). Furthermore, some measures of impulsivity have been found to be positively correlated with ethanol consumption in inbred mouse strains (Logue et al. 1998). The biggest research advantage in this area is the existence of very similar tasks across species. As with human studies, however, it may be difficult for animal studies to distinguish those aspects of impulsivity that are predisposing for AUD phenotypes from those that develop concurrently with or as a result of the disorder. As is the case with withdrawal, a continued focus on identifying the specific, well-

defined facets of impulsivity that seem most important and carefully relating these behaviors both across tasks and species will be crucial for future discoveries.

Dysregulated Alcohol Consumption

As mentioned previously, excessive alcohol consumption is not by itself a criterion for an AUD diagnosis. However, it clearly is a related behavior and is widely considered to be a key trait for any animal system purporting to be a model of disordered drinking. The consilience project group concluded that alcohol consumption further can be broken down into the components of the decision to drink or abstain, the quantity consumed, and the presence or absence of binge drinking (i.e., whether the drinking exceeds levels associated with risk of harm) (Leeman et al. 2010). All of these components then can be assessed in humans through either surveys or experimentally. A wealth of clinical and epidemiological studies have examined various aspects of alcohol consumption, generally reported as drinks per a given period of time, and numerous approaches have been used to assess genetic contributions (e.g., twin and linkage studies). For example, maximum alcohol consumption in a day by fathers has been shown to predict substance abuse in their children (Malone et al. 2002), and linkage studies have implicated high 24-hour consumption as being strongly associated with diagnosis of an AUD (Saccone et al. 2000). In addition to self-reported measures, researchers also use experimental techniques to determine alcohol consumption. These studies are helpful in that specific populations of individuals can be tested (e.g., family history positive and family history negative for AUDs), or behavioral or pharmacological manipulations can be made to determine the effect on subsequent alcohol intake. A combination of these approaches was used to show that the drug naltrexone reduces total drinks during a self-administration paradigm in those with a family history of alcoholism but has no effect on those without familial risk (Krishnan-Sarin et al. 2007). One consideration for experimental consumption studies, however, is that they usually are conducted in a laboratory setting, which may not translate directly to "real-world" drinking.

In animal models, strong evidence exists to implicate genetics as an important factor in voluntary alcohol drinking and alcohol preference: Selected mouse and rat lines have been bred for differences in alcohol consumption, and different inbred mouse strains showed marked differences in consumption measures as well. Perhaps the most classic form of drinking study in rodents presents the animal with continuous access to both an alcohol solution and water. Total consumption is measured (usually over the course of 24 hours), as is preference for or aversion to, the alcohol in relation to water. The majority of high- and low-drinking selected rodent lines have been bred for their intake on some variation of this test (for review, see Spanagel 2000). Despite their ubiquity, a common criticism of continuous-access paradigms is that there is little evidence that animals are reaching pharmacologically significant blood alcohol concentrations (BACs), even in high-drinking genotypes (Dole and Gentry 1984). Without proof that the animals actually are drinking to intoxication, it can be difficult to try to translate results back to the human condition, where intoxication is a key element. One way of promoting high BACs is by presenting alcohol only for a limited period, frequently during the animal's circadian dark. An example of this method is the drinking-in-the-dark procedure, which generally is regarded as a model of binge drinking because animals will consume an intoxicating dose in a relatively short time period (Rhodes et al. 2005). Intake during this test has been shown to differ across inbred mouse strains, and selected lines have been bred for high BACs following the drinking period (Crabbe et al. 2009). An important consideration when interpreting drinking results is the role played by taste in mediating intake and preference. One can envision a scenario wherein an apparent genotype-dependent difference in alcohol consumption actually represents disparate sensitivity to the taste of alcohol rather than to its pharmacological effects. For example, it has been shown that although D2 mice consume very little alcohol in drinking paradigms, they will self-administer alcohol both intravenously (Grahame and Cunningham 1997) and intragastrically (Fidler et al. 2010), suggesting that their limited oral intake may be mediated at least in part by preabsorptive properties of alcohol, such as odor and taste.

In addition to modeling high alcohol consumption, researchers also have attempted to model the compulsive element of drinking that is part of the diagnostic criteria for an AUD (for review, see Vengeliene et al. 2009). "Compulsion" is a somewhat human construct that can be difficult to apply to animals. The escalation of drinking during repeated cycles of dependence and withdrawal (see withdrawal section above) may represent a shift from regulated to dysregulated drinking. Continuing to drink alcohol solutions that have been adulterated with an aversive substance such as quinine may be another example. One possible way of approaching this question is through studies using devaluation of alcohol. If an animal is trained to respond for alcohol, and alcohol subsequently is devalued through pairing with an aversive stimulus (e.g., lithium chloride injection), then continued responding for alcohol could be interpreted as being a result of a habitual or "compulsive" mechanism driving the response. A study by Dickinson and colleagues (2002) used this approach to examine potential habitual components to alcohol self-administration in rats. Rats trained to respond operantly for both food pellets and alcohol solution had either the food or the alcohol devalued with lithium chloride injections. Although devaluation decreased

responding during the conditioning sessions selectively for either food or alcohol, depending on which had been paired with the injection, responding for pellets during extinction was reduced in the food-devalued group relative to control and alcohol-devalued groups. In contrast, extinction responding for alcohol was similar in both the alcohol- and food-devalued groups, although both responded at levels below that of noncontingently injected controls. These results may suggest a more rigid ("habitual") pattern of responding for alcohol than food. Studies of this nature are an interesting avenue of research and could prove useful for enhancing the consilience between the human diagnostic criteria for AUDs and animal models of dysregulated drinking.

One barrier to better consilience for studies of drinking is the disparity between human and animal studies in how intake is reported. In human literature, intake is most commonly recorded as drinks consumed per a certain unit of time, whereas animal intake generally is measured in grams of alcohol per kilogram of body weight. This discrepancy can make it difficult to attempt to relate intake across species. In addition, human studies using self-reported consumption often lack any physiological marker of alcohol effects such as BAC. Some studies have attempted more rigorous approaches by converting reported drinks consumed to a more specific measure such as grams, or by collecting the necessary information for estimating BAC achieved (Miller and DelBoca 1994). Another method that may prove useful for relating human and animal intake is examination of the pattern of how alcohol is consumed (i.e., drinking "microstructure"). In animal studies, lickometer chambers record individual contacts made to the sipper tube and therefore provide continuous consumption data that can be analyzed for measures, such as drinking bout size, duration, or interbout interval (e.g., Ford et al. 2005). These microstructural elements potentially are analogous to similar measures taken in human laboratory studies, such as time between sips and length of time taken to finish a drink. Bout size in particular may be relevant to excessive consumption, as a "gulping" (large bout size) phenotype in primates has been shown to predict risk for heavy drinking (Grant et al. 2008). Rodent studies reinforce the genetic basis of these differences because DBA/2J mice show larger bouts than C57BL/6J mice in an intragastric self-administration procedure (Fidler et al., in press), and most high-drinking rodent genotypes seem to show greater bout size (Samson 2000).

This area of research again must confront the differences between human self-reports and voluntary animal alcohol intake. The particular difficulty of evaluating reward value from behavior is a challenge. Better and more specific definitions of intake in both animal and human studies will be crucial to future progress in this domain.

Low Level of Response to Alcohol

As initially suggested by Schuckit and colleagues in the 1980s, a low level of response to alcohol has been thought to be a potentially predisposing factor for subsequent alcohol abuse. Schuckit evaluated family history–positive and family history–negative individuals on biological measures of alcohol sensitivity (e.g., body sway) as well as their subjective response to alcohol (e.g., self-reported "high") and found that the family history–positive group had overall lower responsiveness to the same dose of alcohol as the family history–negative group (Schuckit 1985). Since these early studies, many more have examined variation in alcohol sensitivity as it pertains to genetics and abuse potential. Subjective response to alcohol has been one of the most widely studied measures, and multiple questionnaires exist for assessing perception of alcohol's effects (e.g., Martin et al. 1993). These questionnaires differ with regard to whether they assess only sedative/anxiolytic effects or if they also include measures of feelings of "activation" in response to alcohol. Although some studies of subjective response have found the same pattern of lower sensitivity in people with a family history of AUDs, others have failed to reproduce this relationship (e.g., McCaul et al. 1990). The role of low level of response in the development of AUDs remains unclear. The contradictory findings in studies of family history–positive and family history–negative individuals may be attributable to the time course of testing in relationship to when alcohol is given. Studies tend to find a low level of response in at-risk populations at peak BACs and on the descending limb of the blood alcohol curve, whereas this same group shows a greater level of response immediately after alcohol administration and during the ascending limb of the curve (for review, see Crabbe et al. 2010; Newlin and Thomson 1990). In addition to subjective response to alcohol, other biological markers of sensitivity have been studied in groups that differ for their risk of developing AUDs. For example, research shows that cortisol response after alcohol consumption is blunted in groups at risk for alcohol abuse (Schuckit et al. 1987), whereas heightened cortisol response is seen in those at low risk (Wall et al. 1994).

Many attempts have been made to model low response in animals as well. However, this is a challenging endeavor given the fact that human studies in this area rely so heavily on self-reported variables. Mild concordance with the human literature has been achieved for the cortisol response to alcohol, with a high-drinking selected rat line showing decreased corticosterone (the rodent analogue of cortisol) response to alcohol relative to the low-drinking line (Apter and Eriksson 2006). These results were contingent upon social isolation of the animals, however, and results from other alcohol-preferring lines and inbred strains have proven inconsistent (Crabbe et al. 2010). Locomotor stimulation in response to alcohol also is used in animal studies as a marker of sensitivity. The FAST and SLOW mouse lines, for example, have been bred for high and low locomotor stimulation respectively

by an intoxicating dose of alcohol (Phillips et al. 2002), and FAST mice have been shown to have greater home-cage drinking than SLOW mice (Risinger et al. 1994). Differences in locomotor stimulation also have been seen in rat lines selected for drinking, with high-drinking lines being stimulated by lower doses than the low drinkers (Rodd et al. 2004). This potential genetic relationship between sensitivity to alcohol's stimulating effects and propensity toward high consumption seems consistent with findings from the human literature that show greater self-reported stimulation to alcohol in high-risk heavy social drinkers relative to low drinkers (King et al. 2011). Many human studies, however, do not specifically address the stimulating effects of alcohol and there are fewer good behavioral endpoints for measuring this in people. A greater focus on including measures of alcohol stimulant effects in human studies will be necessary to translate the extensive locomotor stimulation animal literature across species.

Level of response to alcohol may be a good predictor of risk, but despite many years of research, there is little strict parallelism between the phenotypes studied across species, and this is the logical target for improving consilience in future studies.

Conclusions

Ultimately, relating animal studies to those with humans requires careful consideration of the most relevant traits and tasks to be used. As seen with alcohol withdrawal, the capabilities exist to translate fine-tuned gene mapping of a behavior in mice back to people. This only proves fruitful in a clinical sense, however, if the behavior chosen is relevant to the development or expression of alcoholism in humans. Consequently, in order to continue making strides in the animal-models literature, it will be beneficial to choose the most clinically significant traits and make sure that the tasks used truly are measuring these traits. Likewise, adjustments on the human side can be made to include a greater focus on measuring a set of consistent, well-defined phenotypes that can be readily translated to animal models. For both human and animal researchers, it often can be tempting to gravitate toward tests that look similar in performance across species. However, the more important question is whether the tests are measuring and responding to the same underlying factors in both humans and animals. Designing experiments with this in mind will help lead to even greater discoveries of the genetics underlying alcohol abuse.

Acknowledgements

Preparation of this manuscript was supported by National Institute on Alcohol Abuse and Alcoholism grants AA–13519, AA–10760, and AA–007468, and by the Department of Veterans Affairs and the Achievement Rewards for College Scientists Foundation.

Financial Disclosure

The authors declare that they have no competing financial interests.

References

Andrews, M.M.; Meda, S.A.; Thomas, A.D.; et al. Individuals family history positive for alcoholism show functional magnetic resonance imaging differences in reward sensitivity that are related to impulsivity factors. *Biological Psychiatry* 69(7):675–683, 2011. PMID: 21126735

Apter, S.J., and Eriksson, C.J. The role of social isolation in the effects of alcohol on corticosterone and testosterone levels of alcohol-preferring and non-preferring rats. *Alcohol and Alcoholism* 41(1):33–38, 2006. PMID: 16216823

Balodis, I.M.; Lockwood, K.P.; Magrys, S.A.; and Olmstead, M.C. Preference conditioning in healthy individuals: Correlates with hazardous drinking. *Alcoholism: Clinical and Experimental Research* 34(6):1006–1012, 2010. PMID: 20374214

Becker, H.C. Kindling in alcohol withdrawal. *Alcohol Health & Research World* 22(1):25–33, 1998. PMID: 15706729

Becker, H.C., and Lopez, M.F. Increased ethanol drinking after repeated chronic ethanol exposure and withdrawal experience in C57BL/6 mice. *Alcoholism: Clinical and Experimental Research* 28(12):1829–1838, 2004. PMID: 15608599

Bickel, W.K.; Miller, M.L.; Yi, R.; et al. Behavioral and neuroeconomics of drug addiction: Competing neural systems and temporal discounting processes. *Drug and Alcohol Dependence* 90(Suppl. 1):S85–S91, 2007. PMID: 17101239

Blednov, Y.A.; Borghese, C.M.; McCracken, M.L.; et al. Loss of ethanol conditioned taste aversion and motor stimulation in knockin mice with ethanol-insensitive α2-containing GABA(A) receptors. *Journal of Pharmacology and Experimental Therapeutics* 336(1):145–154, 2011. PMID: 20876231

Blum, K.; Noble, E.P.; Sheridan, P.J.; et al. Allelic association of human dopamine D2 receptor gene in alcoholism. *JAMA: Journal of the American Medical Association* 263(15):2055– 2060, 1990. PMID: 1969501

Bowirrat, A., and Oscar-Berman, M. Relationship between dopaminergic neurotransmission, alcoholism, and reward deficiency syndrome. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics* 132B(1):29–37, 2005. PMID: 15457501

Breese, G.R.; Sinha, R.; and Heilig, M. Chronic alcohol neuroadaptation and stress contribute to susceptibility for alcohol craving and relapse. *Pharmacology & Therapeutics* 129(2):149–171, 2011. PMID: 20951730

Chester, J.A.; Risinger, F.O.; and Cunningham, C.L. Ethanol reward and aversion in mice bred for sensitivity to ethanol withdrawal. *Alcoholism: Clinical and Experimental Research* 22(2): 468–473, 1998. PMID: 9581655

Crabbe, J.C. Consilience of rodent and human phenotypes relevant for alcohol dependence. *Addiction Biology* 15(2):103–108, 2010. PMID: 20148774

Crabbe, J.C.; Bell, R.L.; and Ehlers, C.L. Human and laboratory rodent low response to alcohol: Is better consilience possible? *Addiction Biology* 15(2):125–144, 2010. PMID: 20148776

Crabbe, J.C.; Metten, P.; Rhodes, J.S.; et al. A line of mice selected for high blood ethanol concentrations shows drinking in the dark to intoxication. *Biological Psychiatry* 65(8):662–670, 2009. PMID: 19095222

Cunningham, C.L., and Phillips, T.J. Genetic basis of ethanol reward. In: Maldonado, R., Ed. *Molecular Biology of Drug Addiction*. Totowa, NJ: Humana Press, 2003, pp. 263–294.

Dawes, M.A.; Tarter, R.E.; and Kirisci, L. Behavioral self-regulation: Correlates and 2 year follow-ups for boys at risk for substance abuse. *Drug and Alcohol Dependence* 45(3):165–176, 1997. PMID: 9179518

Dick, D.M.; Smith, G.; Olausson, P.; et al. Understanding the construct of impulsivity and its relationship to alcohol use disorders. *Addiction Biology* 15(2):217–226, 2010. PMID: 20148781

Dickinson, A.; Wood, N.; and Smith, J.W. Alcohol seeking by rats: Action or habit? *Quarterly Journal of Experimental Psychology. B, Comparative and Physiological Psychology* 55(4):331–348, 2002. PMID: 12350285

Dole, V.P., and Gentry, R.T. Toward an analogue of alcoholism in mice: Scale factors in the model. *Proceedings*

of the National Academy of Sciences of the United States of America 81(11):3543–3546, 1984. PMID: 6587369

Fehr, C.; Shirley, R.L.; Belknap, J.K.; et al. Congenic mapping of alcohol and pentobarbital withdrawal liability loci to a <1 centimorgan interval of murine chromosome 4: Identification of Mpdz as a candidate gene. *Journal of Neuroscience* 22(9):3730–3738, 2002. PMID: 11978849

Fidler, T.L.; Dion, A.M.; Powers, M.S.; et al. Intragastric self-infusion of ethanol in high- and low-drinking mouse genotypes after passive ethanol exposure. *Genes, Brain, and Behavior* 10(3):264–275, 2011. PMID: 21091635

Ford, M.M.; Nickel, J.D.; and Finn, D.A. Treatment with and withdrawal from finasteride alter ethanol intake patterns in male C57BL/6J mice: Potential role of endogenous neurosteroids? *Alcohol* 37(1):23–33, 2005. PMID: 16472716

Foroud, T.; Edenberg, H.J.; and Crabbe, J.C. Who is at risk for alcoholism? *Alcohol Research and Health* 33:64–75, 2010.

Franken, I.H.; Van den Berg, I.; and Van Strien, J.W. Individual differences in alcohol drinking frequency are associated with electrophysiological responses to unexpected nonrewards. *Alcoholism: Clinical and Experimental Research* 34(4):702– 707, 2010. PMID: 20121719

Gehlert, D.R.; Cippitelli, A.; Thorsell, A.; et al. 3-(4-Chloro-2-morpholin-4-yl-thiazol-5-yl)-8-(1-ethylpropyl)-2,6-dimethyl-imidazo[1,2-b]pyridazine: A novel brain-penetrant, orally available corticotropin-releasing factor receptor 1 antagonist with efficacy in animal models of alcoholism. *Journal of Neuroscience* 27(10):2718–2726, 2007. PMID: 17344409

Gelernter, J., and Kranzler, H. D2 dopamine receptor gene (DRD2) allele and haplotype frequencies in alcohol dependent and control subjects: No association with phenotype or severity of phenotype. *Neuropsychopharmacology* 20(6):640–649, 1999. PMID: 10327432

Gilman, J.M., and Hommer, D.W. Modulation of brain response to emotional images by alcohol cues in alcohol-dependent patients. *Addiction Biology* 13(3–4):423–434, 2008. PMID: 18507736

Gilpin, N.W.; Richardson, H.N.; Lumeng, L.; and Koob, G.F. Dependence-induced alcohol drinking by alcohol-preferring (P) rats and outbred Wistar rats. *Alcoholism: Clinical and Experimental Research* 32(9):1688–1696, 2008. PMID: 18482158

Grahame, N.J.; Chester, J.A.; Rodd-Henricks, K.; et al. Alcohol place preference conditioning in high- and low-alcohol preferring selected lines of mice. *Pharmacology, Biochemistry, and Behavior* 68(4):805–814, 2001. PMID: 11526980

Grahame, N.J., and Cunningham, C.L. Intravenous ethanol self-administration in C57BL/6J and DBA/2J mice. *Alcoholism: Clinical and Experimental Research* 21(1):56–62, 1997. PMID: 9046373

Grant, K.A.; Leng, X.; Green, H.L.; et al. Drinking typography established by scheduled induction predicts chronic heavy drinking in a monkey model of ethanol self-administration. *Alcoholism: Clinical and Experimental Research* 32(10): 1824–1838, 2008. PMID: 18702645

Green, A.S., and Grahame, N.J. Ethanol drinking in rodents: Is free-choice drinking related to the reinforcing effects of ethanol? *Alcohol* 42(1): 1–11, 2008. PMID: 18164576

Gubner, N.R.; Wilhelm, C.J.; Phillips, T.J.; and Mitchell, S.H. Strain differences in behavioral inhibition in a Go/No-go task demonstrated using 15 inbred mouse strains. *Alcoholism: Clinical and Experimental Research* 34(8):1353–1362, 2010. PMID: 20491731

Heilig, M.; Egli, M.; Crabbe, J.C.; and Becker, H.C. Acute withdrawal, protracted abstinence and negative affect in alcoholism: Are they linked? *Addiction Biology* 15(2): 169–184, 2010. PMID: 20148778

Hitzemann, R.; Hitzemann, B.; Rivera, S.; et al. Dopamine D2 receptor binding, Drd2 expression and the number of dopamine neurons in the BXD recombinant inbred series: Genetic relationships to alcohol and other drug associated phenotypes. *Alcoholism: Clinical and Experimental Research* 27(1):1–11, 2003. PMID: 12543998

Johnsrude, I.S.; Owen, A.M.; Zhao, W.V.; and White, N.M. Conditioned preference in humans: A novel experimental approach. *Learning and Motivation* 30(3):250–264, 1999.

Karpyak, V.M.; Kim, J.H.; Biernacka, J.M.; et al. Sequence variations of the human MPDZ gene and association with alcoholism in subjects with European ancestry. *Alcoholism: Clinical and Experimental Research* 33(4):712–721, 2009. PMID: 19175764

Kendler, K.S.; Prescott, C.A.; Myers, J.; and Neale, M.C. The structure of genetic and environmental risk factors for common psychiatric and substance use disorders in men and women. *Archives of General Psychiatry* 60(9): 929–937, 2003. PMID: 12963675

King, A.C.; de Wit, H.; McNamara, P.J.; and Cao, D. Rewarding, stimulant, and sedative alcohol responses and relationship to future binge drinking. *Archives of General Psychiatry* 68(4):389– 399, 2011. PMID: 21464363

Kliethermes, C.L. Anxiety-like behaviors following chronic ethanol exposure. *Neuroscience and Biobehavioral Reviews* 28(8):837–850, 2005. PMID: 15642625

Koob, G.F., and Le Moal, M. Drug addiction, dysregulation of reward, and allostasis. *Neuropsychopharmacology* 24(2):97–129, 2001. PMID: 11120394

Kosobud, A., and Crabbe, J.C. Ethanol withdrawal in mice bred to be genetically prone or resistant to ethanol withdrawal seizures. *Journal of Pharmacology and Experimental Therapeutics* 238(1):170–177, 1986. PMID: 3723396

Krishnan-Sarin, S.; Krystal, J.H.; Shi, J.; et al. Family history of alcoholism influences naltrexone-induced reduction in alcohol drinking. *Biological Psychiatry* 62(6):694–697, 2007. PMID: 17336941

Le Foll, B.; Gallo, A.; Le Strat, Y.; et al. Genetics of dopamine receptors and drug addiction: A comprehensive review. *Behavioural Pharmacology* 20(1):1–17, 2009. PMID: 19179847

Lê, A.D.; Ko, J.; Chow, S.; and Quan, B. Alcohol consumption by C57BL/6, BALB/c, and DBA/2 mice in a limited access paradigm. *Pharmacology, Biochemistry, and Behavior* 47(2):375–378, 1994. PMID: 8146231

Leeman, R.F.; Heilig, M.; Cunningham, C.L.; et al. Ethanol consumption: How should we measure it? Achieving consilience between human and animal phenotypes. *Addiction Biology* 15(2):109–124, 2010. PMID: 20148775

Logue, S.F.; Swartz, R.J.; and Wehner, J.M. Genetic correlation between performance on an appetitive- signaled nosepoke task and voluntary ethanol consumption. *Alcoholism: Clinical and Experimental Research* 22(9): 1912–1920, 1998. PMID: 9884133

Lopez, M.F.; Grahame, N.J.; and Becker, H.C. Development of ethanol withdrawal-related sensitization and relapse drinking in mice selected for high- or low-ethanol preference. *Alcoholism: Clinical and Experimental Research* 35(5):953– 962, 2011. PMID: 21314693

Malone, S.M.; Iacono, W.G.; and McGue, M. Drinks of the father: Father's maximum number of drinks consumed predicts externalizing disorders, substance use, and substance use disorders in preadolescent and adolescent offspring. *Alcoholism: Clinical and Experimental Research* 26(12): 1823–1832, 2002. PMID: 12500106

Markou, A.; Kosten, T.R.; and Koob, G.F. Neurobiological similarities in depression and drug dependence: A self-medication hypothesis. *Neuropsychopharmacology* 18(3):135–174, 1998. PMID: 9471114

Martin, C.S.; Earleywine, M.; Musty, R.E.; et al. Development and validation of the Biphasic Alcohol Effects Scale. *Alcoholism: Clinical and Experimental Research* 17(1):140–146, 1993. PMID: 8452195

McCaul, M.E.; Turkkan, J.S.; Svikis, D.S.; and Bigelow, G.E. Alcohol and secobarbital effects as a function of familial alcoholism: Acute psychophysiological effects. *Alcoholism: Clinical and Experimental Research* 14(5):704–712, 1990. PMID: 2264598

Metten, P., and Crabbe, J.C. Alcohol withdrawal severity in inbred mouse *(Mus musculus)* strains. *Behavioral Neuroscience* 119(4):911–925, 2005. PMID: 16187819

Miller, W.R., and Del Boca, F.K. Measurement of drinking behavior using the Form 90 family of instruments. *Journal of Studies on Alcohol, Supplement* 12:112–118, 1994. PMID: 7722987

Milner, L.C., and Buck, K.J. Identifying quantitative trait loci (QTLs) and genes (QTGs) for alcohol-related phenotypes in mice. *International Review of Neurobiology* 91:173–204, 2010. PMID: 20813243

Milner, L.C., and Crabbe, J.C. Three murine anxiety models: Results from multiple inbred strain comparisons. *Genes, Brain, and Behavior* 7(4):496– 505, 2008. PMID: 18182070

Newlin, D.B., and Thomson, J.B. Alcohol challenge with sons of alcoholics: A critical review and analysis. *Psychological Bulletin* 108(3):383–402, 1990. PMID: 2270234

Nigg, J.T.; Wong, M.M.; Martel, M.M.; et al. Poor response inhibition as a predictor of problem drinking and illicit drug use in adolescents at risk for alcoholism and other substance use disorders. *Journal of the*

American Academy of Child and Adolescent Psychiatry 45(4):468–475, 2006. PMID: 16601652

Oberlin, B.G., and Grahame, N.J. High-alcohol preferring mice are more impulsive than low-alcohol preferring mice as measured in the delay discounting task. *Alcoholism: Clinical and Experimental Research* 33(7):1294–1303, 2009. PMID: 19389183

Phillips, T.J.; Broadbent, J; Burkhart-Kasch, S; et al. Genetic correlational analyses of ethanol reward and aversion phenotypes in short-term selected mouse lines bred for ethanol drinking or ethanol-induced conditioned taste aversion. *Behavioral Neuroscience* 119(4):892–910, 2005. PMID: 16187818

Phillips, T.J.; Shen, E.H.; McKinnon, C.S.; et al. Forward, relaxed, and reverse selection for reduced and enhanced sensitivity to ethanol's locomotor stimulant effects in mice. *Alcoholism: Clinical and Experimental Research* 26(5):593– 602, 2002. PMID: 12045466

Reich, T.; Edenberg, H.J.; Goate, A.; et al. Genome-wide search for genes affecting the risk for alcohol dependence. *American Journal of Medical Genetics* 81(3):207–215, 1998. PMID: 9603606

Reynolds, B.; Ortengren, A.; Richards, J.B.; and de Wit, H. Dimensions of impulsive behavior: Personality and behavioral measures. *Personality and Individual Differences* 40(2):305–315, 2006.

Rhodes, J.S.; Best, K.; Belknap, J.K.; et al. Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. *Physiology & Behavior* 84(1):53–63, 2005. PMID: 15642607

Risinger, F.O.; Malott, D.H.; Prather, L.K.; et al. Motivational properties of ethanol in mice selectively bred for ethanol-induced locomotor differences. *Psychopharmacology* 116(2):207–216, 1994. PMID: 7862950

Rodd, Z.A.; Bell, R.L.; McKinzie, D.L.; et al. Low-dose stimulatory effects of ethanol during adolescence in rat lines selectively bred for high alcohol intake. *Alcoholism: Clinical and Experimental Research* 28(4):535–543, 2004. PMID: 15100603

Saccone, N.L.; Kwon, J.M.; Corbett, J.; et al. A genome screen of maximum number of drinks as an alcoholism phenotype. *American Journal of Medical Genetics* 96(5):632–637, 2000. PMID: 11054770

Samson, H.H. The microstructure of ethanol drinking: Genetic and behavioral factors in the control of drinking patterns. *Addiction* 95(Suppl. 2):S61– S72, 2000. PMID: 11002903

Schmidt, L.G., and Sander, T. Genetics of alcohol withdrawal. *European Psychiatry* 15(2):135–139, 2000. PMID: 10881211

Schuckit, M.A. Ethanol-induced changes in body sway in men at high alcoholism risk. *Archives of General Psychiatry* 42(4):375–379, 1985. PMID: 3977555

Schuckit, M.A.; Gold, E.; and Risch, C. Plasma cortisol levels following ethanol in sons of alcoholics and controls. *Archives of General Psychiatry* 44(11): 942–945, 1987. PMID: 3675133

Spanagel, R. Recent animal models of alcoholism. *Alcohol Research & Health* 24(2):124–131, 2000. PMID: 11199279

Steinmetz, J.E.; Blankenship, M.R.; Green, J.T.; et al. Evaluation of behavioral disinhibition in P/NP and HAD1/LAD1 rats. *Progress in Neuro-Psychopharmacology & Biological Psychiatry* 24(6):1025–1039, 2000. PMID: 11041542

Stephens, D.N.; Duka, T.; Crombag, H.S.; et al. Reward sensitivity: Issues of measurement, and achieving consilience between human and animal phenotypes. *Addiction Biology* 15(2):145– 168, 2010. PMID: 20148777

Stewart, R.B., and Li, T.K. The neurobiology of alcoholism in genetically selected rat models. *Alcohol Health & Research World* 21(2):169– 179, 1997. PMID: 15704355

Tarter, R.E.; Kirisci, L.; Habeych, M.; et al. Neurobehavior disinhibition in childhood predisposes boys to substance use disorder by young adulthood: Direct and mediated etiologic pathways. *Drug and Alcohol Dependence* 73(2): 121–132, 2004. PMID: 14725951

Thanos, P.K.; Rivera, S.N.; Weaver, K.; et al. Dopamine D2R DNA transfer in dopamine D2 receptor-deficient mice: Effects on ethanol drinking. *Life Sciences* 77(2):130–139, 2005. PMID: 15862598

Vengeliene, V.; Celerier, E.; Chaskiel, L.; et al. Compulsive alcohol drinking in rodents. *Addiction Biology* 14(4): 384–396, 2009. PMID: 19740366

Victor, M., and Adams, R.D. The effect of alcohol on the nervous system. *Research Publications—Association for Research in Nervous and Mental Disease* 32:526–573, 1953. PMID: 13134661

Wall, T.L.; Nemeroff, C.B.; Ritchie, J.C.; and Ehlers, C.L. Cortisol responses following placebo and alcohol in Asians with different ALDH2 genotypes. *Journal of Studies on Alcohol* 55(2):207–213, 1994. PMID: 8189741

Wilhelm, C.J., and Mitchell, S.H. Rats bred for high alcohol drinking are more sensitive to delayed and probabilistic outcomes. *Genes, Brain, and Behavior* 7(7):705–713, 2008. PMID: 18518928

Wills, T.A.; Knapp, D.J.; Overstreet, D.H.; and Breese, G.R. Interactions of stress and CRF in ethanol-withdrawal induced anxiety in adolescent and adult rats. *Alcoholism: Clinical and Experimental Research* 34(9):1603–1612, 2010. PMID: 20586753

Ystrom, E.; Reichborn-Kjennerud, T.; Aggen, S.H.; and Kendler, K.S. Alcohol dependence in men: Reliability and heritability. *Alcoholism: Clinical and Experimental Research* 35(9): 1716–1722, 2011. PMID: 21676009

The Impact of Gene–Environment Interaction on Alcohol Use Disorders

Danielle M. Dick, Ph.D., and Kenneth S. Kendler, M.D.

Danielle M. Dick, Ph.D., *is an associate professor, and* **Kenneth S. Kendler, M.D.,** *is a professor, both in the Department of Psychiatry, Virginia Institute for Psychiatric and Behavioral Genetics, Virginia Commonwealth University, Richmond, Virginia.*

This article describes three types of gene–environment interactions and the challenges inherent in interpreting these interactions. It also reports on what is known about gene–environment interactions in the field of alcohol use disorders (AUDs). Twin studies of the interaction of genetic and environmental influences on AUDs have resulted in relatively consistent findings and have suggested general mechanisms for interaction effects. These studies generally find that environments that exert more social control (e.g., higher parental monitoring, less migratory neighborhoods, etc.) tend to reduce genetic influences, whereas other environments allow greater opportunity to express genetic predispositions, such as those characterized by more deviant peers and greater alcohol availability. Conversely, the gene–environment literature that has been developed surrounding specific genes has focused largely on the role of stress as a moderator of genetic effects. KEY WORDS: Alcohol use disorders (AUDs); genetic factors; environmental factors; genetic and environment interactions; twin studies; statistical models; stress; literature review

This article explores interactions between genetic and environmental effects on alcohol use disorders (AUDs). Two contrasting ideas define what it means to have genes and environment interact. The first approach—the one that this article will focus on—is a statistical perspective. This approach is based on statistical models in which genetic and environmental factors are sometimes measured indirectly (i.e., latent variable modeling—often in twin studies) and sometimes directly via molecular methods (examples of both kinds of interactions are provided below). The statistical approach does not consider the underlying biological process. Rather, it is based on observing processes from afar and modeling them.

The second approach is based on a biological or molecular perspective. The early work by Jacob and Monod on the operon model of gene regulation established that environmental effects can profoundly influence gene expression (Morange 1998). For example, by switching the source of food for bacteria (e.g., from glucose to lactose), researchers can activate a new set of genes that metabolize the lactose molecule. This is another way of thinking about how genes and environment "interact" but one that differs rather dramatically from the statistical viewpoint. From this perspective, the term interact refers to a biological process, measuring environmental exposures in biologically meaningful ways and looking at processes such as gene expression.

Statistical interactions do not equal biological interactions. In fact, any neurobiological system involves multiple gene products interacting with each other, such as components of signaling cascades, neurotransmitters and their receptors, or degradative enzymes. The world of biology seems like nothing but interactions of one molecule with another. Some biologists take this to mean that when we look at the effect of genetic variation, we should see interactions everywhere and that most gene effects involve such interactions. However, this is not true. A large corpus of work in statistical genetics in tractable organisms consistently has shown that most genetic effects look additive (Mather and Jinks 1982). Further explanation of this is beyond the scope of this article. In general use, the term interact sometimes only means "to act together." This is consistent with the technical concept of an additive model in which the main effects of genes and environment interact. In this article, the term interact will refer to its technical statistical meaning.

Examining gene–environment interactions from a statistical perspective is exemplified by the work of the statistician Ronald Fisher and best expressed in the development of the analysis of variance. In this highly influential statistical technique, as explained in any standard statistical textbook, Fisher posited an approach that first took into account main effects. For example, by studying the height of a particular plant 10 weeks after planting, one could examine the effect of the two different plant strains (reflecting genes) and the two different fertilizers (reflecting the environment). This would produce a main effect for each variable. Beyond

this, one would look for a gene–environment (or more technically a "strain by fertilizer") interaction. This interaction would reflect any explanatory power left over after accounting for the main effects. In many such cases, as noted above, no significant interaction is detected. That is, research shows the effects of genes on the phenotype and the effects of environment on the phenotype and no significant interaction. This is what statisticians will call an additive model—one in which the effects of genes and environment just add together.

If research does detect a significant gene-by-environment interaction, the effects of genes and environment on the phenotype (e.g., plant height) are not independent of one another. The impact of genes depends on environmental exposure and the impact of the environment depends on the effect of genes. Note that these two statements are conceptually equivalent. Expressed in yet another way, the central concept of genotype-by-environment interaction is that of conditionality. That is, it is not possible to understand how genes are acting without taking the environment into account, and vice versa.

Types of Gene–Environment Interactions and Challenges With Their Interpretation

This section will review three examples of gene–environment effects, which are illustrated in figure 1. Figure 1 shows five groups differing in level of genetic liability for a particular trait Y (e.g., symptoms of an alcohol use disorder [AUD]), from low to high. The diamonds represent the group with the lowest liability; the asterisks represent the highest-liability group. The x-axis shows the effect of the environment in five increasing categories. Level 1 reflects a very benign environment that conveys no increase at all on trait Y. As the environment becomes more pathogenic—from levels 2 to 5—it has a progressively greater and greater impact on trait Y.

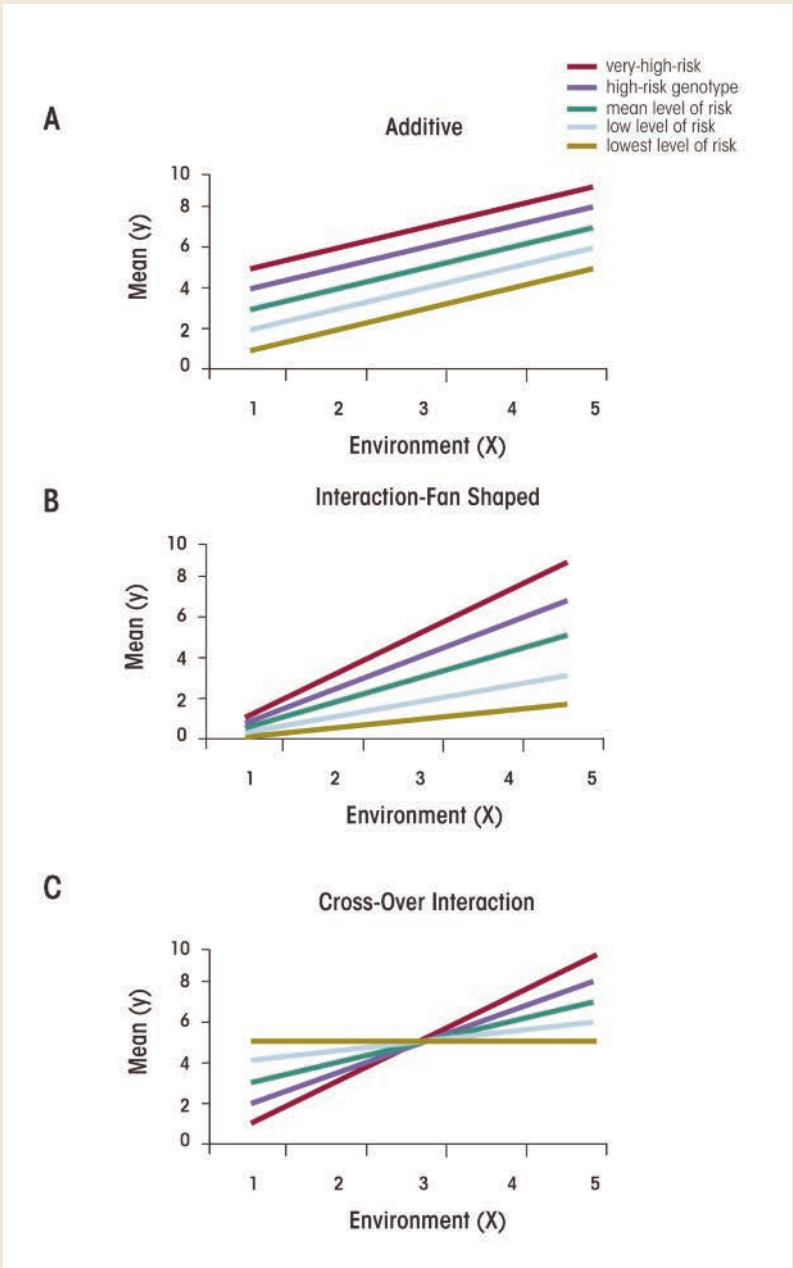


Figure **A–C)** The effect of genes and environment are used to predict the mean level of a quantitative trait Y. The lines depict five different genotypes with varying levels of liability to trait Y (e.g., symptoms of alcohol dependence). The environmental level of risk is depicted on the X-axis and ranges from level 1 (very low risk) to 5 (very high risk). **A)** An additive model of genetic and environmental effects. The key feature of this model is that the lines are all parallel—that is, the increase in the level of trait Y associated with a more adverse environment is the same for all genotypes. **B)** A fan-shaped interaction of genetic and environmental effects on trait Y. **C)** A cross-over interaction of genetic and environmental effects on trait Y.

Panel A in the figure depicts an additive model. The lines all are parallel with one another. Increasing from low- to high-risk environments (i.e., from environments 1 to 5), the increase in the level of Y is the same across all five genotypes. Genes and environment act independently of one another.

Panel B in the figure depicts what is known as a "fan-shaped" interaction. Note that the impact of genes is dependent on the environment, and vice versa. The key characteristic of a fan-shaped interaction is that, in benign environments, the difference in the level of the outcome variable (i.e., Y) as a function of the level of genetic liability is quite modest. That is, genes are not doing that much in a protective environment. However, with increasingly severe environmental exposures, the difference between genotypes increases. (In theory, of course, it does not have to be the case that the genetic differences are more pronounced in adverse environments than in benign environments. It could be that under very adverse conditions the environment becomes all important, but under more normative environmental conditions there is opportunity to see genetic differences.) Genes have a much more potent impact on the phenotype in a stressful environment. Another useful way to conceptualize such fan-shaped interactions is to see that genes in this context do two different things. First, they set the mean level of genetic liability. Second, they affect an individual's sensitivity to the impact of the environment.

Figure 3 depicts a crossover interaction, in which the order of genetic effects changes as a function of the environment. Those at lowest risk in environment 1 are at highest risk in environment 5. One would expect the environment, on average, to have an impact on the phenotype because the average level of risk for individuals in environment 5 (the highest risk environment) will be substantially greater than the average level of risk in the most benign environment (environment 1). However, in general, the main effect on the genotype is limited in this situation, because of a balance between the risk-decreasing effects in benign environments and the risk-increasing effects in malignant environments.

The literature surrounding plant and animal genetics indicates that fan-shaped interactions generally are more common than crossover interactions (Lynch and Walsh 1998; Mather and Jinks 1982). They are more difficult to interpret, however, because a statistical transformation of the scale of measurement can make many fan-shaped interactions disappear. That is, by examining the raw scale scores for a particular trait, it is possible to find significant evidence for a fan-shaped interaction. However, applying statistical analysis (i.e., logarithm or square-root transformation) of the scale scores often causes the interaction to disappear (Lynch and Walsh 1998; Mather and Jinks 1982).

Determining whether the interaction is indeed legitimate is a complicated question. Part of the answer has to do with the degree of "grounding" of the particular scale of measurement that one is examining. In studies of AUD risk, the particular measures are relatively arbitrary and might reflect the number of endorsed *Diagnostic and Statistical Manual, Fourth Edition* (DSM–IV) criteria. In this case, it is difficult to strongly argue that the number of DSM criteria is inherently more real than the square root of those numbers. This adds an extra interpretational difficulty to many analyses of genotype–environment interaction that do not carefully explore the degree to which transformations of the scale of measurement can make the interactions disappear.

A related problem is the common use of logistic regression in the analyses of genotype–environment interaction. Logistic regression is a convenient statistical tool when the dependent measure is dichotomous—such as whether an individual does or does not have a particular disorder. However, logistic regression involves a logarithmic transformation of the probability of being affected. This profoundly changes the nature of relationships between variables, because two variables that multiply as regular numbers will add together when logarithms are applied. The interpretation of interactions that relies solely on logistic regression therefore is rendered relatively treacherous. The interpretation of these results depends in part on a long argument in the epidemiological literature about whether the additive or the multiplicative model of risk is most appropriate.

Eaves (2006) simulated the effect of candidate genes and specific environmental factors in predicting a normally distributed continuous variable using a purely additive model (as in panel A of the figure). The resulting continuous results were dichotomized at a particular threshold value, and the dichotomized data were analyzed by logistic regression. Depending on the nature of the simulation, genotype–environment interaction was detected (spuriously) in 70 to 100 percent of the simulations. These results indicate that genotype–environment studies that detect interactions using logistic regression for dichotomous dependent measures should be interpreted with caution. It is quite challenging in such studies to determine whether the result is valid or an artifact of the statistical measures used. Kendler and Gardner (2010) have further explored this puzzling question of the interpretation of interactions.

Gene–Environment Interaction in the Field of AUDs

Examples of Latent Gene–Environment Interaction

Alcohol research is an area where one might imagine gene–environment interaction effects to be particularly important in etiological models because, by definition, exposure to alcohol is a necessary condition for the eventual development of alcohol-related problems. For example, one of the most widely replicated genetic associations with alcohol dependence is the protective role of a genetic variant responsible for the enzyme aldehyde dehydrogenase (i.e., *ALDH2*).[1] The enzyme produced

by a genetic variant in *ALDH2* is comparatively inactive, interfering with the metabolism of alcohol, which leads to facial flushing and other aversive physiological symptoms when alcohol is consumed (Shen et al. 1997). Accordingly, the association between this gene and risk for alcohol dependence necessarily operates through alcohol exposure. Environments that modify the extent of exposure to alcohol therefore would be predicted to moderate the degree to which genetic variability is important. In the extreme, this becomes obvious. If there is no alcohol in the environment, then genetic risk factors for AUDs cannot, by definition, express themselves.

A growing twin literature provides evidence that a variety of different environmental domains that influence access to alcohol and opportunity to engage in alcohol use moderate the importance of genetic influences. One of the earliest illustrations of gene–environment interaction in the area of substance use research demonstrated that genetic influences on alcohol use were greater among unmarried women, whereas having a marriage-like relationship reduced the impact of genetic influences on drinking (Heath et al. 1989). Religiosity also has been shown to moderate genetic influences on alcohol use among female subjects, with genetic factors playing a larger role among individuals without a religious upbringing (Koopmans et al. 1999).

Adolescent alcohol use also seems to be particularly influenced by gene–environment interactions, as might be expected because most adolescents are moving through a developmental period when adult guardians still exert a fair degree of control over their environment. Genetic influences on adolescent substance use are enhanced in environments with lower parental monitoring (Dick et al. 2007*b*), and easy availability of alcohol (Kendler et al. 2010), as well as in the presence of substance-using friends (Dick et al. 2007*a*; Harden et al. 2008; Kendler et al. 2010). Socioregional or neighborhood-level influences also have been shown to moderate the importance of genetic influences on substance use. Genetic influences for late-adolescent alcohol use (and early-adolescent behavior problems, which are genetically correlated) are enhanced in urban environments, communities characterized by greater migration, and neighborhoods with higher percentages of slightly older adolescents/young adults (Dick et al. 2001, 2009*a*; Rose et al. 2001). These community-based moderation effects presumably reflect differences in the availability of alcohol, role models, neighborhood stability, and community-level monitoring across different areas.

It is likely that many of the important moderating effects of the environment associated with alcohol use and related externalizing behavior reflect differences in social control and/or opportunity, resulting in differential expression of individual predispositions (Shanahan and Hofer 2005). Accordingly, the relevant environments are likely to vary across developmental stage. There is some indication of this in the Finnish twin data, where parental monitoring showed significant moderating effects on substance use starting earlier in adolescence (age 14), whereas the moderating role of peer substance use was not apparent until later in adolescence (age 17). More research in this area is necessary to delineate the developmental periods during which specific environments are critical because alcohol use patterns (and their etiological influences) are dynamic across the transition from adolescence to young adulthood. This also is likely to be true across stages of adulthood, although comparatively little research has been dedicated to this area.

Examples of Gene–Environment Interaction Involving Molecular Variants

As explained above, gene–environment interaction can be detected through the study of genetic influences that are inferred via comparisons of different types of relatives (such as twins) (i.e., latent genetic influences), or through the study of specific measured genes by molecular techniques. Gene–environment interactions modeled latently have the advantage of providing information about the overall genetic effect averaged across the entire genome but tell nothing about the specific underlying biology. Studies of specific genes have the advantage of providing information about the underlying biology, but they are (at this point) largely limited to studying single genes in a system in which there are likely to be hundreds of genes involved.

The literature surrounding specific gene–environment interactions in the area of alcohol use has developed largely independently of the latent gene–environment interaction literature reviewed above. Much of the literature examining measured gene–environment interactions with alcohol use outcomes has focused on stress, which was measured in a variety of ways, a moderator of specific genetic influences. The relationship between stress and alcohol use is complex, with human experimental studies, animal studies, and epidemiological studies all yielding equivocal evidence as to whether stress induces alcohol use (Schwandt et al. 2010; Veenstra et al. 2006). However, the gene–environment interaction literature presupposes that one of the reasons for these disparate findings may be that stress is more likely to induce alcohol use and problems in people who are genetically vulnerable, similar to the literature surrounding the experience of stressful life events and the onset of depression (Kendler et al. 1995).

A number of studies have tested for interactions between alcohol-related outcomes and various measures of stress with the genetic variation for length of the promoter region of the serotonin transporter gene (*5-HTTLPR*) (i.e., whether the genetic variant [allele] for long or short promoter region is associated with stress and alcohol use). Two studies found enhanced risk

[1] By convention, gene names in animals are written in uppercase and lowercase and italicized. Gene names in humans are written in all caps and are italicized, whereas the acronyms for the encoded proteins are all caps but not italicized.

associated with the short allele in the presence of a stressful environment. Covault and colleagues (2007) found that the short allele was associated with more frequent drinking and heavy drinking as well as drug use in college students if they had experienced multiple negative life events in the past year. Kaufman and colleagues (2006) found that the short allele conferred vulnerability to early alcohol use, and that this effect was stronger among maltreated children. Conversely, in the Mannheim Study of Children at Risk, the long allele was associated with more hazardous drinking in males among those exposed to high psychosocial adversity, as defined by early psychosocial stress and/or current life events (Laucht et al. 2009). In a study of Swedish adolescents, having two different alleles (i.e., being heterozygous) at the long/short polymorphism was associated with a higher intoxication frequency in the presence of neutral or bad family relations, which is biologically unlikely (Nilsson et al. 2005). Accordingly, the genetic model associated with the interaction has been inconsistent across studies, and the primary outcomes and measures of the experience of stress have varied considerably.

A more consistent picture has emerged from studies using experimental manipulations of the environment. In a unique prevention study testing for gene–environment interaction associated with the serotonin transporter gene, Brody and colleagues (2009*b*) found that youth carrying the short allele were more likely to initiate high-risk behavior (including alcohol and marijuana use, as well as sexual behavior) over time if they were in the control condition rather than the prevention condition. Similarly, short allele carriers showed increases in substance use over time, but this association was reduced when youth received high levels of involved-supportive parenting (Brody et al. 2009*a*, *b*). Related studies in monkeys indicate that the short allele is associated with higher baseline alcohol consumption (Barr et al. 2004) and increased aggression (Suomi 2006) under conditions of peer rearing (a stressful environment) compared with mother rearing. These studies suggest that experimental manipulation of the environment may be more likely to yield replicable interaction effects than observational designs, as previously has been argued from a statistical perspective (McClelland and Judd 1993). Interaction effects associated with experimental manipulations of the environment also may be more robust because interventions often operate across a variety of environmental domains (e.g., by influencing parenting processes, peer interactions, and equipping individuals with personal tools that are applicable across a variety of settings). Thus, any interaction effects that are detected may be more likely to be replicated for reasons similar to why twin studies, which examine aggregate genetic effects, are more likely to be replicated (discussed further below).

A few studies have evaluated gene–environment interactions with a variant of the gene for the dopamine type 2 receptor (i.e., the *DRD2* Taq1A polymorphism, which actually is located in the neighboring gene *ANKK1*). These studies have suggested that DRD2 A1 carriers show higher alcohol-related problems in the presence of stress (Bau et al. 2000; Madrid et al. 2001) and have higher novelty seeking when their child-rearing environment was assessed as punitive (Keltikangas-Jarvinen et al. 2009). Similarly, there is a small literature surrounding a genetic variant for the enzyme monoamine oxidase (MAO) (i.e., the MAOA polymorphism), adversity, and alcohol-related outcomes. MAO degrades serotonin, dopamine, and norepinephrine, which are all involved in the stress response. One study found a main effect of the MAOA promoter polymorphism on the risk for substance use disorders and an interaction with parenting (Vanyukov et al. 2007). In another study, the MAOA low-activity allele was associated with alcoholism, and particularly with antisocial alcoholism, but only among women experiencing childhood sexual abuse (Ducci et al. 2008). In yet another small study of female adolescents, the long variant increased risk for alcohol-related problems in the presence of an unfavorable environment (as defined by poor family relations or maltreatment/abuse). However, this effect was opposite that reported in the other studies (Nilsson et al. 2008). Accordingly, the association between this genotype and alcohol-related outcomes remains equivocal.

A few notable efforts have been made to extend the measured genotype–environment interaction literature in the field of alcohol-related outcomes in new directions. One such effort tested for moderation effects associated with brain gene expression in rodent models. Evidence in alcohol-preferring rats suggested that variation in the corticotrophin-releasing hormone releasing receptor 1 (*crhr1*) gene was associated with increased sensitivity to relapse into alcohol seeking induced by environmental stress (Bjork et al. 2010). The Mannheim Study of Children at Risk found an association between variants in *crhr1* and higher rates of heavy drinking and more drinking per occasion among 15-year-olds if they had experienced a greater number of negative life events over the previous 3 years (Blomeyer et al. 2008). An extension of this study followed up the adolescents at age 19 and also found that this gene interacted with stressful life events to predict both drinking initiation in adolescence and progression to heavy alcohol use in young adulthood (Schmid et al. 2010).

In addition, Dick and colleagues have attempted to bridge the gap between the latent gene–environment interaction literature and specific measured gene–environment interactions by developing hypotheses about the risk associated with genes. On the basis of twin studies suggesting that genetic influences on adolescent substance use are moderated by parental monitoring (Dick et al. 2007*b*) and peer substance use (Dick et al. 2007*a*), the researchers tested for moderation of the association of two genes associated with adult alcohol dependence in the Collaborative

Studies on Genetics of Alcoholism project. The two genes were for the γ-aminobutyric acid receptor (GABAR) subunit α-2 (*GABRA2*) (Edenberg et al. 2004) and the cholinergic muscarinic 2 receptor (*CHRM2*) (Wang et al. 2004). The researchers found evidence for gene-by-interaction effects in the direction predicted by the twin studies, namely genetic effects were enhanced under conditions of lower parental monitoring (Dick et al. 2009*b*) and higher peer-group antisocial behavior (Latendresse et al. 2011).

Conclusions

Although there is a burgeoning literature surrounding gene–environment interactions in the field of alcohol use and related disorders, far more remains to be understood. In general, the findings from gene-by-environment twin studies have been relatively consistent and have suggested general mechanisms for interaction effects. The common theme that emerges across findings of gene–environment interactions from the twin literature is that environments that exert more social control (e.g., higher parental monitoring, less migratory neighborhoods, etc.) tend to reduce genetic influences, whereas other environments allow greater opportunity to express genetic predispositions, such as those characterized by more deviant peers and greater alcohol availability. Conversely, the gene–environment literature that has been developed surrounding specific genes has focused largely on the role of stress as a moderator of genetic effects. Clearly, there is a disconnect between these literatures. In addition, it is likely that there are other important mechanisms of gene–environment interaction effects in relation to alcohol use and the development of problems. Many other variables, both individual and psychosocial, are known to affect drinking behavior, such as beliefs about alcohol, self-esteem, school attitudes, parental expectancies and messages surrounding alcohol use, and family disruption (Donovan and Molina 2011). It will be important to integrate these literatures, and the broader basis of etiological findings and associated environmental factors, into theoretical models of how gene–environment interaction effects operate with respect to alcohol use.

Another important area for future research is an expansion of the molecular studies of gene–environment interaction beyond a small number of polymorphisms from a handful of genes that are widely studied in the psychological literature (i.e., *5-HTT, MAOA,* and *DRD2*). The existent studies have been based on small samples, and results have been inconsistent. Although a focus on single genes may help advance theoretical models about particular biological pathways of risk, they face the same challenge (and currently have been met with the same fate) as studies of main effects of individual genes. That is, they have been notoriously difficult to replicate consistently. This is in contrast to the generally robust gene–environment interaction effects that have emerged from studies of latent genetic influences and, previous to that, the robustness of heritability estimates. This likely reflects the difference between studying overall genetic effects, versus specific genes in a complex polygenic system. The field of genetics has moved toward creating polygene scores that aggregate across many genes and show predictive power in cases where individual genes cannot be detected (Purcell et al. 2009). Moving studies of measured gene–environment interaction in this direction, to encompass aggregate genetic risk, may be one way to improve replicability of effects and to enhance cross-fertilization between quantitative and molecular genetic research.

This approach has the potential to advance our understanding of gene–environment effects. Similar to the way that evidence for heritability from twin studies for a given outcome was originally used to justify searching for specific genes involved in that outcome, evidence for gene–environment interactions from twin studies also can be used to develop hypotheses to test for gene–environment interactions associated with specific, identified genes. Change in the overall heritability across environmental contexts does not necessarily dictate that any one specific susceptibility gene will operate in a parallel manner. However, a change in heritability suggests that at least a good portion of the involved genes (assuming many genes of approximately equal and small effect) must be operating in that manner for a difference in heritability by environment to be detected. In this sense, one is "loading the dice" when testing for specific candidate gene-by-environment interaction effects with an environment that already has been shown to moderate the overall importance of genetic influences on that outcome. As additional research begins to clarify how specific genetic variants contribute to risk for AUDs, greater cross-talk between the twin literature, gene-identification studies, and studies testing for measured genotype-by-environment interactions will be critical to producing a more systematic research program aimed at understanding gene-by-environment effects for this critical and socially important condition. ■

Acknowledgments

This manuscript was prepared with support from grants AA–15416 and K02–AA–018755 (to Danielle M. Dick) and R37–AA–011408 and P20–AA–017828 (to Kenneth S. Kendler) from the National Institute on Alcohol Abuse and Alcoholism.

Financial Disclosure

The authors declare that they have no competing financial interests.

References

Barr, C.S.; Schwandt, M.L.; Newman, T.K.; and Higley, J.D. The use of adolescent nonhuman primates to model human alcohol intake: Neurobiological, genetic, and psychological variables. *Annals of the New York Academy of Sciences* 1021:221–233, 2004. PMID: 15251892

Bau, C.H.; Almeida, S.; and Hutz, M.H. The TaqI A1 allele of the dopamine D2 receptor gene and alcoholism in Brazil: Association and interaction with stress and harm avoidance on severity prediction. *American Journal of Medical Genetics* 96(3):302–306, 2000. PMID: 10898904

Bjork, K.; Hansson, A.C.; and Sommer, W.H. Genetic variation and brain gene expression in rodent models of alcoholism. Implications for medication development. *International Review of Neurobiology* 91:129–171, 2010. PMID: 20813242

Blomeyer, D.; Treutlein, J.; Esser, G.; et al. Interaction between CRHR1 gene and stressful life events predicts adolescent heavy alcohol use. *Biological Psychiatry* 63(2):146–151, 2008. PMID: 17597588

Brody, G.H.; Beach, S.R.; Philibert, R.A.; et al. Parenting moderates a genetic vulnerability factor in longitudinal increases in youths' substance use. *Journal of Consulting and Clinical Psychology* 77(1):1–11, 2009*a*. PMID: 19170449

Brody, G.H.; Beach, S.R.; Philibert, R.A.; et al. Prevention effects moderate the association of 5-HTTLPR and youth risk behavior initiation: Gene x environment hypotheses tested via a randomized prevention design. *Child Development* 80(3):645–661, 2009*b*. PMID: 19489894

Covault, J.; Tennen, H.; Armeli, S.; et al. Interactive effects of the serotonin transporter 5-HTTLPR polymorphism and stressful life events on college student drinking and drug use. *Biological Psychiatry* 61(5):609–616, 2007. PMID: 16920076

Dick, D.M.; Bernard, M.; Aliev, F.; et al. The role of socioregional factors in moderating genetic influences on early adolescent behavior problems and alcohol use. *Alcoholism: Clinical and Experimental Research* 33(10):1739–1748, 2009*a*. PMID: 19624574

Dick, D.M.; Latendresse, S.J.; Lansford, J.E.; et al. Role of GABRA2 in trajectories of externalizing behavior across development and evidence of moderation by parental monitoring. *Archives of General Psychiatry* 66(6):649–657, 2009*b*. PMID: 19487630

Dick, D.M.; Pagan, J.L.; Holliday, C.; et al. Gender differences in friends' influences on adolescent drinking: A genetic epidemiological study. *Alcoholism: Clinical and Experimental Research* 31(12):2012–2019, 2007*a*. PMID: 17949469

Dick, D.M.; Rose, R.J.; Viken, R.J.; et al. Exploring gene-environment interactions: Socioregional moderation of alcohol use. *Journal of Abnormal Psychology* 110(4): 625–632, 2001. PMID: 11727951

Dick, D.M.; Viken, R.; Purcell, S.; et al. Parental monitoring moderates the importance of genetic and environmental influences on adolescent smoking. *Journal of Abnormal Psychology* 116(1):213–218, 2007*b*. PMID: 17324032

Donovan, J.E., and Molina, B.S. Childhood risk factors for early-onset drinking. *Journal of Studies on Alcohol and Drugs* 72(5):741–751, 2011. PMID: 21906502

Ducci, F.; Enoch, M.A.; Hodgkinson, C.; et al. Interaction between a functional MAOA locus and childhood sexual abuse predicts alcoholism and antisocial personality disorder in adult women. *Molecular Psychiatry* 13(3): 334–347, 2008. PMID: 17592478

Eaves, L.J. Genotype x environment interaction in psychopathology: Fact or artifact? *Twin Research and Human Genetics* 9(1):1–8, 2006. PMID: 16611461

Edenberg, H.J.; Dick, D.M.; Xuei, X.; et al. Variations in GABRA2, encoding the alpha 2 subunit of the GABA(A) receptor, are associated with alcohol dependence and with brain oscillations. *American Journal of Human Genetics* 74(4):705–714, 2004. PMID: 15024690

Harden, K.P.; Hill, J.E.; Turkheimer, E.; and Emery, R.E. Gene-environment correlation and interaction in peer effects on adolescent alcohol and tobacco use. *Behavior Genetics* 38(4):339–347, 2008. PMID: 18368474

Heath, A.C.; Jardine, R.; and Martin, N.G. Interactive effects of genotype and social environment on alcohol consumption in female twins. *Journal of Studies on Alcohol* 50(1):38–48, 1989. PMID: 2927121

Kaufman, J.; Yang, B.Z.; Douglas-Palumberi, H.; et al. Brain-derived neurotrophic factor-5-HTTLPR gene interactions and environmental modifiers of depression in children. *Biological Psychiatry* 59(8):673–680, 2006. PMID: 16458264

Keltikangas-Jarvinen, L.; Pulkki-Raback, L.; Elovainio, M.; et al. DRD2 C32806T modifies the effect of child-rearing environment on adulthood novelty seeking. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics* 150B(3):389–394, 2009. PMID: 18615478

Kendler, K.S., and Gardner, C.O. Interpretation of interactions: Guide for the perplexed. *British Journal of Psychiatry* 197(3):170–171, 2010. PMID: 20807958

Kendler, K.S.; Gardner, C.; and Dick, D.M. Predicting alcohol consumption in adolescence from alcohol-specific and general externalizing genetic risk factors, key environmental exposures and their interaction. *Psychological Medicine* 41(7):1507–1516, 2011. PMID: 20942993

Kendler, K.S.; Kessler, R.C.; Walters, E.E.; et al. Stressful life events, genetic liability, and onset of an episode of major depression in women. *American Journal of Psychiatry* 152(6):833–842, 1995. PMID: 7755111

Koopmans, J.R.; Slutske, W.S.; van Baal, G.C.; and Boomsma, D.I. The influence of religion on alcohol use initiation: Evidence for genotype X environment interaction. *Behavior Genetics* 29(6):445–453, 1999. PMID: 10857249

Latendresse, S.J.; Bates, J.E.; Goodnight, J.A.; et al. Differential susceptibility to adolescent externalizing trajectories: Examining the interplay between CHRM2 and peer group antisocial behavior. *Child Development* 82(6):1797–1814, 2011. PMID: 21883161

Laucht, M.; Treutlein, J.; Schmid, B.; et al. Impact of psychosocial adversity on alcohol intake in young adults: Moderation by the LL genotype of the serotonin transporter polymorphism. *Biological Psychiatry* 66(2):102–109, 2009. PMID: 19358979

Lynch, M., and Walsh, B. *Genetics and Analysis of Quantitative Traits*. Sunderland, MA: Sinauer Associates, 1998.

Madrid, G.A.; MacMurray, J.; Lee, J.W.; et al. Stress as a mediating factor in the association between the DRD2 TaqI polymorphism and alcoholism. *Alcohol* 23(2):117–122, 2001. PMID: 11331109

Mather, K., and Jinks, J.L. *Biometrical Genetics: The Study of Continuous Variation*. London: Chapman & Hall, 1982.

McClelland, G.H., and Judd, C.M. Statistical difficulties of detecting interactions and moderator effects. *Psychological Bulletin* 114(2):376–390, 1993. PMID: 8416037

Morange, M. *A History of Molecular Biology*. Cambridge, MA: Harvard University Press, 1998.

Nilsson, K.W.; Sjoberg, R.L.; Damberg, M.; et al. Role of the serotonin transporter gene and family function in adolescent alcohol consumption. *Alcoholism: Clinical and Experimental Research* 29(4):564–570, 2005. PMID: 15834221

Nilsson, K.W.; Wargelius, H.L.; Sjoberg, R.L.; et al. The MAO-A gene, platelet MAO-B activity and psychosocial environment in adolescent female alcohol-related problem behaviour. *Drug and Alcohol Dependence* 93(1–2): 51–62, 2008. PMID: 18029115

Purcell, S.M.; Wray, N.R.; Stone, J.L.; et al. Common polygenic variation contributes to risk of schizophrenia and bipolar disorder. *Nature* 460(7256):748–752, 2009. PMID: 19571811

Rose, R.J.; Dick, D.M.; Viken And, R.J.; and Kaprio, J. Gene-environment interaction in patterns of adolescent drinking: Regional residency moderates longitudinal influences on alcohol use. *Alcoholism: Clinical and Experimental Research* 25(5):637–643, 2001. PMID: 11371711

Schmid, B.; Blomeyer, D.; Treutlein, J.; et al. Interacting effects of CRHR1 gene and stressful life events on drinking initiation and progression among 19-year-olds. *International Journal of Neuropsychopharmacology* 13(6):703–714, 2010. PMID: 19607758

Schwandt, M.L.; Lindell, S.G.; Chen, S.; et al. Alcohol response and consumption in adolescent rhesus macaques: Life history and genetic influences. *Alcohol* 44(1):67–80, 2010. PMID: 20113875

Shanahan, M.J., and Hofer, S.M. Social context in gene-environment interactions: Retrospect and prospect. *The Journals of Gerontology, Series B: Psychological Sciences and Social Sciences* 60(Spec No. 1):65–76, 2005. PMID: 15863711

Shen, Y.C.; Fan, J.H.; Edenberg, H.J.; et al. Polymorphism of ADH and ALDH genes among four ethnic groups in China and effects upon the risk for alcoholism. *Alcoholism: Clinical and Experimental Research* 21(7):1272–1277, 1997. PMID: 9347089

Suomi, S.J. Risk, resilience, and gene x environment interactions in rhesus monkeys. *Annals of the New York Academy of Sciences* 1094:52–62, 2006. PMID: 17347341

Vanyukov, M.M.; Maher, B.S.; Devlin, B.; et al. The MAOA promoter polymorphism, disruptive behavior disorders, and early onset substance use disorder: Gene-environment interaction. *Psychiatric Genetics* 17(6):323–332, 2007. PMID: 18075472

Veenstra, M.Y.: Lemmens, P.H.: Friesema, I.H.: et al. A literature overview of the relationship between life-events and alcohol use in the general population. *Alcohol and Alcoholism* 41(4):455–463, 2006. PMID: 16627624

Wang, J.C.; Hinrichs, A.L.; Stock, H.; et al. Evidence of common and specific genetic effects: Association of the muscarinic acetylcholine receptor M2 (CHRM2) gene with alcohol dependence and major depressive syndrome. *Human Molecular Genetics* 13(17):1903–1911, 2004. PMID: 15229186

Identifying Gene Networks Underlying the Neurobiology of Ethanol and Alcoholism

Aaron R. Wolen, Ph.D., and Michael F. Miles, M.D., Ph.D.

Aaron R. Wolen, Ph.D., *was a doctoral candidate in the Department of Human and Molecular Genetics, and* **Michael F. Miles, M.D., Ph.D.,** *is a professor in the Departments of Pharmacology and Toxicology and Neurology, Virginia Commonwealth University, Richmond, Virginia.*

For complex disorders such as alcoholism, identifying the genes linked to these diseases and their specific roles is difficult. Traditional genetic approaches, such as genetic association studies (including genome-wide association studies) and analyses of quantitative trait loci (QTLs) in both humans and laboratory animals already have helped identify some candidate genes. However, because of technical obstacles, such as the small impact of any individual gene, these approaches only have limited effectiveness in identifying specific genes that contribute to complex diseases. The emerging field of systems biology, which allows for analyses of entire gene networks, may help researchers better elucidate the genetic basis of alcoholism, both in humans and in animal models. Such networks can be identified using approaches such as high-throughput molecular profiling (e.g., through microarray-based gene expression analyses) or strategies referred to as genetical genomics, such as the mapping of expression QTLs (eQTLs). Characterization of gene networks can shed light on the biological pathways underlying complex traits and provide the functional context for identifying those genes that contribute to disease development. KEY WORDS: **Alcoholism; alcohol use disorders (AUDs); genetics; genetic basis of alcoholism; genetic technology; genetic association studies; quantitative trait loci (QTLs); genetic mapping; gene networks; genomes; genetical genomics; human studies; animal models**

The multiple genetic, environmental, and behavioral factors that play a role in the development of alcohol use disorders (AUDs) make it difficult to identify individual genes linked to these disorders. Nevertheless, some genetic risk factors (i.e., specific variants) associated with AUDs have been identified within many genes, some of which code for proteins involved in known biological pathways. Despite this progress, it has been exceedingly difficult to determine which genes may be the most relevant to developing therapeutic interventions for alcoholism. The major obstacles in treatment development are that gene–disease associations reveal very little about the underlying biology and that any implicated gene variant explains only a tiny proportion of an individual's overall risk for an AUD. Recent work focusing on the study of alcohol-related gene networks is helping to shed light on the molecular factors affecting alcoholism and other complex diseases. This article will provide an overview of approaches used to identify or construct gene networks and describe how systems biology approaches are helping to better understand complex traits such as behavioral responses to beverage alcohol (i.e., ethanol) and alcoholism.

Traditional Approaches to Dissecting Complex Traits

The predominant experimental strategy used by contemporary geneticists to identify the genetic factors involved in complex traits, such as behavioral responses to alcohol, essentially is an expansion of the gene mapping approach proposed by Botstein and colleagues (1980) over 30 years ago. For this approach, investigators scan their samples for genetic variations (i.e., polymorphisms) that segregate with the trait—that is, which are found in samples with the trait more often than would be expected by chance and therefore might contribute to the development of that trait. In recent human studies, this approach typically has been applied in genome-wide association studies (GWASs) of large, population-based samples that comprise both case subjects (i.e., individuals expressing the trait, or phenotype, under investigation) and unaffected control subjects. Hundreds of complex diseases and traits, including susceptibility to AUDs, have been analyzed using GWASs, resulting in the identification of several important links between genetic variants and these diseases (Bierut et al. 2010). Overall, however, the success of this approach has been mixed, and greater progress has been hindered by insufficient

sample sizes, stratified populations, the involvement of rare gene variants (i.e., alleles) that each only have a small effect size, and heterogenous phenotypic constructs (i.e., using different criteria to distinguish cases from controls).[1]

A similar forward-genetics approach that most often is used for studying animal models of complex traits is called quantitative trait locus (QTL) mapping. A quantitative trait is a phenotype that is determined by several genes, each of which has a variable contribution to the trait. The locations of the involved genes on the chromosomes are referred to as QTLs. QTL mapping studies typically are conducted using inbred strains of mice and their various derivatives. For example, the C57BL/6J (B6) and DBA2/J (D2) inbred mice frequently are used in alcohol research because they clearly differ in various responses to alcohol, including development of functional tolerance (Grieve and Littleton 1979), locomotor activation (Phillips et al. 1998), and sensitivity to withdrawal symptoms (Metten and Crabbe 1994). Because the environmental conditions in these experiments can be controlled, any differences observed between the mouse strains in these phenotypes most likely can be attributed to genetic differences. QTL mapping studies then seek to detect the polymorphisms underlying the complex traits of interest by scanning for alleles that co-vary with the traits.

Similar experiments also can be conducted with special derivatives of inbred strains known as recombinant inbred (RI) mice. These animals are derived by cross-breeding two or more distinct parental strains (which often diverge widely for the trait of interest), followed by inbreeding of the offspring for several generations (Bailey 1971). Given the correct breeding strategy, this method results in a panel of RI mouse strains that differ in the degree to which they exhibit a certain phenotype of interest. At the same time, each of the strains effectively is isogenic, meaning that for all genes, the genome carries two identical alleles (i.e., is homozygous). As a result, when two animals from the same RI mouse strain are bred, their offspring will have the exact same genetic makeup (i.e., genotype) as the parents. This makes it possible to directly integrate results generated from disparate experiments, in different laboratories, and at different times if they all use animals from the same RI mouse strain. This feature of RI mouse panels, and inbred animals in general, is particularly valuable for QTL mapping because the expense and time involved with genotyping or sequencing a strain only is incurred once.

The molecular and genetic resources outlined above have greatly increased the power and resolution of QTL mapping for various behaviors or other traits of interest. Yet despite these advances, the DNA regions identified as QTLs typically still are relatively large and may contain several genes; accordingly, few genes have been validated as contributing to quantitative traits (i.e., being quantitative trait genes [QTGs]). This difficulty is attributable largely to the lack of sufficient recombination events in existing mouse panels to reduce the size of DNA segments that typically are inherited together (i.e., haplotype block size) for fine mapping and to the generally small effect size for any single QTG.

Genomic Approaches to Disease Dissection

Because of the technical obstacles impeding their more effective use, both GWASs and QTL mapping studies to date have identified a deluge of disease-associated genetic loci but only few actual causal genes. Moreover, even the most successful studies have failed to place the disease-associated genes in any kind of biological context that would serve to explain the underlying functional biology. Without elucidating the complex interactions of the molecular phenotypes that stand between genetic variation and disease, it will be difficult or impossible to develop new and effective approaches to treating such diseases.

The emerging field of systems biology is tackling this immense challenge by studying networks of genes, proteins, metabolites, and other biomarkers that represent models of genuine biological pathways. Studying complex diseases in terms of gene networks rather than individual genes or genomic loci should aid in uncovering disease genes. With this approach, the effects of multiple genes in the network are combined, producing a stronger signal and reducing the number of statistical tests of association that must be performed.

These benefits effectively were demonstrated in two recent human association studies that modified the typical GWASs strategy by seeking associations only within groups of functionally related genes, rather than across the entire genome. The first of these studies (Ruano et al. 2010) discovered that cognitive ability, a complex phenotype with a large genetic component, was significantly linked to genes encoding molecules called G-proteins that consist of three different subunits (i.e., heterotrimeric G-proteins). The second study (Reimers et al. 2011) found that genes related to signaling pathways involving the neurotransmitters glutamate and γ-aminobutyric acid (GABA) signaling collectively contribute to alcohol dependence.

Network-based approaches to the dissection of complex diseases also can be applied to animal models, yielding experimental results that are more generalizable to humans because the pathways represented by these networks are more evolutionarily conserved than individual genes. This should encourage greater collaboration between researchers studying a common disease in different species. In fact, the biology underlying gene networks is so complex that any hope of deriving novel therapeutics may be entirely contingent on the extent to

[1] This is an issue faced by GWASs researchers when classifying samples as cases or controls. If cases are limited to only individuals who have been diagnosed with an AUD, it becomes difficult to enlist a sufficient number of participants. Moreover, many of the control subjects could very well be undiagnosed alcoholics or people who meet some but not all of the diagnostic criteria for an AUD. As a result, the control group could be polluted with near-cases, diluting any detectable group differences.

which scientists with diverse areas of expertise are willing to share and integrate datasets and make the process of interpretation a collaborative one.

Using High-Throughput Molecular Profiling to Define Disease

As the human and mouse genomes were being assembled using the cutting-edge, high-throughput DNA sequencers that made these endeavors possible, new technologies began to emerge that, for the first time, allowed near-comprehensive profiling of other cellular components. The term profiling refers to the measurement of different types of biological molecules, such as DNA to identify polymorphisms, messenger RNA (mRNA) to determine transcript abundance, proteins to identify certain chemical modifications that occur after the initial protein synthesis, and metabolites to evaluate biochemical processes in the cells. Platforms for high-throughput approaches for all these types of molecular profiling have become increasingly commonplace. Concurrently, methods for analyzing data produced by these technologies constantly are evolving, yielding results that are simultaneously more sensitive and more specific. As a result, researchers are better able to appreciate systems-level changes associated with disease.

Of these various high-throughput profiling techniques, microarray-based gene expression platforms have featured most prominently in biomedical research to date. Through an unbiased profiling of the transcriptome—that is, a measurement of all mRNA molecules produced within a cell or tissue sample—microarray expression studies allow researchers to identify patterns of gene expression associated with a disease. In some cases, such patterns can better define a complex phenotype by identifying disease subtypes. For example, microarray analysis of breast cancer tumors identified gene expression signatures that predict patient prognosis and therefore help physicians tailor treatment regimens (van't Veer et al. 2002). From a basic research perspective, microarray expression profiles can help tease apart the complex interactions that underlie the development of a disease by implicating a subset of genes whose regulation is altered with the disease. With this information, it may become feasible to reconstruct the underlying biological pathways and enhance understanding of disease etiology.

Genomic approaches have been applied to alcoholism directly by studying postmortem human brain tissue isolated from alcoholics and matched control subjects using gene expression microarrays. This has revealed novel information about changes in the brain's transcriptome that are associated with chronic ethanol consumption. One of the findings was a significant deregulation of genes encoding proteins that synthesize and maintain myelin, the substance that forms a sheath surrounding the long extensions (i.e., axons) of nerve cells and that is essential for effective nerve signal transmission (Lewohl et al. 2000; Mayfield et al. 2002). However, the nature of these studies makes it impossible to determine whether such gene expression deviations actually are risk factors that contribute to AUDs or simply represent molecular consequences of excessive alcohol consumption that are unrelated to the behaviors constituting alcoholism.

Animal models can assist greatly in this analysis by allowing for experiments that are far more detailed and informative but too invasive to ever be performed with humans. Although animal models could never replicate a phenotype as complex as alcoholism, they can mimic certain facets of the trait, which then can be associated with specific expression signatures using gene expression microarrays. For example, a genetic predisposition for alcoholism may entail a stronger-than-average preference for alcoholic beverages. This particular facet of alcoholism is captured by rodent models that selectively were bred to maximize a penchant for or aversion to ethanol, such as the aptly named high-alcohol preference (HAP) and low-alcohol preference (LAP) mice (Grahame et al. 1999). In order to identify genes that may alter the perceived desirability of ethanol, gene expression microarrays were used to compare the brain transcriptomes of HAP and LAP mice, as well as of several other inbred mouse strains that drastically differ in ethanol preference (Mulligan et al. 2006). This important study identified a diverse array of molecular pathways associated with differences in ethanol preference. Some of the genes that had the largest effect sizes were related to neuronal function and to the maintenance of the cells' normal internal conditions (i.e., cellular homeostasis).

Another important facet of a genetic predisposition to alcoholism is a comparatively blunted sensitivity to the effects of ethanol. Studies have shown that people who initially are less sensitive to acute ethanol exposure are more likely to have a family history of alcoholism and are at greater risk for developing an AUD (Schuckit 1984, 1994). As mentioned earlier, the B6 and D2 inbred mice frequently are used in genetic studies of ethanol sensitivity. For this reason, Kerns and colleagues (2005) used microarray expression studies to dissect the effect of acute ethanol exposure on the brain's transcriptome using the B6 and D2 inbred mouse strains. The investigators analyzed three brain regions involved in a brain system called the mesocorticolimbic reward pathway, which is involved in mediating the rewarding properties of alcohol and other drugs. For each region analyzed, the study identified a specific set of genes (i.e., a gene module) whose expression was altered in response to acute ethanol exposure. These gene modules contained greater-than-expected numbers of genes involved in several signaling pathways (i.e., retinoic acid signaling, neuropeptide expression, and glucocorticoid signaling). Moreover, similar to the microarray studies of postmortem human alcoholic brains (Lewohl et al. 2000; Mayfield et al. 2002), several genes involved in myelination robustly were altered by alcohol exposure, particularly in the prefrontal cortex (Kerns et al. 2005).

In examining the responses to acute or chronic alcohol exposure in rodent brains, these and numerous other genomic studies have enhanced the understanding of the "ethanol transcriptome" and provided a more comprehensive picture of the genes and molecular pathways that contribute to specific facets of AUDs than what is possible with studies of postmortem human brains (Daniels and Buck 2002; Mulligan et al. 2011; Rimondini et al. 2002; Saito et al. 2004; Treadwell and Singh 2004). Moreover, these studies effectively have demonstrated how gene expression microarrays can help close the information gap that exists between DNA variation and complex diseases. However, prioritizing the long lists of genes produced by comparative microarray studies conducted in either species has proven exceedingly difficult. As the costs associated with validating a given gene's role in driving a complex trait are considerable, an effective strategy for prioritizing candidate genes is crucial. Investigators therefore have used more systems-level approaches that combine genetic, genomic, and pharmacological methods to better delineate gene networks causally related to ethanol behaviors. Networks allow us to infer relationships between genes and determine which are most important.

The Gene Network As a Modern Genetic Map

The previous section mentioned several studies that used gene-expression microarrays to define lists of genes responding to ethanol or otherwise relevant to AUDs. Although these studies have provided important biological insights, the question of how such lists can be used to further advance understanding of a complex disease is not easily answered. Network-based approaches can greatly improve the interpretability of differential gene-expression results by providing information about the relationships between genes.

Networks are systems of interconnected components. For example, the World Wide Web is a global network of computers sharing documents connected by hyperlinks; road maps are renderings of city networks connected by highways; social networks are groups of people connected through friendships; cellular signaling pathways are groups of proteins connected through molecular interactions; et cetera. Placing such complex systems within a network framework makes it possible to formally analyze the relationships that constitute these systems. Gene networks typically are visualized as mathematical graphs—that is, a collection of vertices and edges, where genes are represented by nodes and the lines connecting the nodes indicate that some relationship exists between the genes.

Many published network analyses of gene groups use information about pre-existing biological relationships, which may be derived from sources such as literature co-citation analysis (i.e., genes mentioned together in a scientific abstract), protein–protein interaction databases, or gene ontology groupings. Some commercial tools are available for such studies, such as Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA). However, although these sources provide categories for interpreting the genomic data, they also force such interpretation into the mold of pre-existing information, thereby partially defeating the goal of genomic studies.

Genomic data collected with high-throughput molecular profiling presents the opportunity to derive novel gene–gene interactions. The maturity of gene expression microarrays relative to similar technologies designed to measure other molecular phenotypes on a genomic scale has meant that gene networks primarily are rendered as gene coexpression networks. In the context of gene coexpression networks, links between nodes typically indicate that the expression levels for two genes are strongly correlated with one another across whatever conditions an experiment entails (e.g., across tissues, time points, treatments, or individuals). Each link in a gene network essentially represents a testable hypothesis that can be validated through follow-up molecular experiments. Indeed, coexpression networks have been used to identify protein interactions that are novel (Scott et al. 2005) and conserved across species (Stuart et al. 2003).

Various novel and innovative methods exist for generating gene coexpression networks. Although a comprehensive review of these methods is beyond the scope of this article, a few select methods are described in more detail in the sidebar. In their simplest form, however, gene coexpression networks can be constructed by calculating Pearson correlations between all gene pairs and applying a cutoff threshold to determine which genes should be connected. The simplicity of this approach makes it an appealing choice for conducting a first round of analyses.

Wolen and colleagues (in press) have attempted to better define the mesocorticolimbic reward pathway's transcriptional response to acute ethanol exposure by expanding the original B6/D2 study (Kerns et al. 2005) to include members of the BXD family of recombinant inbred mouse strains. The naturally occurring DNA polymorphisms that distinguish each BXD strain cause heritable changes in gene expression, making it possible to identify genetically coregulated transcripts across the BXD family. Microarray expression data from the prefrontal cortex of BXD family animals were used to look for evidence of coregulation among the 307 ethanol-responsive genes identified in the original B6/D2 study (see figure 1). The analysis identified several groups of intercorrelated gene modules, indicating this gene set is comprised of several gene networks (figure 1).

A variety of calculations can be used to gauge the relative importance of a particular gene to the network as a whole (Horvath and Dong 2008). The simplest measurement of node importance is determined by the degree of "connectivity"—that is, the number of

other genes the node is connected to in the network. However, a gene's "position" in the network also is an important consideration. For example, a gene that served as the sole connection between two otherwise independent gene networks would rank fairly low on a priority scale based on connectivity alone, despite being an important channel of intermodule communication. A measurement of "betweenness centrality" (Girvan and Newman, 2002) can highlight such a gene by determining the frequency with which a node is included in the shortest paths between all possible node combinations.

Figure 2 highlights six subnetworks taken from the larger coexpression network depicted in figure 1. The network comprising genes known as *Mbp*, *Mobp*, *Mal*, and *Plp1* is of particular interest, because these genes all play a role in the formation and stabilization of myelin. In addition, a parallel analysis was conducted using microarray data only from the prefrontal cortex of the same BXD strains after they had received an injection of 1.8 g/kg ethanol into their abdominal cavity. This analysis revealed that the myelin gene network persisted but underwent minor topographical modifications. Most notably, additional connections were detected between *Plp1* and two additional genes called *Mog* and *Lpar1*. The absence of *Mog* in the original network probably was an artifact of the method used to form these networks, and the gene likely should have been included. *Plp1* and *Lpar1*, in contrast, were effectively unrelated at baseline and only showed evidence of coregulation after ethanol treatment, suggesting this is a genuine molecular response to ethanol (see figure 2B).

The *Lpar1* gene encodes a receptor for lysophosphatidic acid (LPA), a signaling molecule containing phosphate and lipid components (i.e., a phospholipid). Regulation of *Lpar1* is critical for proper nerve-cell formation (i.e., neurogenesis), including in a brain region called the hippocampus in adults (Matas-Rico et al. 2008). In addition, *Lpar1* regulates the breakdown of the myelin sheath (i.e., demyelination) that occurs after nerve injury (Inoue

Constructing Gene Networks

Various methods exist for generating gene networks. As mentioned in the main text, the simplest method for constructing gene coexpression networks involves calculating Pearson correlations for all pair-wise genes and applying a hard threshold to determine which genes should be connected. The robustness of these networks, initially referred to as "relevance networks," can be assessed through an approach called permutation testing (Butte et al. 2000). A more rigorous method for constructing gene coexpression networks utilizes a graph theoretical approach to identify densely intercorrelated gene modules called paracliques (Baldwin et al. 2005). Paracliques represent gene–gene interaction networks with extensive, but not perfect, strong expression correlations between all genes in the network (http://grappa.eecs.utk.edu/grappa/root). Paracliques can contain members with missing links. Therefore, paracliques provide an attractive compromise by augmenting coexpression with genes whose correlational relationships to a network are strong, but permissibly imperfect with a proportion of the network. This proportion, called the proportional glom factor, is a user-defined parameter.

A potential limitation of both relevance networks and paracliques is that they rely on hard thresholds to classify the relationship between genes as either connected or unconnected. The dichotomy imposed by this approach may be artificially limiting these networks, causing biologically meaningful relationships to be overlooked (Carter et al. 2004). For example, the absence of the *Mog* gene from the myelin network described in the main article and depicted in figure 2A following ethanol treatment is symptomatic of this limitation. An approach called weighted gene coexpression network analysis (WGCNA) is an increasingly popular method that avoids these potential pitfalls by utilizing a "soft-thresholding" approach to generate networks that conform to a scale-free topology (Zhang and Horvath 2005). Scale-free networks follow the power distribution they are named for, comprising many nodes that have sparse connections and a few that are highly interconnected. In addition to providing an accurate model for metabolic networks (Jeong et al. 2000), neural networks of the roundworm *Caenorhabditis elegans* (Watts and Strogatz 1998), and the World Wide Web (Albert and Jeong 1999), the scale-free topology also typifies gene coexpression networks (van Noort et al. 2004). Some researchers recently have used WGCNA to define correlated gene modules associated with blood alcohol levels using the "drinking-in-the-dark" paradigm of excessive ethanol consumption in B6 mice (Mulligan et al. 2011). WGCNA also can be implemented as a freely available package (Langfelder and Horvath 2008) for the R Statistical Environment and provides an excellent set of tutorials (available at genetics.ucla.edu/labs/horvath/CoexpressionNetwork/Rpackages/WGCNA).

Lpar1 regulates the breakdown of the myelin sheath (i.e., demyelination) that occurs after nerve injury (Inoue et al. 2004). The fact that *Lpar1* is brought into this network by ethanol exposure suggests the intriguing possibility that this gene may play a role in the loss of white matter[2] commonly observed in long-term alcoholic patients (Kril and Halliday 1999). This example illustrates how studying ethanol-induced changes in gene-network topology can produce testable hypotheses relevant to the neurobiology of alcoholism. Obviously, alterations in the gene network occurring after acute ethanol exposure might not always be relevant to alterations in brain structure and function (i.e., neural plasticity) or toxic effects that occur with chronic exposure, such as in alcoholism. Therefore, findings regarding networks relevant to one ethanol behavioral phenotype should be considered "specific" to that phenotype unless other genetic, pharmacological, or behavioral data suggests links to other aspects of ethanol's actions in animal models or humans. More generally, this example demonstrates how systems-level methods, like gene coexpression analysis, can help greatly expand the information content of gene expression microarray studies by filling in information about the gene–gene relationships.

Bridging the Gap Between Genomics and Gene Mapping

Genetical Genomics

Another important early advancement toward a more systems-level approach to identifying disease-associated genes was the application of gene mapping methods to high-throughput molecular data in order to identify causal links between molecular phenotypes and genomic regions. Like classical physiological or behavioral phenotypes, genetic factors influencing high-throughput measures of transcript, protein, and metabolite abundance can be identified by QTL mapping. To date, such analyses mostly have been applied to gene-expression microarrays, mapping gene expression QTLs (eQTLs). This largely is related to technical constraints, because whole-proteome expression profiling currently cannot be done with the same degree of sensitivity, coverage, and throughput as mRNA profiling.

The strategy of performing genetic linkage analysis on genome-wide molecular profiles was formalized and termed "genetical genomics" by Jansen and Nap (2001). This proposal primarily focused on gene-expression microarrays and posited that mapping eQTLs would enable researchers to construct robust gene networks as well as link these networks to metabolic or other phenotypes. The investigators also suggested that eQTL mapping could greatly aid in the identification of candidate genes underlying classical QTLs for disease traits. The first study to carry out QTL analysis across genome-wide gene expression microarrays was conducted using an experimental cross between two strains of the yeast *Saccharomyces cerevisiae* (Brem et al. 2002). Subsequently, several investigations applied the approach to mammalian systems (Schadt et al. 2003; York et al. 2005), including brain gene expression (Chesler et al. 2003, 2005).

These early genetical genomics studies also characterized the two major classes of eQTLs, labeled *cis* and *trans* eQTLs, which differ with respect to the position of the eQTL relative to the gene whose expression is altered (figure 3).

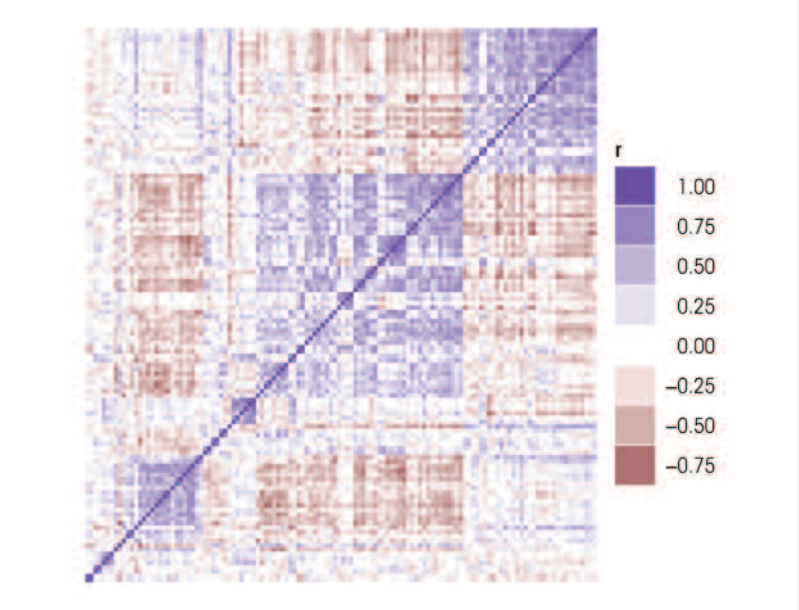


Figure 1 Correlation heatmap depicting patterns of co-expression among genes previously identified as being regulated by acute ethanol (Kerns et al. 2005). Each colored square represents the Pearson correlation (r) between a pair of genes, calculated using microarray expression data of prefrontal cortex tissue collected from B6, D2, and 27 BXD mouse strains. The blue and red colors indicate the strength and direction of the gene–gene correlation. Hierarchical clustering was applied to group genes based on the similarity of their expression profile across this dataset. In doing so, modules of co-expressed genes are revealed as cohesive blocks along the diagonal.

[2] The term "white matter" refers to brain structures made up primarily of nerve fibers that are enclosed by the myelin sheaths and therefore have a whitish appearance. Conversely, the term "gray matter" refers to brain structures composed mainly of the bodies of nerve cells, which have no myelin sheath, resulting in a grayish appearance.

A *cis* eQTL is located at the same site of the genome as the gene under study. In contrast, a *trans* eQTL can be located elsewhere in the genome, away from the gene whose expression is altered. A good example of how a *trans* eQTL could manifest involves transcription factors (TFs). These are proteins that bind with regulatory DNA regions that are located in front of a gene. Only when the TF binds to the corresponding DNA sequence can the first step in the process of gene expression—transcription—begin. The interaction between the TF and the DNA involves a certain part of the TF called the TF DNA–binding domain that allows the TF to recognize and bind with specific regulatory DNA sequences. Through this mechanism, certain TFs only may activate the transcription of specific sets of genes. Accordingly, a polymorphism at the DNA-binding domain of a certain TF can affect the TF's ability to recognize and bind its recognition sites, causing altered expression of all genes regulated by this TF. In other words, the abundance of all transcripts from those genes would co-vary with the TF polymorphism. Such a case might be recognized by a clustering of *trans* eQTLs at the site of the causal polymorphism, sometimes referred to as a regulatory hotspot. The identification of *trans* eQTL clusters can be a powerful approach for identifying key regulators underlying a complex trait of interest.

Figure 4 depicts the eQTL results for the same list of 307 ethanol-responsive genes identified in the B6/D2 study that earlier was used to construct coexpression networks. This analysis revealed that these coexpression networks share common eQTLs that drive this coordinated expression. Furthermore, the strongest eQTLs underlying many of these genes mapped to one end of chromosome 7, forming a *trans* eQTL cluster. These findings provide preliminary evidence that acute ethanol–responsive genes comprise a handful of gene coexpression networks in the prefrontal cortex and that a key regulator of these networks resides on chromosome 7. A more extensive analysis of this type has recently been completed (Wolen et al., in press).

The genes comprising *trans* eQTL clusters often have biological functions that have been conserved among species, suggesting that these hotspots may have a biological relevance. Accordingly, the search for *trans* eQTLs may allow researchers to identify biological functions associated with complex traits through defining quantitative trait gene networks (QTGNs). Mozhui and colleagues (2008) have, for example, dissected a *trans* eQTL cluster on distal mouse chromosome 1 and identified a candidate gene (*Fmn2*) that they

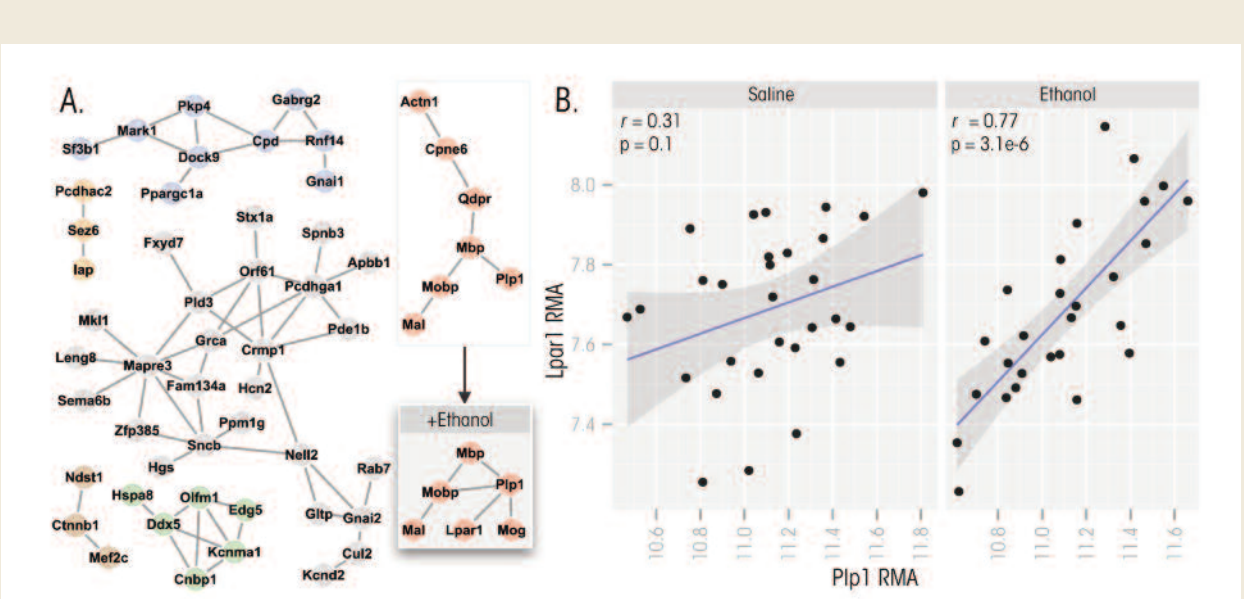


Figure 2 **A)** Gene networks that are regulated by acute alcohol exposure were identified in the same prefrontal cortex dataset used in Figure 1. Gene networks were generated by applying a hard threshold of 0.75 to the gene correlation matrix. The inset box contains a cognate of the myelin network (red) that was generated with expression data from the same strains following ethanol treatment. The ethanol-induced modifications of this network include the addition of a novel connection between *Plp1* and *Lpar1*. **B)** Scatterplots illustrating the correlation between *Plp1* and *Lpar1* at baseline and following ethanol treatment. The effective absence of any correlation between these genes at baseline suggests that this relationship is driven by ethanol exposure.

propose has a major influence on the expression of linked gene networks. Moreover, a diverse group of phenotypic QTLs seemed to be located in the same region, including several related to ethanol.

Genetical Genomics Studies to Identify Gene Variants Increasing Disease Risk

The integration of eQTL and classical QTL data enables identification of key markers of disease-causing variants. The effectiveness of this approach was demonstrated by a genetical genomics analysis of liver expression data from a population of mice placed on a high-fat diet (Schadt et al. 2003). The purpose of this diet was to model an obesity-like phenotype, which was measured using fat-pad mass (FPM). QTL mapping for FPM revealed a significant QTL on chromosome 2 that also harbored over 400 eQTLs. By scanning this region for *cis* eQTL-linked genes that also were strongly correlated with FPM, the researchers were able to identify two novel obesity candidate genes.

Saba and colleagues (2006) used a similar approach to identify candidate genes for alcohol preference and acute functional tolerance to alcohol. This large-scale study included rodent strains selectively bred for ethanol phenotypes (i.e., HAP and LAP mice) as well as a subset of the BXD family of recombinant inbred mice. Applying microarray expression profiles using mRNAs obtained from the entire brain, the investigators identified independent lists of genes whose expression differed between the HAP and LAP strains and between the BXD strains with high and low levels of acute functional tolerance. The genetic regulation of these gene lists then was mapped using BXD expression and genotypic data. High-priority candidate genes were highlighted by screening for differentially expressed genes with *cis* eQTLs that overlapped previously mapped behavioral QTLs for either alcohol preference (Belknap and Atkins 2001) or acute functional tolerance (Kirstein et al. 2002).

The rationale for prioritizing candidate QTGs on the basis of their having *cis* eQTLs located at the same sites as classical QTLs is based on the hypothesis that the variability of a complex phenotype is linked to a particular locus because the causal gene is being produced in variable quantities through a

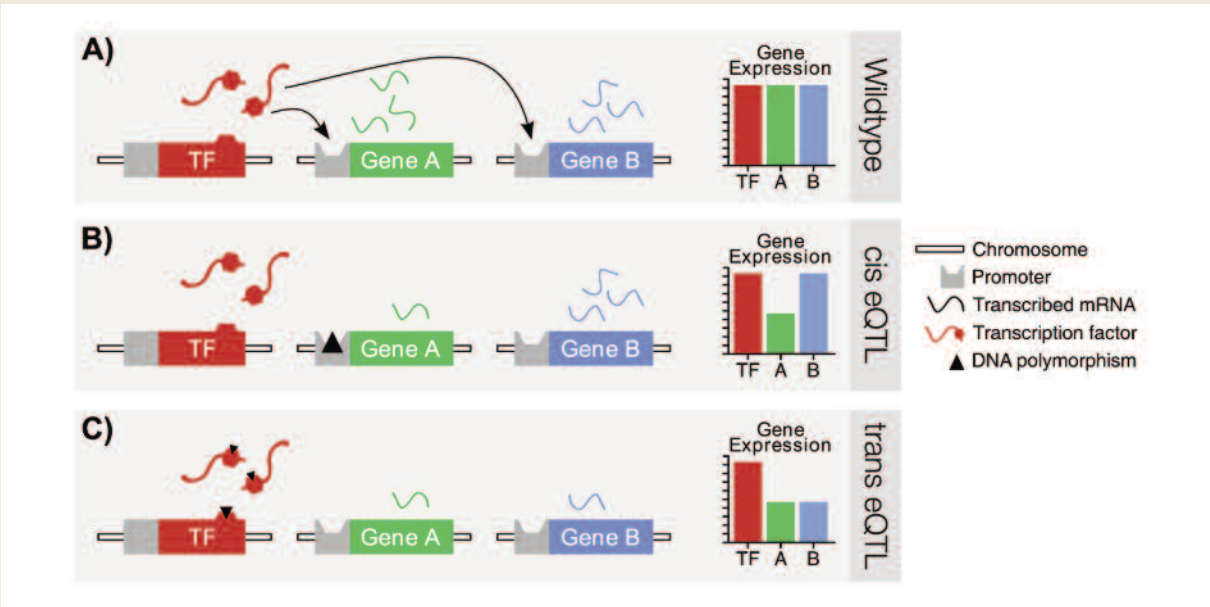


Figure 3 Illustration of the concept of *cis* and *trans* expression quantitative trait loci (eQTLs). **A)** The left-most gene (red) codes for a transcription factor (TF) protein that activates the transcription of genes A (green) and B (blue) by binding to their respective promoters (gray). In the wildtype, or "normal," scenario all genes are transcribed at their full potential, as indicated by the bar graph on the right. **B)** A variant (i.e., polymorphism) (triangle) in gene A's promoter region hinders TF binding, causing a reduction in the rate at which gene A is transcribed, while gene B is unaffected. Thus, gene A is being regulated by a *cis* eQTL because its level of expression is associated with a nearby polymorphism located on the same chromosome. **C)** A polymorphism in the TF gene's DNA binding region (hexagon)—the region of the TF protein that binds to gene promoters—hinders binding with all downstream promoters, regardless of whether the regulated gene is located near the TF gene, like gene A, or located on an entirely different chromosome, like gene B. In fact, all genes regulated by this TF would be linked to a *trans* eQTL at the site of this TF polymorphism.

cis-acting polymorphism. This hypothesis is somewhat of an oversimplification and leaves out several important caveats. Nevertheless, increasing evidence supports the importance of gene-expression variability in regulating complex traits. In fact, recent evidence indicates that SNPs associated with a variety of complex traits are more likely to contain *cis* eQTLs than normally would be expected (Nicolae et al. 2010). This indicates that the importance of expression variability in complex trait regulation is not limited to genetic model systems and that it may be possible for GWASs and QTL mapping studies to improve their track record by incorporating expression data.

Dissecting Complex Diseases Through Integrating Genomic Approaches

The discussion above has illustrated how traditional QTL mapping and GWASs approaches can benefit from systems-biological approaches by filling in critical information about the molecular phenotypes that stand between DNA variation and complex disease (figure 5). The incorporation of data from high-throughput molecular profiling technologies, such as gene expression microarrays, can better define a disease by identifying groups of genes that respond to or covary with disease-associated traits. Network analysis of disease-associated genes allows investigators to move beyond static gene lists, partially reconstruct the underlying molecular pathways, and prioritize genes based on their importance to the larger network. Applying QTL mapping to each gene's expression trait makes it possible to identify the genomic regions that regulate each gene's expression and uncover the existence of regulatory hotspots that exert enormous influence over a gene network. The series of studies discussed below has demonstrated how effective these methods are for dissecting complex traits, particularly when they are integrated.

Zhu and colleagues (2004) followed up the genetical genomics analysis of liver expression data from mice on a high-fat diet that was mentioned earlier (Schadt et al. 2003) by generating gene networks from the same microarray gene-expression dataset. This analysis included two distinct approaches to network construction: The first strategy formed networks on the basis of the covariation among gene-expression traits—that is, genes whose expression changed in the same manner were considered linked. In the second strategy, gene-network interactions were determined on the basis of the similarity of their eQTL profiles. Thus, the networks were constructed once without and once with the benefit of genotypic and eQTL data. Because links within gene networks represent causal relationships, analyses of these links can help researchers predict how a system will respond to the perturbation of a specific gene. Zhu and colleagues (2004) tested this hypothesis by measuring differential expression in response to a pharmacological substance that interfered with the function of a central gene in both predicted networks. They found that the eQTL profile network was a significantly better predictor of which genes would be affected by the pharmacological perturbation than the network constructed with expression data alone. Therefore, integrating both gene-expression and genotypic information into network construction greatly enhances the predictive value of gene networks.

The ultimate goal of systems-level analyses of complex diseases is to uncover

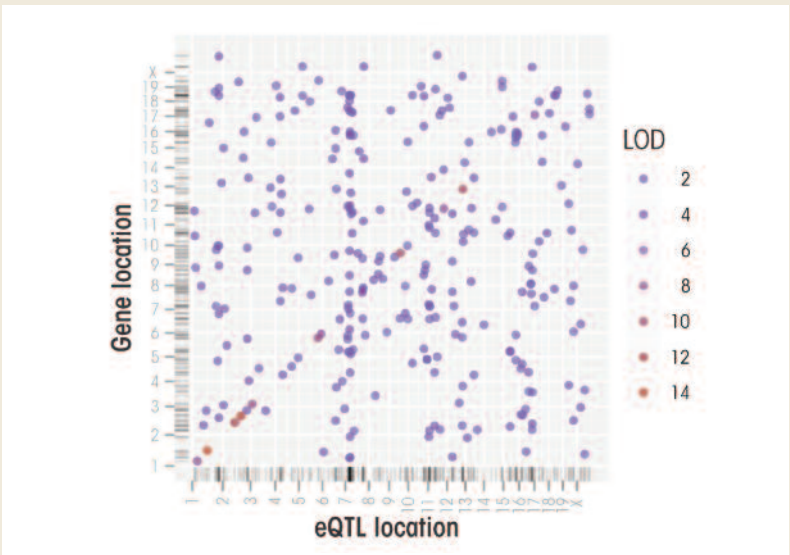


Figure 4 Genome–genome plot of peak expression quantitative trait loci (eQTLs) for the same dataset used in figure 1. Each point indicates the chromosomal position of a gene versus the position of its peak eQTL. Point color is used to communicate the strength of association between a gene and its eQTL, measured by logarithm of the odds (LOD) score. A LOD score is a ratio that measures that probability that a gene is linked to genetic markers, versus the probability that it is not. Thus, the higher a LOD score, the more likely a gene's expression level genuinely is regulated by an eQTL. Points plotted along the diagonal likely represent *cis* eQTLs, which also tend to have stronger LOD scores. Perpendicular tick-marks along both axes show the distribution of data points. Along the x-axis the dense clustering of tick-marks toward the proximal tip of chromosome 7 indicates the presence of a *trans* eQTL cluster, suggesting this region may harbor an important regulator of the gene co-expression modules seen in figure 1.

information necessary to establish a correlation between disease phenotype, mRNA abundance, and the underlying DNA polymorphism or causal gene network. Schadt and colleagues (2005) demonstrated this approach of integrating genotypic data, gene-expression data, and disease endophenotypes,[3] using the same liver expression dataset mentioned above, and a novel network construction technique termed likelihood-based causality model selection (LCMS). The investigators first identified all QTLs associated with a classical phenotype and then winnowed the list of potentially associated gene-expression traits on the basis of their correlation or eQTL overlap with the phenotype of interest. Candidate genes then were ranked by applying the LCMS technique, which uses the eQTL data to establish causal relationships between DNA loci and transcripts as well as between transcripts and phenotypes and finally identifies a model that best fits the data.

By ranking genes according to their performance in these models, the investigators identified several novel obesity candidate genes as well as uncovered additional support for the involvement of a gene called *Hsd11b1* that previously had been implicated in obesity risk (Rask et al. 2002). Because this gene seemed to be relevant to the phenotype they were investigating, the researchers then sought to reconstruct the gene network in which *Hsd11b1* participates by performing the LCMS procedure with *Hsd11b1* as the trait of interest. The resulting network was able to successfully predict genes that would be affected by inhibition of *Hsd11b1*. A similar approach has been used by other investigators to identify transcriptional networks associated with ethanol sensitivity behavior in the fruit fly *Drosophila melanogaster* (Morozova et al. 2011). This progression from phenotype to gene network to candidate gene and back to a gene network is a striking example of the promise that combining genetical genomics and gene-network analysis provides for understanding complex traits such as alcoholism.

As previously mentioned, such network-based techniques also have been applied to provide novel insight into the functional consequences associated with ethanol exposure in the mesocorticolimbic reward pathway. Preliminary results have identified several gene-coexpression networks that are robustly altered by ethanol in a tightly coordinated fashion (Wolen et al., in press). These studies used the BXD panel, so that the genetic regulation of ethanol-induced expression changes and behavioral responses also could be examined. Similar to results shown in figure 1, this analysis has revealed that ethanol-responsive gene networks are regulated by a small number of loci that largely are specific to a given network. At least one of these loci overlaps a previously mapped QTL for loss of righting reflex, a measure of acute ethanol sensitivity (Bennett et al. 2002; Markel et al. 1997). This work suggests that focusing on identifying gene networks both greatly reduces the complexity of whole-genome expression data and provides a wealth of hypotheses regarding both functional implications and regulatory mechanisms relevant to ethanol's action.

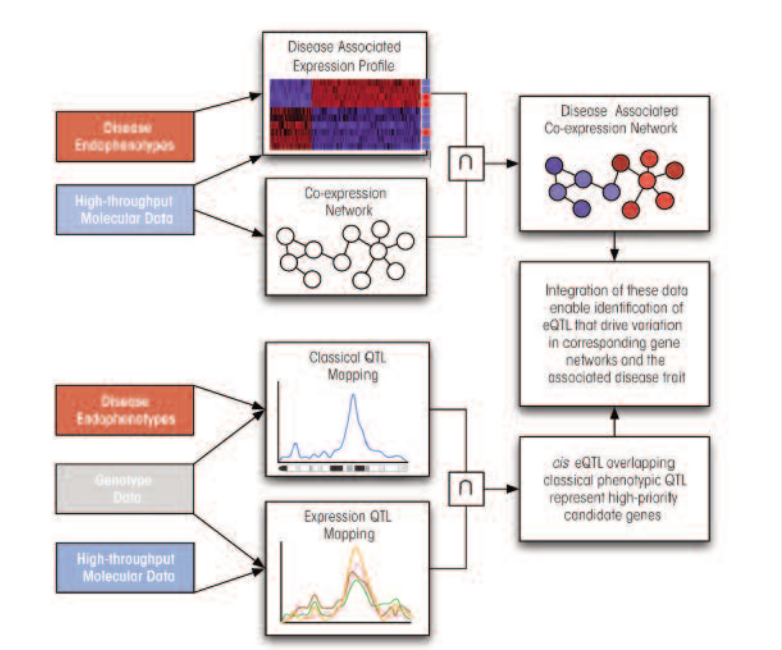


Figure 5 Diagram of how the genomic approaches discussed here can be integrated to identify gene networks and candidate genes for complex traits such as alcoholism. The information flow indicates how gene networks, expression quantitative trait locus (eQTL) and behavioral QTL analyses can be used together to identify candidate genes as potential targets for intervention. Note that resulting networks or candidate genes are entirely derived from experiments rather than relying on prior knowledge. In some cases, use of biomedical literature on gene–gene interactions can be used to augment such experimentally-derived networks.

[3] An endophenotype is a heritable trait or characteristic that is thought to be an intermediate phenotype between a genetic predisposition and a clinical disorder; for example, certain neurobiological characteristics have been noted in people with alcoholism and may be used as endophenotypes to identify people at risk for alcoholism. Endophenotypes are thought to be useful for gene identification under the assumption that they are simpler and closer to the genetic underpinnings of the disorder.

expression profiles associated with ethanol or alcoholism has provided modern neuroscience with a wealth of molecular information regarding ethanol's effects on the body. At the same time, alcohol researchers must make sense of a plethora of weak genetic signals and large lists of genes whose expression changes in response to alcohol. Newer approaches, such as exome[4] sequencing studies and certain approaches to analyzing gene expression (e.g., RNA-Seq analyses), promise added clarity but also may deliver even more confusing data. By combining behavioral, genetic, and genomic studies through genetical genomics and gene-network analysis designs, researchers may be able to construct gene networks rich in functional relations to ethanol behaviors. Additional refinements in ethanol-related gene-network structures and causal relation of such networks to aspects of ethanol-induced behaviors will provide a new generation of candidate genes for therapeutic intervention in alcoholism. ■

Acknowledgements

The authors would like to thank Drs. Elissa Chesler, Michael Langston, and Robert Williams for discussions and collaborations that contributed to this manuscript, as well the anonymous reviewers for their helpful feedback. This work was supported by National Institute on Alcohol Abuse and Alcoholism (NIAAA) grants P20 AA–017828, U01 AA–016667 and U01 AA–016662 to MFM and National Institute of Mental Health (NIMH) training grant MH–20030 supporting ARW.

Financial Disclosure

The authors declare that they have no competing financial interests.

[4] The exome is the part of the genome formed by the coding portions of genes (i.e., exons) that provide the genetic blueprint used in the synthesis of proteins and other functional gene products. It is the functionally most relevant part of the genome and, therefore, most likely to contribute to the phenotype of an organism.

References

Albert, R., and Jeong, H. Internet: Diameter of the World-Wide Web. *Nature* 401:130–131, 1999.

Bailey, D.W. Recombinant-inbred strains: An aid to finding identity, linkage, and function of histocompatibility and other genes. *Transplantation* 11(3):325–327, 1971. PMID: 5558564

Baldwin, N.E.; Chesler, E.J.; Kirov, S.; et al. Computational, integrative, and comparative methods for the elucidation of genetic coexpression networks. *Journal of Biomedicine & Biotechnology* 2005(2):172–180, 2005. PMID: 16046823

Belknap, J.K., and Atkins, A.L. The replicability of QTLs for murine alcohol preference drinking behavior across eight independent studies. *Mammalian Genome* 12(12):893–899, 2001. PMID: 11707775

Bennett, B.; Beeson, M.; Gordon, L.; and Johnson, T.E. Reciprocal congenics defining individual quantitative trait loci for sedative/hypnotic sensitivity to ethanol. *Alcoholism: Clinical and Experimental Research* 26(2):149–157, 2002. PMID: 11964553

Bierut, L.J.; Agrawal, A.; Bucholz, K.K.; et al. A genome-wide association study of alcohol dependence. *Proceedings of the National Academy of Sciences of the United States of America* 107(11):5082–5087, 2010. PMID: 20202923

Botstein, D.; White, R.L.; Skolnick, M.; and Davis, R.W. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics* 32(3):314–331, 1980. PMID: 6247908

Bottomly, D.; Walter, N.A.; Hunter, J.E.; et al. Evaluating gene expression in C57BL/6J and DBA/2J mouse striatum using RNA-Seq and microarrays. *PLoS One 6*(3):e17820, 2011. PMID: 21455293

Brem, R.B.; Yvert, G.; Clinton, R.; and Kruglyak, L. Genetic dissection of transcriptional regulation in budding yeast. *Science* 296(5568):752–755, 2002. PMID : 11923494

Butte, A.J.; Tamayo, P.; Slonim, D.; et al. Discovering functional relationships between RNA expression and chemotherapeutic susceptibility using relevance networks. *Proceedings of the National Academy of Sciences of the United States of America* 97(22): 12182–12186, 2000. PMID: 11027309

Carter, S.L.; Brechbühler, C.M.; Griffin, M.; and Bond, A.T. Gene co-expression network topology provides a framework for molecular characterization of cellular state. *Bioinformatics* 20(14): 2242–2250, 2004. PMID: 15130938

Chen, H., and Sharp, B.M. Content-rich biological network constructed by mining PubMed abstracts. *BMC Bioinformatics* 5:147, 2004. PMID: 15473905

Chesler, E.J.; Lu, L.; Shou, S.; et al. Complex trait analysis of gene expression uncovers polygenic and pleiotropic networks that modulate nervous system function. *Nature Genetics* 37(3):233– 242, 2005. PMID: 15711545

Chesler, E.J.; Lu, L.; Wang, J.; et al. WebQTL: Rapid exploratory analysis of gene expression and genetic networks for brain and behavior. *Nature Neuroscience* 7(5):485–486, 2004. PMID : 15114364

Chesler, E.J.; Wang, J.; Lu, L.; et al. Genetic correlates of gene expression in recombinant inbred strains: A relational model system to explore neurobehavioral phenotypes. *Neuroinformatics* 1(4):343–357, 2003. PMID: 15043220

Daniels, G.M., and Buck, K.J. Expression profiling identifies strain-specific changes associated with ethanol withdrawal in mice. *Genes, Brain, and Behavior* 1(1):35–45, 2002. PMID: 12886948

DeFries, J.C.; Wilson, J.R.; Erwin, V.G.; and Petersen, D.R. LS × SS recombinant inbred strains of mice: Initial characterization. *Alcoholism: Clinical and Experimental Research* 13(2):196– 200, 1989. PMID: 2658655

Eisen, M.B.; Spellman, P.T.; Brown, P.O.; and Botstein, D. Cluster analysis and display of genome-wide expression patterns. *Proceedings of the National Academy of Sciences of the United States of America* 95(25): 14863–14868, 1998. PMID: 9843981

Girvan, M., and Newman, M.E. Community structure in social and biological networks. *Proceedings of the National Academy of Sciences of the United States of America* 99(12):7821–7826, 2002. PMID: 12060727

Grahame, N.J.; Li, T.K.; and Lumeng, L. Limited access alcohol drinking in high- and low-alcohol preferring selected lines of mice. *Alcoholism: Clinical and Experimental Research* 23(6):1015– 1022, 1999. PMID: 10397285

Grieve, S.J., and Littleton, J.M. Age and strain differences in the rate of development of functional tolerance to ethanol by mice. *Journal of Pharmacy and Pharmacology* 31(10):696–700, 1979. PMID: 41043

Horvath, S., and Dong, J. Geometric interpretation of gene coexpression network analysis. *PLoS Computational Biology* 4(8):e1000117, 2008. PMID: 18704157

Inoue, M.; Rashid, M.H.; Fujita, R.; et al. Initiation of neuropathic pain requires lysophosphatidic acid receptor signaling. *Nature Medicine* 10(7):712–718, 2004. PMID: 15195086

Jansen, R.C., and Nap, J.P. Genetical genomics: The added value from segregation. *Trends in Genetics* 17(7):388–391, 2001. PMID: 11418218

Jeong, H.; Tombor, B.; Albert, R.; et al. The large-scale organization of metabolic networks. *Nature* 407(6804): 651–654, 2000. PMID: 11034217

Kerns, R.T.; Ravindranathan, A.; Hassan, S.; et al. Ethanol-responsive brain region expression networks: Implications for behavioral responses to acute ethanol in DBA/2J versus C57BL/6J mice. *Journal of Neuroscience* 25(9): 2255–2266, 2005. PMID: 15745951

Kirstein, S.L.; Davidson, K.L.; Ehringer, M.A.; et al. Quantitative trait loci affecting initial sensitivity and acute functional tolerance to ethanol-induced ataxia and brain cAMP signaling in BXD recombinant inbred mice. *Journal of Pharmacology and Experimental Therapeutics* 302(3):1238–1245, 2002. PMID: 12183685

Kril, J.J., and Halliday, G.M. Brain shrinkage in alcoholics: A decade on and what have we learned? *Progress in Neurobiology* 58(4):381– 387, 1999. PMID: 10368034

Langfelder, P., and Horvath, S. WGCNA: An R package for weighted correlation network analysis. *BMC Bioinformatics* 9:559, 2008. PMID: 19114008

Lewohl, J.M.; Wang, L.; Miles, M.F.; et al. Gene expression in human alcoholism: Microarray analysis of frontal cortex. *Alcoholism: Clinical and Experimental Research* 24(12):1873–1882, 2000. PMID: 11141048

Markel, P.D.; Bennett, B.; Beeson, M.; et al. Confirmation of quantitative trait loci for ethanol sensitivity in long-sleep and short-sleep mice. *Genome Research* 7(2):92–99, 1997. PMID: 9049627

Matas-Rico, E.; Garcia-Diaz, B.; Llebrez-Zayas, P.; et al. Deletion of lysophosphatidic acid receptor LPA1 reduces neurogenesis in the mouse dentate gyrus. *Molecular and Cellular Neurosciences* 39(3):342–355, 2008. PMID: 18708146

Mayfield, R.D.; Lewohl, J.M.; Dodd, P.R.; et al. Patterns of gene expression are altered in the frontal and motor cortices of human alcoholics. *Journal of Neurochemistry* 81(4):802–813, 2002. PMID: 12065639

Metten, P., and Crabbe, J.C. Common genetic determinants of severity of acute withdrawal from ethanol, pentobarbital and diazepam in inbred mice. *Behavioural Pharmacology* 5(4 and 5):533–547, 1994. PMID: 11224305

Morozova, T.V.; Mackay, T.F.; and Anholt, R.R. Transcriptional networks for alcohol sensitivity in Drosophila melanogaster. *Genetics* 187(4): 1193–1205, 2011. PMID: 21270389

Mozhui, K.; Ciobanu, D.C.; Schikorski, T.; et al. Dissection of a QTL hotspot on mouse distal chromosome 1 that modulates neurobehavioral phenotypes and gene expression. *PLoS Genetics* 4(11): e1000260, 2008. PMID: 19008955

Mulligan, M.K.; Ponomarev, I.; Hitzemann, R.J.; et al. Toward understanding the genetics of alcohol drinking through transcriptome meta-analysis. *Proceedings of the National Academy of Sciences of the United States of America* 103(16):6368–6373, 2006. PMID: 16618939

Mulligan, M.K.; Rhodes, J.S.; Crabbe, J.C.; et al. Molecular profiles of drinking alcohol to intoxication in C57BL/6J mice. *Alcoholism: Clinical and Experimental Research* 35(4):659–670, 2011. PMID: 21223303

Nicolae, D.L.; Gamazon, E.; Zhang, W.; et al. Trait-associated SNPs are more likely to be eQTLs: Annotation to enhance discovery from GWASs. *PLoS Genetics* 6(4): e1000888, 2010. PMID: 20369019

Phillips, T.J.; Brown, K.J.; Burckhart-Kasch, S.; et al. Alcohol preference and sensitivity are markedly reduced in mice lacking dopamine D2 receptors. *Nature Neuroscience* 1(7):610–615, 1998. PMID: 10196569

Rask, E.; Walker, B.R.; Söderberg, S.; et al. Tissue-specific changes in peripheral cortisol metabolism in obese women: Increased adipose 11beta-hydroxysteroid dehydrogenase type 1 activity. *Journal of Clinical Endocrinology and Metabolism* 87(7):3330–3336, 2002. PMID: 12107245

Reimers, M. A.; Riley, B. P.; Kalsi, G.; et al. Pathway based analysis of genotypes in relation to alcohol dependence. *Pharmacogenomics Journal,* 2011, April 5 [Epub ahead of print]. PMID: 21468025

Rimondini, R.; Arlinde, C.; Sommer, W.; and Heilig, M. Long-lasting increase in voluntary ethanol consumption and transcriptional regulation in the rat brain after intermittent exposure to alcohol. *FASEB Journal* 16(1):27–35, 2002. PMID: 11772933

Ruano, D.; Abecasis, G.R.; Glaser, B.; et al. Functional gene group analysis reveals a role of synaptic heterotrimeric G proteins in cognitive ability. *American Journal of Human Genetics* 86(2):113–125, 2010. PMID: 20060087

Saba, L.; Bhave, S.V.; Grahame, N.; et al. Candidate genes and their regulatory elements: Alcohol preference and tolerance. *Mammalian Genome* 17(6):669–688, 2006. PMID: 16783646

Saito, M.; Szakall, I.; Toth, R.; et al. Mouse striatal transcriptome analysis: Effects of oral self-administration of alcohol. *Alcohol* 32(3):223–241, 2004. PMID: 15282116

Schadt, E.E.; Lamb, J.; Yang, X.; et al. An integrative genomics approach to infer causal associations between gene expression and disease. *Nature Genetics* 37(7):710–717, 2005. PMID: 15965475

Schadt, E.E.; Monks, S.A.; Drake, T.A.; et al. Genetics of gene expression surveyed in maize, mouse and man. *Nature* 422(6929):297–302, 2003. PMID: 12646919

Schuckit, M. A. Subjective responses to alcohol in sons of alcoholics and control subjects. *Archives of General Psychiatry* 41(9):879–884, 1984. PMID: 6466047

Schuckit, M.A. Low level of response to alcohol as a predictor of future alcoholism. *American Journal of Psychiatry* 151(2):184–189, 1994. PMID: 8296886

Scott, R.E.; White-Grindley, E.; Ruley, H.E.; et al. P2P-R expression is genetically coregulated with components of the translation machinery and with PUM2, a translational repressor that associates with the P2P-R mRNA. *Journal of Cellular Physiology* 204(1):99–105, 2005. PMID: 15617101

Stuart, J.M.; Segal, E.; Koller, D.; and Kim, S.K. A gene-coexpression network for global discovery of conserved genetic modules. *Science* 302 (5643):249–255, 2003. PMID: 12934013

Tabakoff, B.; Saba, L.; Printz, M.; et al. Genetical genomic determinants of alcohol consumption in rats and humans. *BMC Biology* 7:70, 2009. PMID: 19874574

Treadwell, J.A., and Singh, S.M. Microarray analysis of mouse brain gene expression following acute ethanol treatment. *Neurochemical Research* 29(2):357–369, 2004. PMID: 15002731

van't Veer, L.J.; Dai, H.; van de Vijver, M.J.; et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 415(6871): 530–536, 2002. PMID: 14654526

van Noort, V.; Snel, B.; and Huynen, M.A. The yeast coexpression network has a small-world, scale-free architecture and can be explained by a simple model. *EMBO Reports* 5(3):280–284, 2004. PMID: 14968131

Watts, D.J., and Strogatz, S. H. Collective dynamics of "small-world" networks. *Nature* 393(6684): 440–442, 1998. PMID: 9623998

Wickham, H. *ggplot2: Elegant Graphics for Data Analysis.* New York: Springer, 2009.

Williams, R.W.; Bennett, B.; Lu, L.; et al. Genetic structure of the LXS panel of recombinant inbred mouse strains: A powerful resource for complex trait analysis. *Mammalian Genome* 15(8):637– 647, 2004. PMID: 15457343

Williams, R.W.; Gu, J.; Qi, S.; and Lu, L. The genetic structure of recombinant inbred mice: High-resolution consensus maps for complex trait analysis. *Genome Biology* 2(11):RESEARCH0046, 2001. PMID: 11737945

Wolen, A.R.; Phillips, C.A.; Langston, M.A.; et al. Genetic dissection of acute ethanol respoonsive gene networks in prefrontal cortex; Functional and mechanistic implications. *PLoS,* in press.

York, T.P.; Miles, M.F.; Kendler, K.S.; et al. Epistatic and environmental control of genome-wide gene expression. *Twin Research and Human Genetics* 8(1):5–15, 2005. PMID: 15836804

Zhang, B., and Horvath, S. A general framework for weighted gene co-expression network analysis. *Statistical Applications in Genetics and Molecular Biology* 4:Article17, 2005. PMID: 16646834

Zhu, J.; Lum, P.Y.; Lamb, J.; et al. An integrative genomics approach to the reconstruction of gene networks in segregating populations. *Cytogenetic and Genome Research* 105(2–4):363–374, 2004. PMID: 15237224

Epigenetics—Beyond the Genome in Alcoholism

Bela G. Starkman; Amul J. Sakharkar, Ph.D.; and Subhash C. Pandey, Ph.D.

Bela G. Starkman *is a graduate student in the Departments of Anatomy and Cell Biology and Psychiatry;* **Amul J. Sakharkar, Ph.D.,** *is a research assistant professor in the Department of Psychiatry; and* **Subhash C. Pandey, Ph.D.,** *is a professor and director of Neuroscience Alcoholism Research in the Department of Psychiatry and Department of Anatomy and Cell Biology at the University of Illinois, Chicago, Illinois. Dr. Pandey also is a VA career scientist at the Jesse Brown Veterans Affairs Medical Center, Chicago, Illinois.*

Genetic and environmental factors play a role in the development of alcoholism. Whole-genome expression profiling has highlighted the importance of several genes that may contribute to alcohol abuse disorders. In addition, more recent findings have added yet another layer of complexity to the overall molecular mechanisms involved in a predisposition to alcoholism and addiction by demonstrating that processes related to genetic factors that do not manifest as DNA sequence changes (i.e., epigenetic processes) play a role. Both acute and chronic ethanol exposure can alter gene expression levels in specific neuronal circuits that govern the behavioral consequences related to tolerance and dependence. The unremitting cycle of alcohol consumption often includes satiation and self-medication with alcohol, followed by excruciating withdrawal symptoms and the resultant relapse, which reflects both the positive and negative affective states of alcohol addiction. Recent studies have indicated that behavioral changes induced by acute and chronic ethanol exposure may involve chromatin remodeling resulting from covalent histone modifications and DNA methylation in the neuronal circuits involving a brain region called the amygdala. These findings have helped identify enzymes involved in epigenetic mechanisms, such as the histone deacetylase, histone acetyltransferase, and DNA methyltransferase enzymes, as novel therapeutic targets for the development of future pharmacotherapies for the treatment of alcoholism. **KEY WORDS: Alcoholism; alcohol use disorders; genome; epigenome; genetic factors; epigenetics; DNA; brain; amygdala; neuronal circuits; chromatin; histone; DNA methylation; histone deacetylase; histone acetyltranferase; DNA methyltransferase; treatment**

Alcohol is one of the most widely used addictive drugs, and continued use and abuse can lead to the development of tolerance and dependence (Koob 2003*a*; Tabakoff et al. 1986). Numerous studies have shown that both genetic and environmental risk factors play a role in the development of alcoholism (Ducci and Goldman 2008; Edenberg and Foroud 2006; Farris et al. 2010). Genetic studies in both humans and animal models of alcoholism (Contet et al. 2011; Crabbe et al. 2006; Pignataro et al. 2009; Spanagel et al. 2010; Tabakoff et al. 2009) have identified several genes that may be critical in the pathophysiology of alcoholism (see figure 1). Recently, researchers have identified mechanisms that result in heritable changes in gene expression but are caused by other processes than changes in the underlying DNA sequence (i.e., epigenetic mechanisms) as a promising area of research to better understand the molecular mechanisms of human diseases, including psychiatric and alcohol use disorders (AUDs) (Moonat et al. 2010; Tsankova et al. 2007). This article reviews some of the epigenetic mechanisms that seem to play a role in the development of AUDs.

What Is Epigenetics?

The genome encompasses the complete set of genetic material (i.e., DNA) that determines the development of an organism and all its traits and characteristics (i.e., the phenotype). Changes (i.e., mutations) in the DNA can lead to the development of various diseases, including AUDs. In comparison, the epigenome, as first defined by Waddington (1942), refers to chemical modifications that occur within a genome without changing the DNA sequence (Holliday 2006; Murrell et al. 2005; Waddington 1942). Epigenetic alterations include the direct addition of methyl groups to (i.e., methylation of) DNA and the chemical modification of the proteins around which the DNA is wrapped (i.e., histone proteins) to form the chromosomes. Both of these mechanisms work in concert to remodel the structure of the protein–DNA complex (i.e., the chromatin) and regulate gene

[1] Gene expression is the process of converting the genetic information encoded in the DNA into actual gene products (i.e., proteins). This process involves two steps. In the first step, called transcription, the genetic information comprising one gene is copied into an intermediary molecule called messenger RNA (mRNA). After the mRNA is processed further using a variety of posttranscriptional modifications, it is used as a template to generate proteins in a process called translation.

expression[1] (Kornberg 1974; Olins and Olins 1974; Hsieh and Gage 2005).

Chromatin is made up of units called nucleosomes, which consist of approximately 147 base pairs of DNA wrapped around a complex of eight histone proteins that comprise the histone core (figure 2) (Jenuwine and Allis 2001; Smith 1991). This core structure, which also is referred to as the histone fold domain, consists of a central heterotetramer of histones H3 and H4 and two heterodimers[2] of histones H2A and H2B (Luger et al. 1997; Smith 1991). The histone proteins assemble at one end called the carboxy (C) terminal to form the histone core, with the other end, in the amino (*N*)-terminal "tail" region, projecting out from the histone core (figure 2). This *N*-terminal region is made up mainly of the amino acids lysine and arginine (Hsieh and Gage 2005; Mersfelder and Parthun 2006). The majority of epigenetic histone modifications occur at the *N*-terminal tail of the histones and include methylation as well as addition of acetyl groups (i.e., acetylation), phosphate groups (i.e., phosphorylation), binding of a small molecule called ubiquitin (i.e., ubiquitylation), or addition of a molecule called ADP ribose (i.e., ADP-ribosylation) (Jenuwine and Allis 2001; Smith 1991). The most recognized and well established of these epigenetic modifications include histone acetylation and histone and DNA methylation, which control the accessibility of chromatin to essential transcriptional proteins mediating the first step in the conversion of the genetic information encoded in the DNA into mRNA (i.e., transcription), which then serves as template for the synthesis of protein products in a process called translation. By controlling DNA accessibility, these epigenetic modifications play a crucial role in the regulation of gene transcription (Abel and Zukin 2008; Feng and Fan 2009).

Another epigenetic regulatory mechanism involves microRNAs (miRNAs), a class of noncoding RNAs approximately 21 to 23 building blocks (i.e., nucleotides) in length, which are involved in the posttranscriptional modulation of gene expression and function (Bartel 2009). Altered regulation of these specific epigenetic mechanisms plays a pivotal role during the onset of disease (Bonasio et al. 2010). This review discusses the implications of abnormal chromatin remodeling and miRNAs as major factors in several brain disease processes, including AUDs. It also considers the impact of current epigenetic research on the alcohol field and the therapeutically significant function of compounds called histone deacetylase (HDAC) inhibitors in the prevention and treatment of alcoholism.

Role of Histone Modifications in the Brain During Alcoholism

Histone Acetylation

Histone modification involving acetylation is regulated by two types of enzymes—histone acetyltransferases (HATs), which add acetyl groups to histones, and HDACs, which remove acetyl groups from histones. Both types of enzymes dynamically interact to regulate the remodeling of the chromatin architecture and gene expression (Abel and Zukin 2008; Hsieh and Gage 2005).

Histone Acetyltransferases. Several studies have indicated that regulation of histone acetylation is important during formation of memories in a brain region called the hippocampus (Guan et al. 2009; Levenson et al. 2004). Furthermore, alterations in histone acetylation are associated with age-dependent memory impairment in mice (Peleg et al. 2010). One mechanism through which histone acetylation and thus chromatin remodeling may be regulated involves a protein called cyclic-AMP responsive–element binding (CREB) protein. This protein helps modulate the transcription of certain genes by binding

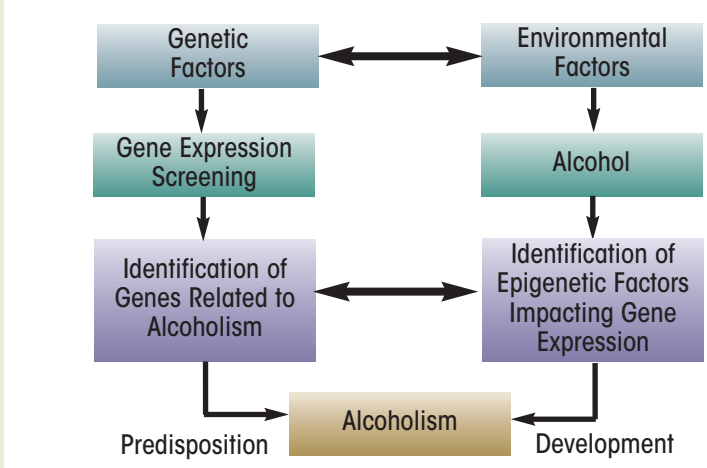


Figure 1 A hypothetical model for the interactions of genetic and environmental factors in the predisposition to and development of alcoholism. Whole genome expression profiling in human and animal models has identified several potential candidate genes that may be associated with increased risk for alcoholism and alcohol abuse. Emerging evidence suggests that genomic function can be modulated by alcohol-induced epigenetic modification of histones (acetylation and methylation) and DNA methylation, which can lead to the development of alcoholism. Both genetic and epigenetic factors may interact in the underlying mechanisms of alcoholism.

[2] Heterotetramers consist of four subunits that are not all identical, and heterodimers consist of two subunits that are not identical.

to a specific sequence on the DNA after it has been activated by phosphorylation. Phosphorylated CREB (pCREB) then recruits another transcriptional co-factor called CREB-binding protein (CBP), which contains intrinsic HAT activity. CBP, along with another molecule called p300, enzymatically remodels the nucleosome by transferring acetyl groups to histones, thereby allowing the chromatin to exist in a relaxed conformation that is accessible for transcription (figure 3) (Chrivia et al. 1993; Hsieh and Gage 2005; Liu et al. 2008; Martinez-Balbas et al. 1998).

CBP is essential for both short-term and long-term memory formation and consolidation (Chen et al. 2010; Korzus et al. 2004). A recent study in a mouse model of Alzheimer's disease expressing abnormal CREB functioning demonstrated that delivery of the CBP gene into the brain increased the levels of a molecule known as brain-derived neurotrophic factor (BDNF), which correlated with improved learning and memory (Caccamo et al. 2010). Of interest, alterations in both memory capacity and responses to stress also have been observed in mice that lack a molecule called p300/CBP-associated factor (PCAF), which has a similar function to CBP and also has HAT activity (Kalkhoven 2004; Maurice et al. 2008).

Correction of deficits in CBP function increasingly has become an important target in the treatment of neurological disorders, including Rubinstein-Taybi syndrome (RSTS), Huntington's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (Klevytska et al. 2010; Rouaux et al. 2004; Selvi et al. 2010). Mutations in the CBP gene are prevalent in RSTS (Alarcon et al. 2004; Bartsch et al. 2005; Roelfsema et al. 2005). For example, exon deletions in the genes that encode p300 and CBP have been detected in children with RSTS (Tsai et al. 2011). The changes in CBP function described in these neurological disorders are similar to neuroadaptations observed in alcoholism; therefore, CBP–HAT may be an important factor in the development of alcoholism (see figures 4 and 5).

It is well established that CREB protein functioning is important during long-term memory formation, synaptic plasticity, and addiction (Abel and Zukin 2008; Alberini 2009; Carlezon et al. 2005; Pandey et al. 2004). CREB mediates the activity of several signaling cascades, including cyclic-AMP–dependent protein kinase A, Ca^{2+}/calmodulin-dependent protein kinases (CaMKs), and mitogen-activated protein kinase (Lonze and Ginty 2002; Pandey 2004). Studies from several laboratories have suggested a critical role for CREB in the regulation of alcohol's effects and alcohol-related behaviors:

- Alcohol-induced damage to nerve cells (i.e., neurodegeneration) in the hippocampus was associated with decreased CREB functioning (Crews and Nixon 2009).

- Sensitivity to alcohol's effects is associated with CREB transcriptional activity in another part of the brain called the cerebellum (Acquaah-Mensah et al. 2006). In addition, chronic ethanol exposure decreases CREB phosphorylation in the rat cerebellum (Yang et al. 1998).

- Abnormal CREB functioning in the amygdala—an important brain region implicated in regulating emotions—has been linked with a predisposition to anxiety[3] and alcoholism (Pandey et al. 2004, 2005; Wand 2005). Amygdaloid pCREB and CBP levels both are significantly increased by acute ethanol exposure and decreased in rats undergoing withdrawal after chronic ethanol exposure (Pandey et al. 2008*a,b*). These results suggest that changes in CBP levels may be involved in the dynamic chromatin remodeling in the amygdala caused by acute and chronic ethanol exposure (see figures 4 and 5).

CREB signaling also helps regulate the expression of genes implicated in alcohol addiction, such as BDNF and neuropeptide Y (NPY) (Janak et al.

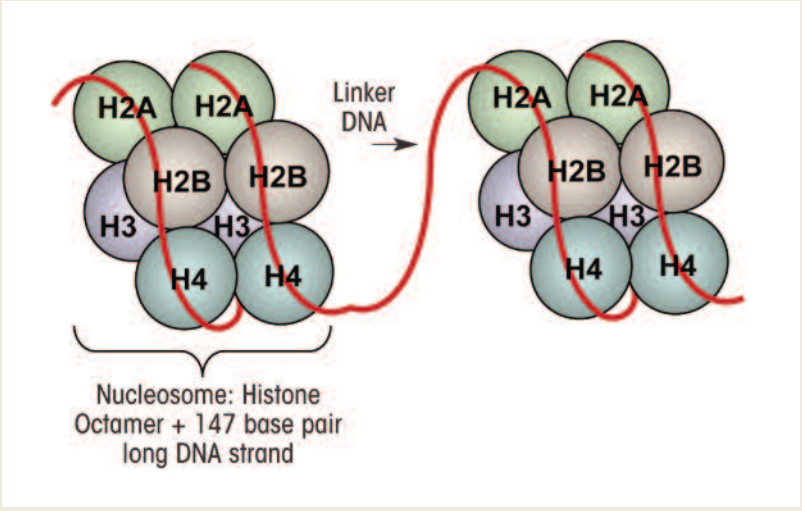


Figure 2 Schematic representation of units of chromatin known as nucleosomes. Each nucleosome is comprised of 147 base pairs of DNA wrapped around the histone octamer made up of a heterotetramer of histones H3 and H4 and two heterodimers of H2A and H2B. Two nucleosomes are connected by the linker DNA.

[3] Anxiety is an early withdrawal symptom that plays a major role in relapse, helping to maintain a dependent state in certain alcoholics (Cooper et al. 1995; Koob 2003a; Kushner et al. 1990; Menzaghi et al. 1994).

2006; Moonat et al. 2010; Pandey 2003; Valdez and Koob 2004). Lower NPY mRNA and protein levels have been found in the central (CeA) and medial nucleus of amygdala (MeA) of alcohol-preferring (P) rats compared with alcohol nonpreferring (NP) rats, suggesting a role for NPY in anxiety-like and alcohol-drinking behaviors (Pandey et al. 2005; Suzuki et al. 2004). NPY infusion into the CeA produced anxiety-reducing (i.e., anxiolytic) effects and decreased alcohol intake in P rats, most likely by leading to increased CaMK IV–dependent CREB phosphorylation in the CeA (Pandey et al. 2005; Zhang et al. 2010). Furthermore, P rats that had never been exposed to alcohol (i.e., were alcohol naïve) exhibited lower expression of other CREB target genes, such as BDNF and activity-regulated cytoskeleton-associated protein (Arc) in the CeA and MeA compared with NP rats (Moonat et al. 2011; Prakash et al. 2008). Finally, acute ethanol exposure increased CREB phosphorylation levels as well as the expression of BDNF and Arc in these amygdaloid structures of P, but not NP, rats (Moonat et al. 2011; Pandey et al. 2005).

Gamma-aminobutyric acid (GABA) is the main inhibitory brain signaling molecule (i.e., neurotransmitter), and alcohol is thought to produce many of its effects by facilitating GABA receptor functioning (Koob 2003*a*; Koob et al. 1998; Kumar et al. 2009). GABA activity in the amygdala is enhanced by exposure to alcohol and then decreased during alcohol withdrawal (Clapp et al. 2008; Koob 2003*a*). CREB seems to play a role in this process. A recent study showed that expression of one type of GABA receptors (i.e., the $GABA_A$ receptor) was dependent on the formation of CREB heterodimers (Hu et al. 2008). Despite the tremendous amount of work that has been done on CREB, the epigenetic mechanisms for the regulation of gene expression via CREB and CBP interactions and its overall implications in alcoholism have not been well established and need to be investigated further.

Histone Deacetylases. HDACs are enzymes involved in covalent histone modifications that oppose the activity of HATs by inducing the removal of acetyl groups, thereby resulting in a condensed chromatin conformation and decreased gene expression levels in the cell (Grunstein 1997; Hsieh and Gage 2005; Turner 2002). Four distinct families of HDACs have been described, including class I (HDACs 1, 2, 3, and 8), class II (HDACs 4, 5, 6, 7, 9, and 10), class III (sirtuins), and class IV (HDAC 11). Class I HDACs mostly are found in the cell nucleus, whereas class II HDACs can be localized in the cytosol and/or nucleus. The class IV HDAC is located in the nucleus. Class I, II, and IV HDACs require zinc for proper functioning (i.e., are Zn^{2+} dependent). In contrast, class III HDACs require a molecule called nicotinamide adenosine dinucleotide (i.e., are NAD^+ dependent) (Grayson et al. 2010; Kelly and Marks 2005; Lee et al. 2008).

Because of their epigenetic effects on chromatin structure and the role of epigenetic factors in the development of many tumors, HDAC inhibitors have been considered promising anticancer drugs for several years. Several of these agents currently are in clinical trials, and one of them, vorinostat (suberoylanilide hydroxamic acid), has been approved by the U.S. Food and Drug Administration for the treatment of a type of blood cancer (i.e, cutaneous T-cell lymphoma) (Dickinson et al. 2010; Dokmanovic and Marks 2005; Kelly and Marks 2005; Lee at al. 2008). Recent neuroscience research also advocates HDAC inhibitors as novel treatments for psychiatric and neurodegenerative disorders (Abel and Zukin 2008; Kanzantsev and Thompson 2008; Tsankova et al. 2007). For example, inhibitors of class I HDACs were able to reverse contextual memory deficits in a mouse model of AD; more specifically, HDAC 2 was involved in the negative regulation of learning and memory and related gene expression involved in synaptic plasticity (Guan et al. 2009; Kilgore et al. 2010). In addition, HDAC inhibitor treatment targeting HDAC 2 and HDAC 5 may have antidepressant effects (Covington et al. 2009; Tsankova et al. 2006). Finally, several studies assessing the modulatory effects of HDAC inhibition on cocaine abuse have indicated that these agents also may be beneficial in treating addictive processes related to drugs of abuse (Febo et al. 2009; Malvaez et al. 2010; Romieu et al. 2008; Wang et al. 2010).

Despite the abundance of research on the effects of HDAC inhibition on psychiatric disorders and drugs of

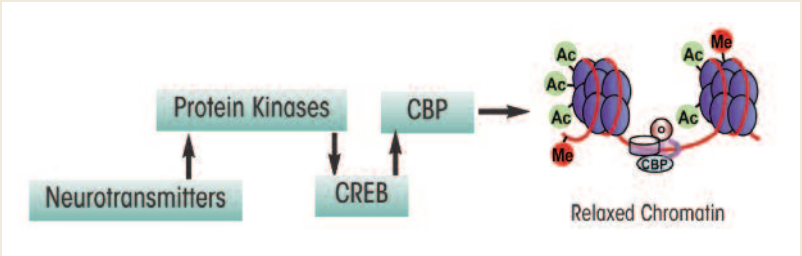


Figure 3 A schematic representation of the signaling pathway showing the possible role for cyclic-AMP responsive element binding (CREB) protein and CREB-binding protein (CBP) in the epigenetic regulation of gene expression. CREB is activated after its phosphorylation (pCREB) by different protein kinases, which further recruits CBP to bind to the DNA. CBP also has intrinsic histone acetyltransferase (HAT) activity by which it increases histone aceylation and relaxes the chromatin, which in turn makes the DNA more accessible to the transcriptional machinery and thereby facilitates gene transcription.

abuse, the evaluation of HDAC inhibition in alcoholism research still is in its infancy. Emerging evidence from animal and human studies indicates that negative emotionality (e.g., anxiety or depression) is important in alcohol dependence, and sensitivity to anxiety may serve as a risk factor for AUDs (Cooper et al.1995; Koob 2003*a*; Kushner et al. 1990; Pandey 2003). Alcohol has anxiolytic effects in humans, and rapid tolerance to these effects may stimulate alcohol drinking in alcoholics (Lipscomb et al. 1980; Moberg and Curtin 2009). Accordingly, rapid tolerance to alcohol's anxiolytic effects may be crucial in the promotion of alcohol drinking and thereby contributes to the pathogenesis of alcohol dependence. In this context, recent discoveries involving the role of HDACs in alcohol tolerance and dependence are very promising. For example, studies have suggested that HDAC-induced chromatin remodeling in the amygdala may regulate the development of anxiety-like behaviors during ethanol withdrawal after chronic exposure and also may be paramount in rapid tolerance to the anxiolytic effects of ethanol in rats. The studies also found that HDAC activity was inhibited by acute ethanol in the amygdala of rats, and the expression of rapid tolerance to the anxiolytic effects of ethanol was reversed by treatment with a potent HDAC inhibitor, trichostatin A (TSA) (Pandey et al. 2008*a*; Sakharkar et al. 2012). Conversely, HDAC activity was increased in the amygdala of alcohol-withdrawn rats, which correlated with decreased histone acetylation and reduced NPY gene expression in the CeA and MeA of rats (Pandey et al. 2008*a*). Treatment with TSA during ethanol withdrawal prevented the development of anxiety-like behaviors and corrected the deficits in histone acetylation and NPY expression. These data suggest that epigenetic modifications involving HDACs result in a refinement of amygdaloid chromatin structure, which may be a contributing factor that alters the expression of genes implicated in alcohol tolerance and dependence (figures 4 and 5).

Histone Methylation

Methylation of the chromatin (i.e., of both histones and DNA) is another important mechanism by which the cell regulates transcriptional activity (Holliday 2006). Histone methylation is mediated by histone methyltransferases (HMTs), whereas histone demethylation occurs via histone demethylases (HDMs) (Agger et al. 2008; Cheung and Lau 2005; Margueron et al. 2005; Shukla et al. 2008; Wysocka et al. 2006). Histone methylation is important in the regulation of memory formation (Gupta et al. 2010). Aberrant histone methylation at lysine residues has been implicated in several different psychi-

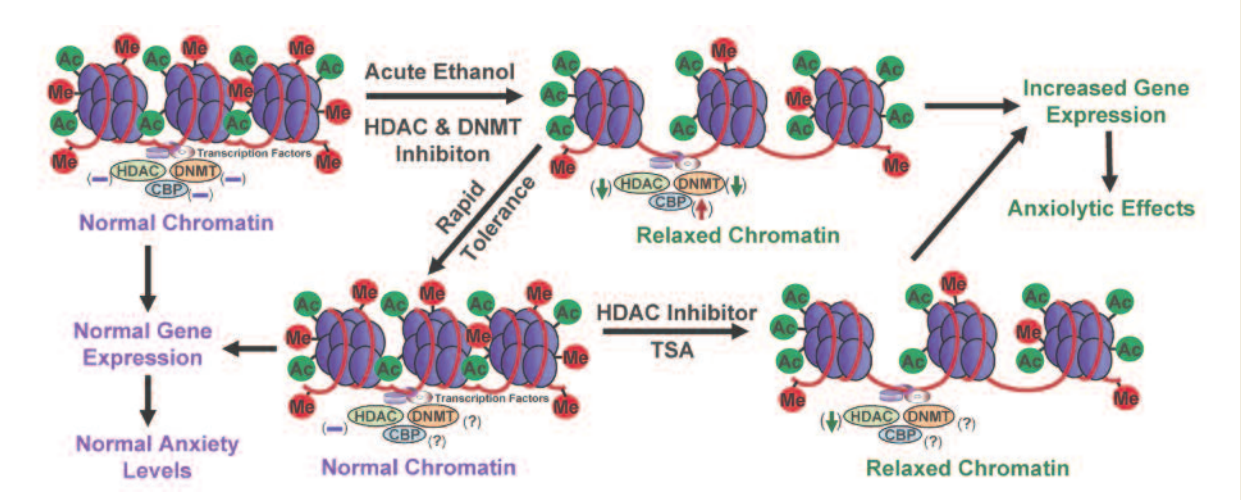


Figure 4 A schematic diagram depicting possible epigenetic mechanisms acting in neuronal circuits of the amygdala that may contribute to rapid tolerance to the anxiolytic effects of ethanol. Acute ethanol exposure can inhibit both histone deacetylase (HDAC) and DNA methyltransferase (DNMT) activities (Pandey et al. 2008*a*). This inhibition correlates with increased amygdaloid levels of CREB-binding protein (CBP), which has histone acetyltransferase (HAT) activity. The observed changes in HDAC activity and CBP levels also correlate with increased histone acetylation (H3-K9 and H4-K8) in the central and medial nucleus of amygdala, resulting in a relaxed chromatin structure. As a result, the transcriptional machinery can more easily access the DNA, leading to increased gene expression. Increased amygdaloid expression of certain genes, such as neuropeptide Y or brain-derived neurotrophic factor, may mediate the anxiolytic effects of acute ethanol exposure (Pandey et al. 2008*a, b*). Cellular tolerance at the level of HDAC-induced chromatin remodeling in the amygdala may be operative in rapid tolerance to the anxiolytic effects of ethanol (Sakharkar et al. 2012).

NOTE: (↓) = decrease; (↑) = increase; (–) = normal; (?) = unknown; Me = methylation site; Ac = acetylation site.

atric disorders (Akbarian and Huang 2009). For instance, mutations within genes encoding specific HMTs and HDMs have been associated with autism and mental retardation, respectively (Balemans et al. 2010; Tahiliani et al. 2007). In addition, chromatin remodeling at GABAergic gene promoters by a certain HMT may be involved in prefrontal cortex dysfunction during schizophrenia (Huang et al. 2007). Histone methylation, specifically involving histone H3, also has been linked with the expression levels for the receptor for the neurotransmitter glutamate, which has been implicated in schizophrenia because a hallmark of this disease is altered glutamate receptor functioning (Chavez-Noriega et al. 2002; Conn et al. 2009; Stadler et al. 2005). Tian and colleagues (2009) recently demonstrated that stimulation of a specific glutamate receptor (i.e., the *N*-methyl-D-aspartate [NMDA] receptor[4]) was associated with a decrease in histone H3 methylation and a concomitant increase in H3 acetylation at lysine 9 in rat hippocampal neurons. These findings also were linked with increased transcriptional activity of CREB and CBP at the BDNF gene promoter region 1. Taken together, these studies suggest that histone methylation may play an important role in the preservation of neuronal function by regulating expression of synaptic plasticity-associated genes.

Epigenetic Modifications in Peripheral Tissues During Alcoholism

Histone Acetylation and Methylation

Alcohol exposure not only affects the brain, leading to tolerance and dependence, but also can have serious harmful effects in other organs. For example, alcohol has deleterious effects on the liver that are associated with epigenetic changes (Shukla et al. 2008). Alcoholic liver disease is a debilitating condition associated with chronic alcohol abuse (Beier and McClain 2010). Studies in intact organisms (i.e., in vivo studies) have shown an increase in histone acetylation in the liver, lungs, spleen, and testes of rats acutely treated with ethanol (Kim and Shukla 2006). A study in isolated rat liver cells (i.e., primary rat hepatocytes) indicated that ethanol induced the acetylation of histone H3 at lysine 9 (Park et al. 2003). Also, by activating HAT, ethanol exposure caused increased histone acetylation (H3–lysine 9) in a promoter region of the gene encoding the alcohol-metabolizing enzyme alcohol dehydrogenase 1 (ADH 1) in rat hepatocytes (Park et al. 2005). In addition, exposure of hepatocytes to ethanol was associated with distinct histone H3 methylation patterns at lysine 9 and lysine 4, which correlated with increases and decreases, respectively, in gene expression (Pal-Bhadra et al. 2007).

Researchers recently demonstrated that another protein-modifying pathway (i.e., the ubiquitin–proteasome pathway[5]) is involved in epigenetic mechanisms regulating liver injury in alcoholic liver disease (Oliva et al. 2009). The investigators found that chronic ethanol feeding significantly inhibited the ubiquitin–proteasome pathway in rats, which was associated with an increase in histone acetylation and a decrease in histone methylation. Taken together, these data clearly suggest a promising epigenetic target for the treatment of ethanol-induced liver injury.

DNA Methylation in the Brain and Periphery During Alcoholism

In addition to histone methylation, DNA methylation is an important epigenetic regulatory mechanism of gene expression that often can result in gene silencing (Comb and Goodman 1990; Feng and Fan 2009). DNA methylation is more specific than histone methylation and occurs in specific DNA sequences[6] (Antequera 2003; Bestor 2000; Okano et al. 1999). DNA methylation within regulatory regions (i.e., promoters) of a gene has long been known to attenuate gene expression. More recently, however, DNA methylation within the gene also has been shown to regulate tissue- and cell-specific gene expression (Feng and Fan 2009; Maunakea et al. 2010).

DNA methylation patterns are established and maintained by enzymes called DNA methyltransferases (DNMTs); these patterns then are recognized by specific proteins (Robertson 2005; Sharma et al. 2010). Three main types of DNMTs regulate DNA methylation. For example, DNMT 1 maintains DNA methylation patterns, whereas DNMT 3a and DNMT 3b are involved in the de novo methylation of DNA (Okano et al. 1999). Similar to histones, DNA also undergoes removal of methyl groups (i.e., demethylation), and some enzymes involved in this process putatively have been identified (Bhattacharya et al. 1999; Metivier et al. 2008; Ooi and Bestor 2008).

DNMTs are abundantly expressed in postmitotic nerve cells (i.e., neurons) and are important for normal learning and memory (Feng et al. 2010). For example, an experimental approach in which animals learned to exhibit fear in a specific situation (i.e., contextual fear conditioning) was shown to increase the expression of DNMT 3a and 3b, but not DNMT 1 in the adult hippocampus, whereas infusion of a DNMT inhibitor blocked memory formation (Miller and Sweatt 2007). Furthermore, La Plant and colleagues (2010) found that DNMT3a expres-

[4] NMDA receptors mediate excitatory neurotransmission in the brain. These ubiquitous receptors play a role in the development of neurons and synaptic plasticity (Wheal et al. 1998). Modulation of NMDA receptor function may mediate the development of tolerance to alcohol as well as alcohol withdrawal symptoms (Kumari and Ticku 2000).

[5] The ubiquitin–proteasome pathway is a series of reactions by which unwanted or defective proteins are marked for degradation by the addition of ubiquitin molecules and subsequently destroyed by large protein complexes known as proteasomes. Accurate functioning of this pathway is essential for many cellular processes, the cell cycle, and regulation of gene expression.

[6] Specifically, DNA methlylation occur at the 5′-position of cytosines found in cytosine–guanosine (CpG) dinucleotides within CpG-rich DNA regions (i.e., CpG islands).

sion was enhanced in a brain region called the nucleus accumbens by chronic cocaine use and chronic social stress, suggesting an important role for this enzyme in regulating emotional behavior and cocaine addiction.

DNA methylation also may play a role in psychiatric disorders, including alcohol and other drug dependence. For example, schizophrenia often is characterized by dysfunction of the GABA-using (i.e., GABAergic) signaling systems (Costa et al. 2003; Wassef et al. 2003). GABAergic neurons express high levels of DNMT 1, and excessive methylation (i.e., hypermethylation) of the promoter regions of genes involved in the GABA system may be a possible target for treating schizophrenia (Costa et al. 2007, 2009).

To date, no studies have assessed how DNMT enzymes are regulated during adaptive changes to ethanol exposure in the brain. Preliminary investigations from the author's laboratory have suggested that acute ethanol exposure can significantly inhibit DNMT activity in the amygdala of rats. Other studies have shown that alcoholics have lower DNMT 3a and 3b expression in whole blood cells, with greater reductions found with higher blood ethanol concentrations (Bonsch et al. 2006). In addition, several other genes seem to be regulated through epigenetic mechanisms involving DNA methylation:

- The protein α-synuclein has been implicated in alcohol craving, and both the protein and mRNA levels of α-synuclein are elevated with chronic alcohol administration (Bonsch et al. 2005*a*; Walker and Grant 2006). Studies of human alcoholics found DNA hyperme-

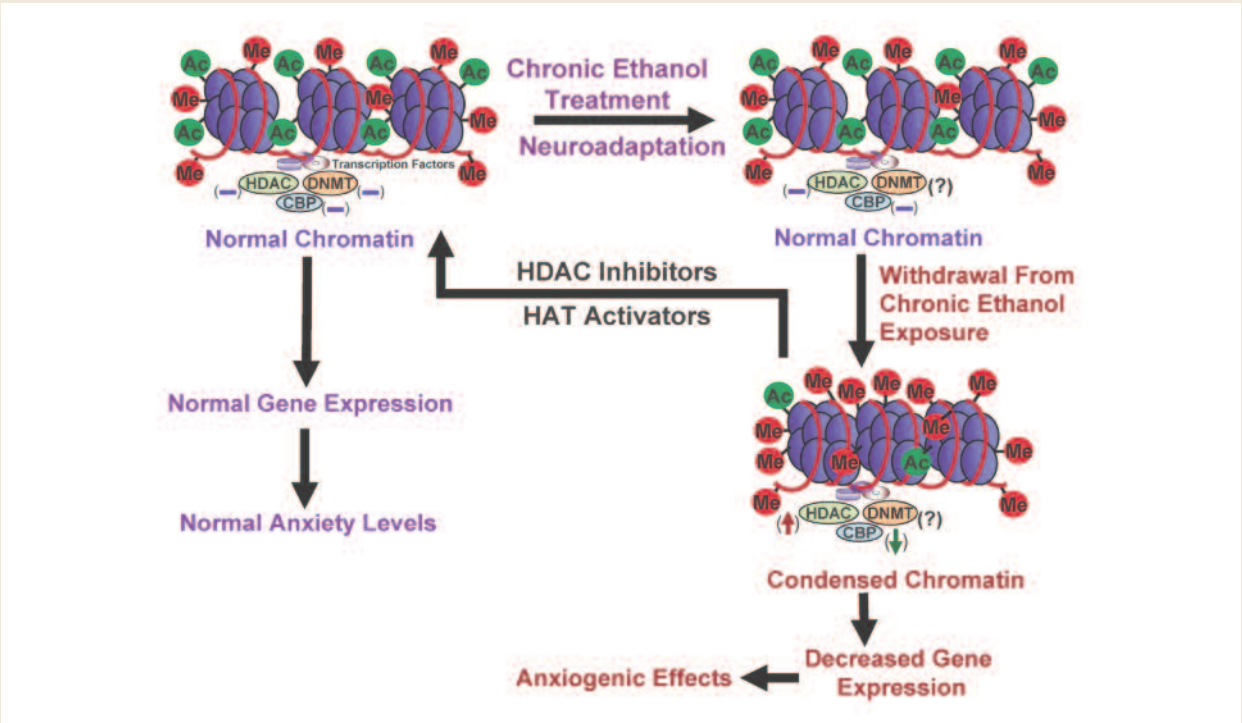


Figure 5 A representative model of possible epigenetic mechanisms acting in neuronal circuits of the amygdala that may contribute to the development of anxiety-like behaviors during ethanol withdrawal after chronic exposure in rats. Chronic ethanol exposure and the concomitant neuroadaptations in the amygdala of rats do not significantly affect levels of histone acetylation, CREB-binding protein (CBP), or histone deacetylase (HDAC) activity, because these were altered by acute ethanol exposure (see figure 4). However, withdrawal after chronic ethanol exposure is associated with increased HDAC activity and decreased levels of CBP and associated histone acetylation (Pandey et al. 2008*a*). As a result, the chromatin configuration may become more condensed, which limits accessibility of the transcriptional machinery to the DNA. This may result in decreased gene expression levels of neuropeptide Y and brain-derived neurotrophic factor, both of which have been linked to increased anxiety-like behaviors (i.e., have anxiogenic effects) following withdrawal from chronic ethanol exposure (Pandey et al. 2008*a,b*). HDACs and histone acetyltransferases (HATs) are promising targets in the possible reversal of these anxiogenic consequences of withdrawal. Thus, pharmacological treatment using potent HDAC inhibitors or HAT activators may lead to the normalization of reduced histone acetylation and subsequent stabilization of gene expression and anxiety levels.

NOTE: (↓) = decrease; (↑) = increase; (–) = normal; (?) = unknown; Me = methylation site; ac = acetylation site

thylation of the promoter region for the α-synuclein gene in a type of white blood cells (i.e., peripheral mononuclear cells), which may be responsible for reduced craving under active alcohol-drinking conditions (Bonsch et al. 2005*b*; Bleich and Hillemacher 2009).

- Methylation also affects transcription of a gene encoding the endogenous opioid prodynorphin, a small protein (i.e., polypeptide) hormone involved in brain signaling that exists in several variants (i.e., alleles) that influence a person's risk of alcoholism. A DNA variation (i.e., single nucleotide polymorphism) of this gene exists that overlaps with a CpG dinucleotide, which is subject to methylation. Methylation of this dinucleotide affects prodynorphin transcription in the prefrontal cortex of alcoholics (Taqi et al. 2011). Moreover, people who carried a nonrisk allele of the prodynorphin gene but in whom the CpG dinucleotide was methylated had a higher probability of developing alcohol dependence.

- Expression of the gene encoding a protein called homocysteine-induced endoplasmic reticulum protein (Herp) is lower in blood cells of alcoholics compared with healthy controls. This difference is related to promotor hypermethylation within the Herp gene in alcoholics (Bleich and Hillemacher 2009).

- The expression of the genes encoding the NMDA receptors, which as mentioned earlier are activated by the neurotransmitter glutamate, also seems to be regulated by DNA methylation. Thus, a clinical study demonstrated that in alcoholic patients the degree of methylation at the promoter region of the gene encoding the NMDA 2B receptor subtype (*NR2B*) during withdrawal negatively correlated with the severity of alcohol consumption (Biermann et al. 2009). Moreover, in primary cultured neurons, chronic intermittent ethanol exposure resulted in demethylation of a regulatory region of the *NR2B* gene, which correlated with increased *NR2B* gene expression (Qiang et al. 2010).

All of these findings suggest that ethanol-induced changes in DNA methylation may be an important factor in the regulation of gene expression that occurs during the complex processes of alcoholism pathogenesis.

DNA methylation represses gene expression via the actions of a protein called methyl CpG-binding protein-2 (MeCP2), which selectively binds to methylated DNA, thereby blocking transcription. Mutations in MeCP2 have been linked with a neurodevelopmental disorder, Rett syndrome (Bienvenu and Chelly 2006). Moreover, MeCP2 has been implicated in regulating behavioral responses to drugs of abuse (Feng and Nestler 2010). For example, changes in MeCP2 in the nucleus accumbens have been shown to contribute to the neural and behavioral responses to psychostimulants (Deng et al. 2010). MeCP2 also can control BDNF expression and cocaine intake (Im et al. 2010). Through its effects on BDNF expression, MeCP2 also may be important in regulating alcohol's addictive properties (He et al. 2010), and BDNF expression is altered in various brain regions by acute and chronic ethanol exposure (Jeanblanc et al. 2009; Moonat et al. 2011; Pandey et al.2008*b*).

Role of DNA Methylation in Fetal Alcohol Spectrum Disorders

Fetal alcohol spectrum disorders (FASD) are developmental abnormalities related to the effects of in utero alcohol exposure on the developing fetus (Jones and Smith 1973; Jones et al. 1973). Several recent studies have emphasized how alcohol exposure can result in aberrant epigenetic regulatory mechanisms during development, leading to FASD. For example, alcohol consumption by the mother altered DNA methylation profiles in mouse embryos, resulting in neurofacial deficits and growth retardation, both of which are hallmarks of FASD (Liu et al. 2009). A recent study linked chronic alcohol use in men with lower-than-normal methylation (i.e., hypomethylation) of paternal sperm DNA, suggesting that genes from alcoholic males transferred through fertilization may result in offspring with FASD features (Ouko et al. 2009). Likewise, Weaver and colleagues (2004) demonstrated that maternal behavior was able to produce stable alterations in DNA methylation and chromatin structure in the hippocampus of their offspring that persisted into adulthood. However, this epigenetic maternal programming was reversible in adult offspring through methyl supplementation, suggesting that DNA methylation patterns that are formed early in life may not be permanent (Weaver et al. 2005). Finally, alcohol exposure seems to interfere with normal DNA methylation patterns of neural stem cell genes and to attenuate neural stem cell differentiation (Zhou et al. 2011). Taken together, these findings imply that epigenetic processes may play an important role in the mechanisms underlying FASD (Miranda 2011).

miRNAs and Gene Expression in Alcoholism

In addition to mRNA, which is generated during gene expression and represents the coding sequences of the genes, several classes of noncoding RNA molecules exist that have regulatory functions. One of these classes is miRNA, which has been implicated in the regulation of gene expression and synaptic plasticity (Siegel et al. 2011). miRNAs control gene expression by interfering with the intricate processes of mRNA translation into a protein product and/or mRNA decay (Bartel 2009; Ghildiyal and Zamore 2009). Recent findings suggest that complex interactions occur between miRNAs and the epigenetic machinery (Bonasio et al. 2010). Likewise, other molecules known as large intergenic noncoding

(linc) RNAs interact with chromatin-modifying complexes and regulate gene expression (Khalil et al. 2009).

Several studies have implicated miRNAs in the cellular effects of ethanol use and abuse (Miranda 2010; Pietrzykowski 2010). For example, miRNA-9 (miR-9) posttranscriptionally regulates big potassium (BK) channel mRNA variants that encode different types of BK channels with different sensitivities to alcohol (Pietrzykowski et al. 2008). Acute ethanol exposure increased the expression of miR-9 in neurons isolated from certain regions of the adult rodent brain (i.e., the supraoptic nucleus and striatum), which correlated with reduced BK channel expression (Pietrzykowski et al. 2008). Alcohol-related BK channel dysfunction may play an important role in the development of alcohol tolerance (Cowmeadow et al. 2005; Pietrzykowski 2008). On the other hand, physiologically relevant ethanol concentrations, similar to those attained by alcoholics, can significantly suppress the expression of four other miRNAs (i.e., miR-21, miR-335, miR-153, and miR-9) (Sathyan et al. 2007). Together, these studies highlight the emerging role of miRNAs as mediators of epigenetic modifications that may be involved in ethanol's actions.

Conclusions

Epigenetics is an emerging area of research in the neuroscience field. The work done so far indicates that chromatin remodeling resulting from histone modifications in the amygdala may be important in the effects of both acute and chronic ethanol exposure (see figures 4 and 5). Other evidence has identified DNA methylation as a critical regulator of gene expression levels, which also may play a critical role in alcoholism. However, the specific effects of different types of HDAC in alcohol's action or the regulation of various DNMTs in the brain by alcohol exposure still remain unclear. It also would be interesting to determine the role of other important enzymes involved in remodeling the chromatin structure, such as DNA and histone demethylases and HMTs, in alcohol's effects. Currently, the alcohol-related regulation of histone and DNA methylation in the brain and other organ systems are not well understood. Future studies will provide a more comprehensive picture of epigenetic mechanisms regulated by alcohol in order to increase our understanding of overall genomic function in alcoholism (figure 1). Nonetheless, these early research findings clearly provide evidence that compounds that inhibit HDACs may be promising future therapeutic agents in the treatment of alcoholism (figures 4 and 5). ■

Acknowledgements

The work described here from the laboratory of Dr. Pandey was supported by National Institute on Alcohol Abuse and Alcoholism grants AA–016690, AA–019971, AA–010005, and AA–013341 and by the Department of Veterans Affairs (Merit Review Grant; Research Career Scientist Award).

Financial Disclosure

The authors declare that they have no competing financial interests.

References

Abel, T., and Zukin, R.S. Epigenetic targets of HDAC inhibition in neurodegenerative and psychiatric disorders. *Current Opinion in Pharmacology* 8:57–64, 2008. PMID: 18206423

Acquaah-Mensah, G.K.; Misra, V.; and Biswal, S. Ethanol sensitivity: A central role for CREB transcription regulation in the cerebellum. *BMC Genomics* 7:308, 2006. PMID: 17147806

Agger, K.; Christensen, J.; Cloos, P.A.; and Helin, K. The emerging functions of histone demethylases. *Current Opinion in Genetics and Development* 18:159–168, 2008. PMID: 18281209

Akbarian, S., and Huang, H.S. Epigenetic regulation in human brain: Focus on histone lysine methylation.*Biological Psychiatry* 65:198–203, 2009. PMID: 18814864

Alarcon, J.M.; Malleret, G.; Touzani, K.; et al. Chromatin acetylation, memory, and LTP are impaired in CBP+/- mice: A model for the cognitive deficit in Rubenstein-Taybi Syndrome and its amelioration. *Neuron* 42:947–959, 2004. PMID: 15207239

Alberini, C.M. Transcription factors in long-term memory and synaptic plasticity. *Physiological Reviews* 89:121–145, 2009. PMID: 19126756

Antequera, F. Structure, function, and evolution of CpG island promoters. *Cellular and Molecular Life Sciences* 60:1647–1658, 2003. PMID: 14504655

Balemans, M.C.; Huibers, M.M.; Eikelenboom, N.W.; et al. Reduced exploration, increased anxiety, and altered social behavior: Autistic-like features of euchromatin histone methyltransferase 1 heterozygous knockout mice. *Behavioural Brain Research* 208:47–55, 2010. PMID: 19896504

Bartel, D.P. MicroRNAs: Target recognition and regulatory functions. *Cell* 136:215–233, 2009. PMID: 19167326

Bartsch, O.; Schmidt, S.; Richter, M.; et al. DNA sequencing of CREBBP demonstrates mutations in 56% of patients with Rubinstein-Taybi syndrome (RSTS) and in another patient with incomplete RSTS. *Human Genetics* 117:485–493, 2005. PMID: 16021471

Beier, J.I., and McClain, C.J. Mechanisms and cell signaling in alcoholic liver disease. *Biological Chemistry* 391:1249–1264, 2010. PMID: 20868231

Bestor, T.H. The DNA methyltransferases of mammals. *Human Molecular Genetics* 9:2395–2402, 2000. PMID: 11005794

Bhattacharya, S.K.; Ramchandani, S.; Cervoni, N.; and Szyf, M. A mammalian protein with specific demethylase activity for mCpG DNA. *Nature* 397:579–583,1999. PMID:10050851

Bienvenu, T., and Chelly, J. Molecular genetics of Rett syndrome: When DNA methylation goes unrecognized. *Nature Reviews. Genetics* 7:415–426, 2006. PMID: 16708070

Biermann, T.; Reulbach, U.; Lenz, B.; et al. N-methyl-D-aspartate 2b receptor subtype (NR2B) promoter methylation in patients during alcohol withdrawal. Journal of Neural Transmission 116:615–622, 2009. PMID: 19350219

Bleich, S., and Hillemacher, T. Homocysteine, alcoholism and its molecular networks. *Pharmacopsychiatry* 42(Suppl. 1): S102–S109, 2009. PMID: 19434547

Bonasio, R.; Tu, S.; and Reinberg, D. Molecular signals of epigenetic states. *Science* 330:612–616, 2010. PMID: 21030644

Bonsch, D.; Greifenberg, V.; Bayerlein, K.; et al. α-Synuclein protein levels are increased in alcoholic patients and are linked to craving. *Alcoholism: Clinical and Experimental Research* 29:763–765, 2005*a*. PMID: 15897720

Bonsch, D.; Lenz, B.; Fiszer, R.; et al. Lowered DNA methyltransferase (DNMT-3b) mRNA expression is associated with genomic DNA hypermethylation in patients with chronic alcoholism. *Journal of Neural Transmission* 113:1299–1304, 2006. PMID: 16463117

Bonsch, D.; Lenz, B.; Kornhuber, J.; and Bleich, S. DNA hypermethylation of the alpha synuclein promoter in patients with alcoholism. *NeuroReport* 16:167–170, 2005*b*. PMID: 15671870

Caccamo, A.; Maldonado, M.A.; Bokov, A.F.; et al. CBP gene transfer increases BDNF levels and ameliorates learning and memory deficits in a mouse model of Alzheimer's disease. *Proceedings of the National Academy of Sciences of the United States of America* 107:22687–22692, 2010. PMID: 21149712

Carlezon, W.A., Jr.; Duman, R.S.; and Nestler, E.J. The many faces of CREB. *Trends in Neurosciences* 28:436–445, 2005. PMID: 15982754

Chavez-Noriega, L.E.; Schaffhauser, H.; and Campbell, U.C. Metabotropic glutamate receptors: Potential drug targets for the treatment of schizophrenia. *Current Drug Targets CNS and Neurological Disorders* 1:261–281, 2002. PMID: 12769619

Chen, G.; Zou, X.; Watanabe, H.; et al. CREB binding protein is required for both short-term and long-term memory formation. *Journal of Neuroscience* 30:13066–13077, 2010. PMID: 20881124

Cheung, P.; and Lau, P. Epigenetic regulation by histone methylation and histone variants. *Molecular Endocrinology* 19:563–573, 2005. PMID: 15677708

Chrivia, J.C.; Kwok, R.P.; Lamb, N.; et al. Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* 365:855–859, 1993. PMID: 8413673

Clapp, P. Bhave, S.V.; and Hoffman, P.L. How adaptation of the brain to alcohol leads to dependence: A pharmacological perspective. *Alcohol Research & Health* 31:310–339, 2008. PMID: 20729980

Comb, M., and Goodman, H.M. CpG methylation inhibits proenkephalin gene expression and binding of the transcription factor AP-2. *Nucleic Acids Research* 18:3975–3982, 1990. PMID: 1695733

Conn, P.J.; Lindsley, C.W.; and Jones, C.K. Activation of metabotropic glutamate receptors as a novel approach for the treatment of schizophrenia. *Trends in Pharmacological Sciences* 30:25–31, 2009. PMID: 19058862

Contet, C.; Gardon, O; Filliol, D.; et al. Identification of genes regulated in mouse extended amygdala by excessive ethanol drinking associated with dependence. *Addiction Biology* 16:615-619, 2011. PMID:21392173

Cooper, M.L.; Frone, M.R.; Russel, M.; and Mudar, P. Drinking to regulate positive and negative emotions: A motivational model of alcohol use. *Journal of Personality and Social Psychology* 69:990–1005, 1995. PMID: 7473043

Costa, E.; Chen, Y.; Dong, E.; et al. GABAergic promoter hypermethylation as a model to study the neurochemistry of schizophrenia vulnerability. *Expert Review of Neurotherapeutics* 9:87–98, 2009. PMID: 19102671

Costa, E.; Dong, E.; Grayson, D.R.; et al. Reviewing the role of DNA (cytosine-5) methyltransferase overexpression in the cortical GABAergic dysfunction associated with psychosis vulnerability. *Epigenetics* 2:29–36, 2007. PMID: 17965595

Costa, E.; Grayson, D.R.; and Guidotti, A. Epigenetic downregulation of GABAergic function in schizophrenia: Potential for pharmacological intervention? *Molecular Interventions* 3:220–229, 2003. PMID: 14993449

Covington, H.E 3rd.; Maze, I.; Laplant, Q.C.; et al. Antidepressant actions of histone deacetylase inhibitors. *Journal of Neuroscience* 29:11451–11460, 2009. PMID: 19759294

Cowmeadow, R.B.; Krishnan, H.R.; and Atkinson, N.S. The slowpoke gene is necessary for rapid ethanol tolerance in Drosophila. *Alcoholism: Clinical and Experimental Research* 29:1777–1786, 2005. PMID: 16269907

Crabbe, J.C.; Phillips, T.J.; Harris, R.A.; et al.. Alcohol-related genes: Contributions from studies with genetically engineered mice. *Addiction Biology* 11:195–269, 2006. PMID: 16961758

Crews, F.T., and Nixon, K. Mechanisms of neurodegeneration and regeneration in alcoholism. *Alcohol and Alcoholism* 44:115–127, 2009. PMID: 18940959

Deng, J.V.; Rordriguiz, R.M.; Hutchinson, A.N.; et al. MeCP2 in the nucleus accumbens contributes to neural and behavioral responses to psychostimulants. *Nature Neuroscience* 13:1128–1136, 2010. PMID: 20711186

Dickinson, M.; Johnstone, R.W.; and Prince, H.M. Histone deacetylase inhibitors: Potential targets responsible for their anti-cancer effect. *Investigational New Drugs* 28(Suppl 1):S3–S30, 2010. PMID: 21161327

Dokmanovic, M., and Marks, P.A. Prospects: Histone deacetylase inhibitors. *Journal of Cellular Biochemistry* 96:293–304, 2005. PMID: 16088937

Ducci, F., and Goldman, D. Genetic approaches to addiction: Genes and alcohol.*Addiction* 103:1414–1428, 2008. PMID: 18422824

Edenberg, H. J., and Foroud, T. The genetics of alcoholism: Identifying specific genes through family studies. *Addiction Biology* 11:386–396, 2006. PMID: 16961766

Farris, S.P.; Wolen, A.R.; and Miles, M.F. Using expression genetics to study the neurobiology of ethanol and alcoholism. *International Review of Neurobiology* 91:95–128, 2010. PMID: 20813241.

Febo, M.; Akbarian, S.; Schroeder, F.A.; and Ferris, C.F. Cocaine-induced metabolic activation in cortico-limbic circuitry is increased after exposure to the histone deacetylase inhibitor, sodium butyrate. *Neuroscience Letters* 465:267–271, 2009. PMID: 19638299

Feng, J., and Fan, G. The role of DNA methylation in the central nervous system and neuropsychiatric disorders. *International Review of Neurobiology* 89:67–84, 2009. PMID: 19900616

Feng, J., and Nestler, E.J. MeCP2 and drug addiction. *Nature Neuroscience* 13: 1039–1041, 2010. PMID: 20740030

Feng, J.; Zhou, Y.; Campbell, S.L.; et al. Dnmt1 and Dnmt3a maintain DNA methylation and regulate synaptic function in adult forebrain neurons. *Nature Neuroscience* 13:423–430, 2010. PMID: 20228804

Ghildiyal, M., and Zamore, P.D. Small silencing RNAs: An expanding universe. *Nature Reviews. Genetics* 10:94–108, 2009. PMID: 19148191

Grayson, D.R.; Kundakovic, M.; and Sharma, R.P. Is there a future for histone deacetylase inhibitors in the pharmacotherapy of psychiatric disorders? *Molecular Pharmacology* 77:126–135, 2010. PMID: 19917878

Grunstein, M. Histone acetylation in chromatin structure and transcription. *Nature* 389:349–352, 1997. PMID: 9311776

Guan, J.S.; Haggarty, S.J.; Giacometti, E.; et al. HDAC2 negatively regulates memory formation and synaptic plasticity. *Nature* 459:55–60, 2009: PMID: 19424149

Gupta, S.; Kim, S.Y.; Artis, S.; et al. Histone methylation regulates memory formation. *Journal of Neuroscience* 30:3589–3599, 2010. PMID: 20219993

He, D.Y.; Neasta, J.; and Ron, D. Epigenetic regulation of BDNF expression via the scaffolding protein Rack1. *Journal of Biological Chemistry* 285:19043–19050, 2010. PMID: 20410295

Holliday, R. Epigenetics: A historical overview. *Epigenetics* 1:76–80, 2006. PMID: 17998809

Hsieh, J., and Gage, F.H. Chromatin remodeling in neural development and plasticity. *Current Opinion in Cell Biology* 17:664–671, 2005. PMID: 16226449

Hu, Y.; Lunde, I.V.; Gravielle, M.C.; et al. Surface expression of GABAA receptors is transcriptionally controlled by the interplay of cAMP-response element-binding protein and its binding partner inducible cAMP early repressor. *Journal of Biological Chemistry* 283:9328–9340, 2008. PMID: 18180303

Huang, H.S.; Matevossian, A.; Whittle, C.; et al. Prefrontal dysfunction in schizophrenia involves mixed-lineage leukemia 1-regulated histone methylation at GABAergic gene promoters. *Journal of Neuroscience* 27:11254–11262, 2007. PMID: 17942719

Im, H.I.; Hollander, J.A.; Bali, P.; and Kenny, P.J. MeCP2 controls BDNF expression and cocaine intake through homeostatic interactions with microRNA-212. *Nature Neuroscience* 13:1120–1127, 2010. PMID: 20711185

Janak, P.H.; Wolf, F.W.; Heberlein, U.; et al. BIG news in alcohol addiction: New findings on growth factor pathways BDNF, insulin, and GDNF. *Alcoholism: Clinical and Experimental Research* 30:214–221, 2006. PMID: 16441270

Jeanblanc, J.; He, D.Y.; Carnicella, S.; et al. Endogenous BDNF in dorsolateral striatum gates alcohol drinking. *Journal of Neuroscience* 29:13494–13502, 2009. PMID: 19864562

Jenuwein, T., and Allis, C.D. Translating the histone code. *Science* 293:1074–1080, 2001. PMID: 11498575

Jones, K.L., and Smith, D.W. Recognition of fetal alcohol syndrome in early infancy. *Lancet* 302:999–1001, 1973. PMID: 4127281

Jones, K.L.; Smith, D.W.; Ulleland, C.N.; and Streissguth, P. Pattern of malformation in offspring of chronic alcoholic mothers. *Lancet* 1:1267–1271, 1973. PMID: 4126070

Kalkhoven, E. CBP and p300: HATs for different occasions. *Biochemical Pharmacology* 68:1145–1155, 2004. PMID: 15313412

Kazantsev, A.G., and Thompson, L.M. Therapeutic application of histone deacetylase inhibitors for central nervous system disorders. *Nature Reviews. Drug Discovery* 7:854–868, 2008. PMID: 18827828

KELLY, W.K., AND MARKS, P.A. Drug insight: Histone deacetylase inhibitors-development of the new targeted anticancer agent suberoylanilide hydroxamic acid. *Nature Clinical Practice. Oncology* 2:150–157, 2005. PMID: 16264908

KHALIL, A.M.; GUTTMAN, M.; HUARTE, M.; ET AL. Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. *Proceedings of the National Academy of Sciences of the United States of America* 106:11667–11672, 2009. PMID: 19571010

KILGORE, M.; MILLER, C.A.; FASS, D.M.; ET AL. Inhibitors of class 1 histone deacetylases reverse contextual memory deficits in a mouse model of Alzheimer's disease. *Neuropsychopharmacology* 35:870–880, 2010. PMID: 20010553

KIM, J.S., AND SHUKLA, S.D. Acute *in vivo* effect of ethanol (binge drinking) on histone H3 modifications in rat tissues. *Alcohol and Alcoholism* 41:126–132, 2006. PMID: 16314425

KLEVYTSKA, A.M.; TEBBENKAMP, A.T.; SAVONENKO, A.V.; AND BORCHELT, D.R. Partial depletion of CREB-binding protein reduces life expectancy in a mouse model of Huntington disease. *Journal of Neuropathology and Experimental Neurology* 69:396–404, 2010. PMID: 20448484

KOOB, G.F. Alcoholism: Allostasis and beyond. *Alcoholism: Clinical and Experimental Research* 27:232–243, 2003*a*. PMID: 12605072

KOOB, G.F. Neuroadaptive mechanisms of addiction: Studies on the extended amygdala. *European Neuropsychopharmacology* 13:442–452, 2003*b*. PMID: 14636960

KOOB, G.F.; ROBERTS, A.J.; SCHULTEIS, G.; ET AL. Neurocircuitry targets in ethanol reward and dependence. *Alcoholism: Clinical and Experimental Research* 22:3–9, 1998. PMID: 9514280

KORNBERG, R.D. Chromatin structure: A repeating unit of histones and DNA. *Science* 184:868–871, 1974. PMID: 4825889

KORZUS, E.; ROSENFELD, M.G.; AND MAYFORD, M. CBP histone acetyltransferase activity is a critical component of memory consolidation. *Neuron* 42:961–972, 2004. PMID: 15207240

KUMAR, S.; PORCU, P.; WERNER, D.F.; ET AL. The role of GABA(A) receptors in the acute and chronic effects of ethanol: A decade of progress. *Psychopharmacology (Berlin)* 205:529–564, 2009. PMID: 19455309

KUMARI, M., AND TICKU, M.K. Regulation of NMDA receptors by ethanol. *Progress in Drug Research* 54:152–189, 2000. PMID: 10857388

KUSHNER, M.G.; SHER, K.J., AND BEITMAN, B.D. The relation between alcohol problems and the anxiety disorders. *American Journal of Psychiatry* 147: 685–695, 1990. PMID: 2188513

LAPLANT, Q.; VIALOU, V.; COVINGTON, H.E., 3RD; ET AL. Dnmt3a regulates emotional behavior and spine plasticity in the nucleus accumbens. *Nature Neuroscience* 13:1137–1143, 2010. PMID: 20729844

LEE, M.J.; KIM, Y.S.; KUMMAR, S.; ET AL. Histone deacetylase inhibitors in cancer therapy. *Current Opinion in Oncology* 20:639–649, 2008. PMID: 18841045

LEVENSON, J.M.; O'RIORDAN, K.J.; BROWN, K.D.; ET AL. Regulation of histone acetylation during memory formation in the hippocampus. *Journal of Biological Chemistry* 279:40545–40559, 2004. PMID: 15273246

LIPSCOMB, T.R.; NATHAN, P.E.; WILSON, G.T.; AND ABRAMS, D.B. Effects of tolerance on the anxiety-reducing function of alcohol. *Archives of General Psychiatry* 37:577–582, 1980. PMID: 7377915

LIU, X.; WANG, L.; ZHAO, K.; ET AL. The structural basis of protein acetylation by the p300/CBP transcriptional coactivator. *Nature* 451:846–850, 2008. PMID: 18273021

LIU, Y.; BALARAMAN, Y; WANG, G.; ET AL. Alcohol exposure alters DNA methylation profiles in mouse embryos at early neurulation. *Epigenetics* 4:500–511, 2009. PMID: 20009564

LONZE, B.E., AND GINTY, D.D. Function and regulation of CREB family transcription factors in the nervous system. *Neuron* 35:605–623, 2002. PMID: 12194863

LUGER, K.; MADER, A.W.; RICHMOND, R.K.; ET AL. Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* 389:251–260, 1997. PMID: 9305837

MALAVAEZ, M., SANCHIS-SEGURA, C.; VO, D.; ET AL. Modulation of chromatin modification facilitates extinction of cocaine-induced conditioned place preference. *Biological Psychiatry* 67:36–43, 2010. PMID: 19765687

MARGUERON, R.; TROJER, P.; AND REINBERG, D. The key to development: Interpreting the histone code? *Current Opinion in Genetics & Development* 15:163–176, 2005. PMID: 15797199

MARTINEZ-BALBAS, M.A.; BANNISTER, A.J.; MARTIN, K.; ET AL. The acetyltransferase activity of CBP stimulates transcription. *EMBO Journal* 17:2886–2893,1998. PMID: 9582282

MAUNAKEA, A.K.; NAGARAJAN, R.P.; BILENKY, M.; ET AL. Conserved role of intragenic DNA methylation in regulating alternative promoters. *Nature* 466:253–257, 2010. PMID: 20613842

MAURICE, T.; DUCLOT, F.; MEUNIER, J.; ET AL. Altered memory capacities and response to stress in p300/CBP-associated factor (PCAF) histone acetylase knockout mice. *Neuropsychopharmacology* 33:1584–1602, 2008. PMID: 17805310

MENZAGHI, F.; RASSNICK, S.; HEINRICHS, S; ET AL. The role of corticotropin-releasing factor in the anxiogenic effects of ethanol withdrawal. *Annals of the New York Academy of Sciences* 739:176–184, 1994. PMID: 7832471

MERSFELDER, E.L., AND PARTHUN, M.R. The tale beyond the tail: Histone core domain modifications and the regulation of chromatin structure. *Nucleic Acids Research* 34:2653–2662, 2006. PMID: 16714444

METIVIER, R.; GALLAIS, R.; TIFFOCHE, C.; ET AL. Cyclical DNA methylation of a transcriptionally active promoter. *Nature* 452:45–50, 2008. PMID: 18322525

MILLER, C.A., AND SWEATT, J.D. Covalent modification of DNA regulates memory formation. *Neuron* 53:857–869, 2007. PMID: 17359920

MIRANDA, R.C. Commentary: Will analyzing the epigenome yield cohesive principles of ethanol teratology? *Alcoholism: Clinical and Experimental Research* 35:1201–1203, 2011. PMID: 21554338

MIRANDA, R.C.; PIETRZYKOWSKI, A.Z.; TANG, Y.; ET AL. MicroRNAs: Master regulators of ethanol abuse and toxicity? *Alcoholism: Clinical and Experimental Research* 34:575–587, 2010. PMID: 20102566

MOBERG, C.A., AND CURTIN, J.J. Alcohol selectively reduces anxiety but not fear: Startle response during unpredictable versus predictable threat. *Journal of Abnormal Psychology* 118:335–347, 2009. PMID: 19413408

MOONAT, S.; SAKHAKAR, A.J.; ZHANG, H.; AND PANDEY, S.C. The role of amygdaloid brain-derived neurotrophic factor, activity-regulated cytoskeletal-associated protein and dendritic spines in anxiety and alcoholism. *Addiction Biology* 16:238–250, 2011. PMID: 21182574

MOONAT, S.; STARKMAN, B.G.; SAKHARKAR, A.; AND PANDEY, S.C. Neuroscience of alcoholism: Molecular and cellular mechanisms. *Cellular and Molecular Life Sciences* 67:73–88, 2010. PMID: 19756388

MURRELL, A.; RAKYAN, V.K.; AND BECK, S. From genome to epigenome. *Human Molecular Genetics* 14(Spec No 1):R3–R10, 2005. PMID: 15809270

OKANO, M., BELL, D.W.; HABER, D.A.; AND LI, E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 99:247–257, 1999. PMID: 10555141

OLINS, A.L., AND OLINS, D.E. Spheroid chromatin units (v bodies). *Science* 183:330–332, 1974. PMID: 4128918

OLIVA, J.; DEDES, J.; LI, J.; ET AL. Epigenetics of proteasome inhibition in the liver of rats fed ethanol chronically. *World Journal of Gastroenterology* 15:705–712, 2009. PMID: 19222094

OOI, S.K., AND BESTOR, T.H. The colorful history of active DNA demethylation. *Cell* 133:1145–1148, 2008. PMID: 18585349

OUKO, L.A.; SHANTIKUMAR, K.; KNEZOVICH, J.; ET AL. Effect of alcohol consumption on CpG methylation in the differentially methylated regions of H19 and IG-DMR in male gametes: Implications for fetal alcohol spectrum disorders. *Alcoholism: Clinical and Experimental Research* 33:1615–1627, 2009. PMID: 19519716

PAL-BHADRA, M.; BHADRA, U.; JACKSON, D.E.; ET AL. Distinct methylation patterns in histone H3 at Lys-4 and Lys-9 correlate with up- & down-regulation of genes by ethanol in hepatocytes. *Life Sciences* 81:979–987, 2007. PMID: 17826801

PANDEY, S.C. Anxiety and alcohol abuse disorders: A common role for CREB and its target, the neuropeptide Y gene. *Trends in Pharmacological Sciences* 24:456–460, 2003. PMID: 12967770

PANDEY, S.C. The gene transcription factor cyclic AMP-responsive element binding protein: Role in positive and negative affective states of alcohol addiction. *Pharmacology & Therapeutics* 104:47–58, 2004. PMID: 15500908

PANDEY, S.C.; ROY, A; ZHANG, H.; AND XU, T. Partial deletion of the cAMP response element-binding protein gene promotes alcohol-drinking behaviors. *Journal of Neuroscience* 24:5022–5030, 2004. PMID: 15163695

Pandey, S.C.; Ugale, R.; Zhang, H.; et al. Brain chromatin remodeling: A novel mechanism of alcoholism. *Journal of Neuroscience* 28:3729–3737, 2008*a*. PMID: 18385331

Pandey, S.C., Zhang, H.; Roy, A.; and Xu, T. Deficits in amygdaloid cAMP-responsive element-binding protein signaling play a role in genetic predisposition to anxiety and alcoholism. *Journal of Clinical Investigation* 115:2762–2773, 2005. PMID: 16200210

Pandey, S.C., Zhang, H.; Ugale, R.; et al. Effector immediate-early gene arc in the amygdala plays a critical role in alcoholism. *Journal of Neuroscience* 28:2589–2600, 2008*b*. PMID: 18322102

Park, P.H.; Lim, R.W.; and Shukla, S.D. Involvement of histone acetyltransferase (HAT) in ethanol-induced acetylation of histone H3 in hepatocytes: Potential mechanism for gene expression. *American Journal of Physiology. Gastrointestinal and Liver Physiology* 289:G1124–G1136, 2005. PMID: 16081763

Park, P.H.; Miller, R; and Shukla, S.D. Acetylation of histone H3 at lysine 9 by ethanol in rat hepatocytes. *International Journal of Biochemical and Biophysical Research Communications* 306:501–504, 2003. PMID: 12804592

Peleg, S.; Sananbenesi, F.; Zovoilis, A.; et al. Altered histone acetylation is associated with age-dependent memory impairment in mice. *Science* 328:753–756, 2010. PMID: 20448184

Pignataro, L.; Varodayan, F.P.; Tennenholz, L.E.; and Harrison, N.L. The regulation of neuronal gene expression by alcohol. *Pharmacology & Therapeutics* 124:324–335, 2009. PMID: 19781570

Pietrzykowski, A.Z. The role of microRNAs in drug addiction: A big lesson from tiny molecules. *International Review of Neurobiology* 91:1–24, 2010. PMID: 20813238

Pietrzykowski, A.Z.; Friesen, R.M.; Martin, G.E.; et al. Posttranscriptional regulation of BK channel splice variant stability by miR-9 underlies neuroadaptation to alcohol. *Neuron* 59:274–287, 2008. PMID: 18667155

Prakash, A.; Zhang, H.; and Pandey, S.C. Innate differences in the expression of brain-derived neurotrophic factor in the regions within the extended amygdala between alcohol preferring and nonpreferring rats. *Alcoholism: Clinical and Experimental Research* 32:909–920, 2008. PMID: 18445109

Qiang, M.; Denny, A.D.; and Ticku, M.K. Chronic intermittent ethanol treatment selectively alters *N*-methyl-D-aspartate receptor subunit surface expression in cultured cortical neurons. *Molecular Pharmacology* 72:95–102, 2007. PMID: 17440117

Quiang, M.; Denny, A.; Chen, J.; et al. The site specific demethylation in the 5'-regulatory area of NMDA receptor 2B subunit gene associated with CIE-induced up-regulation of transcription. *PLoS One* 5:e8798, 2010. PMID: 20098704

Robertson, K.D. DNA methylation and human disease. *Nature Reviews. Genetics* 6:597–610, 2005. PMID: 16136652

Roelfsema, J.H.; White, S.J.; Ariyurek, Y.; et al. Genetic heterogeneity in Rubinstein-Taybi syndrome: Mutations in both the CBP and EP300 genes cause disease. *American Journal of Human Genetics* 76:572–580, 2005. PMID: 15706485

Romieu, P.; Host, L.; Gobaille, S.; et al. Histone deacetylase inhibitors decrease cocaine but not sucrose self-administration in rats. *Journal of Neuroscience* 28:9342– 9348, 2008. PMID: 18799668

Rouaux, C.; Loeffler, J.P.; and Boutillier, A.L. Targeting CREB-binding protein (CBP) loss of function as a therapeutic strategy in neurological disorders. *Biochemical Pharmacology* 68:1157–1164, 2004. PMID: 15313413

Sakharkar, A.J.; Zhang, H.; Tang, L.; et al. Histone deacetylases (HDAC)-induced histone modifications in the amygdala: A role in rapid tolerance to the anxiolytic effects of ethanol. *Alcoholism: Clinical and Experimental Research* 36:61-71, 2012. PMID: 21790673

Sathyan, P.; Golden, H.B.; and Miranda, R.C. Competing interactions between micro-RNAs determine neural progenitor survival and proliferation after ethanol exposure: Evidence from an ex vivo model of the fetal cerebral cortical neuroepithelium. *Journal of Neuroscience* 27:8546–8557, 2007. PMID: 17687032

Selvi, B.R.; Cassel, J.C.; Kundu, T.K., and Boutillier, A.L. Tuning acetylation levels with HAT activators: Therapeutic strategy in neurodegenerative diseases. *Biochimica et Biophysica Acta. Gene Regulatory Mechanisms* 1799:840–853, 2010. PMID: 20833281

Sharma, R.P.; Gavin, D.P.; and Grayson, D.R. CpG methylation in neurons: Message, memory, or mask? *Neuropsychopharmacology* 35:2009–2020, 2010. PMID: 20631690

Shukla, S. D.; Velazquez, J.; French, S.W.; et al. Emerging role of epigenetics in the actions of alcohol. *Alcoholism: Clinical and Experimental Research* 32:1525–1534, 2008. PMID: 18616668

Siegel, G.; Saba, R.; and Schratt, G. microRNAs in neurons: Manifold regulatory roles at synapse. *Current Opinion in Genetics and Development* 21:491–497, 2011. PMID: 21561760

Smit, M.M. Histone structure and function. *Current Opinion in Cell Biology* 3:429–437, 1991. PMID: 1892654

Spanagel, R.; Bartsch, D.; Brors, B.; et al. An integrated genome research network for studying the genetics of alcohol addiction. *Addiction Biology* 15:369–379, 2010. PMID: 21040237

Stadler, F.; Kolb, G.; Rubusch, L.; et al. Histone methylation at gene promoters is associated with developmental regulation and region-specific expression of ionotropic and metabotropic glutamate receptors in human brain. *Journal of Neurochemistry* 94:324–336, 2005. PMID: 15998284

Suzuki, R.; Lumeng, L.; McBride, W.J.; et al. Reduced neuropeptide Y mRNA expression in the central nucleus of amygdala of alcohol preferring (P) rats: Its potential involvement in alcohol preference and anxiety. *Brain Research* 1014:251–254, 2004. PMID: 15213011

Tabakoff, B.; Cornell, N.; and Hoffman, P.L. Alcohol tolerance. *Annals of Emergency Medicine* 15:1005–1012, 1986. PMID: 3526989

Tabakoff, B.; Saba, L.; Printz, M.; et al. Genetical genomic determinants of alcohol consumption in rats and humans. *BMC Biology* 7:70, 2009. PMID: 19874574

Tahiliani, M.; Mei, P.; Fang, R.; et al. The histone H3K4 demethylase SMCX links REST target genes to X-linked mental retardation. *Nature* 447:601–605, 2007. PMID: 17468742

Taqi, M.M.; Bazov, I.; Watanabe, H.; et al. Prodynorphin CpG-SNPs associated with alcohol dependence: Elevated methylation in the brain of human alcoholics. *Addiction Biology* 16:499–509, 2011. PMID: 21521424

Tian, F.; Hu, X.Z.; Wu, X.; et al. Dynamic chromatin remodeling events in hippocampal neurons are associated with NMDA receptor-mediated activation of Bdnf gene promoter 1. *Journal of Neurochemistry* 109: 1375–1388, 2009. PMID: 19476549

Tsai, A.C.; Dossett, C.J.; Walton, C.S.; et al. Exon deletions of the EP300 and CREBBP genes in two children with Rubinstein-Taybi syndrome detected by aCGH. *European Journal of Human Genetics* 19:43–49, 2011. PMID: 20717166

Tsankova, N.M.; Berton, O.; Renthal, W.; et al. Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. *Nature Neuroscience* 9:519–525, 2006. PMID: 16501568

Tsankova, N.; Renthal, W.; Kumar, A.; and Nestler, E.J. Epigenetic regulation in psychiatric disorders. *Nature Reviews. Neuroscience* 8:355–367, 2007. PMID 17453016

Turner, B.M. Cellular memory and the histone code. *Cell* 111:285–291, 2002. PMID: 12419240

Valdez, G.R., and Koob, G.F. Allostasis and dysregulation of corticotropin-releasing factor and neuropeptide Y systems: Implications for the development of alcoholism. *Pharmacology, Biochemistry, and Behavior* 79:671–689, 2004. PMID: 15582675

Waddington, C. The epigenotype. *Endeavour* 1:18–20, 1942.

Walker, S.J., and Grant, K.A. Peripheral blood α-Synuclein mRNA levels are elevated in cynomolgus monkeys that chronically self-administer ethanol. *Alcohol* 38:1–4, 2006. PMID: 16762686

Wand, G. The anxious amygdala: CREB signaling and predisposition to anxiety and alcoholism. *Journal of Clinical Investigation* 115:2697–2699, 2005. PMID: 16200206

Wang, L.; Lv, Z; Hu, Z.; et al. Chronic cocaine-induced H3 acetylation and transcriptional activation of CaMKIIalpha in the nucleus accumbens is critical for motivation for drug reinforcement. *Neuropsychopharmacology* 35:913–928, 2010. PMID: 20010550

Wassef, A.; Baker, J.; and Kochan, L.D. GABA and schizophrenia: A review of basic science and clinical studies. *Journal of Clinical Psychopharmacology* 23:601–640, 2003. PMID: 14624191

Weaver, I.C.; Champagne, F.A.; Brown, S.E.; et al. Reversal of maternal programming of stress responses in adult offspring through methyl supplementation: Altering epigenetic marking later in life. *Journal of Neuroscience* 25:11045–11054, 2005. PMID: 16306417

Weaver, I.C.; Cervoni, N.; Champagne, F.A.; et al. Epigenetic programming by maternal behavior. *Nature Neuroscience* 7:847–854, 2004. PMID: 15220929

Wheal, H.V.; Chen, Y.; Mitchell, J.; et al. Molecular mechanisms that underlie structural and functional changes at the postsynaptic membrane during synaptic plasticity. *Progress in Neurobiology* 55:611–640, 1998. PMID 9670221

Wysocka, J.; Allis, C.D.; and Coonrod, S. Histone arginine methylation and its dynamic regulation. *Frontiers in Bioscience* 11:344–355, 2006. PMID: 16146736

Yang, X.; Horn, K.; Baraban, J.M.; and Wand, G.S. Chronic ethanol administration decreases phosphorylation of cyclic AMP response element binding protein in granule cells of rat cerebellum. *Journal of Neurochemistry* 70:224–232, 1998. PMID: 9422366

Zhang, H.; Sakharkar, A.J.; Shi, G.; et al. Neuropeptide Y signaling in the central nucleus of amygdala regulates alcohol-drinking and anxiety-like behaviors of alcohol-preferring rats. *Alcoholism: Clinical and Experimental Research* 34:451–461, 2010. PMID: 20028368

Zhou, F.C.; Balaraman, Y.; Teng, M.; et al. Alcohol alters DNA methylation patterns and inhibits neural stem cell differentiation. *Alcoholism: Clinical and Experimental Research* 35:735–746, 2011. PMID: 21223309

Using Genetically Engineered Animal Models in the Postgenomic Era to Understand Gene Function in Alcoholism

Matthew T. Reilly, Ph.D.; R. Adron Harris, Ph.D.; and Antonio Noronha, Ph.D.

Matthew T. Reilly, Ph.D., *is a program director, and* **Antonio Noronha, Ph.D.,** *is director of the Division of Neuroscience and Behavior, National Institute on Alcohol Abuse and Alcoholism, Bethesda, Maryland.*

R. Adron Harris, Ph.D., *is M. June and J. Virgil Waggoner Chair in Molecular Biology and director of the Waggoner Center for Alcohol and Addiction Research, University of Texas at Austin, Austin, Texas.*

Over the last 50 years, researchers have made substantial progress in identifying genetic variations that underlie the complex phenotype of alcoholism. Not much is known, however, about how this genetic variation translates into altered biological function. Genetic animal models recapitulating specific characteristics of the human condition have helped elucidate gene function and the genetic basis of disease. In particular, major advances have come from the ability to manipulate genes through a variety of genetic technologies that provide an unprecedented capacity to determine gene function in the living organism and in alcohol-related behaviors. Even newer genetic-engineering technologies have given researchers the ability to control when and where a specific gene or mutation is activated or deleted, allowing investigators to narrow the role of the gene's function to circumscribed neural pathways and across development. These technologies are important for all areas of neuroscience, and several public and private initiatives are making a new generation of genetic-engineering tools available to the scientific community at large. Finally, high-throughput "next-generation sequencing" technologies are set to rapidly increase knowledge of the genome, epigenome, and transcriptome, which, combined with genetically engineered mouse mutants, will enhance insight into biological function. All of these resources will provide deeper insight into the genetic basis of alcoholism. **KEY WORDS: Alcoholism; genetic; genetic basis of alcoholism; neuroscience; gene function; genetic engineering; genetic technology; gene knockout technology; gene sequencing; gene functions; phenotype; genome; epigenome; transcriptome; animal studies; knockout mice**

During the first decade of the new millennium, remarkable advances in technology allowed investigators in all areas of biological research to collect massive amounts of genetic data at an unprecedented rate. The genomics revolution, which began with the sequencing of the human genome, was the basis for efforts such as the 1000 Genomes Project (www.1000genomes.org) that strive to compile a comprehensive catalogue of genetic variation in humans. A catalogue of genetic variation across multiple species also was borne out of this effort. Indeed, sequencing of the genome of the canonical research mouse strain, called C57BL/6, followed by the sequencing of other inbred mouse strains, has opened major opportunities for a fundamental understanding of how an organism's genetic makeup (i.e., genotype) is related to its observable characteristics (i.e., phenotype). Sophisticated tools for creating genetically engineered animal models of human diseases also have reached a point where community-centered efforts have begun to eclipse previous efforts of individual laboratories. These genomic advances, coupled with major progress in genetic-engineering technology, are set to significantly enhance understanding of the genetic basis of human disease, including the genetic basis of alcoholism.

An inherited predisposition for alcoholism has been suspected for hundreds of years because of the observation that alcoholism tends to run in families. However, this familial pattern is not direct proof of a genetic vulnerability, because it also could be explained by a shared environment. It was not

until the 1970s that this notion was scientifically tested in a systematic fashion, when Goodwin and colleagues (1973) studied the drinking histories of 55 adopted-out sons of alcoholics and 78 adopted-out sons of nonalcoholics, all of whom were adopted within the first 6 weeks of life. It is worth noting that the sons of alcoholics in this study had no knowledge that their biological parents had alcoholism. The results of this analysis were striking: The biological sons of alcoholics who had been adopted by nonrelated foster families were four times as likely to become alcoholics compared with the sons of nonalcoholics. Similar lines of research in twin and family studies convincingly have demonstrated that genetic factors account for between 50 to 60 percent of the vulnerability to alcoholism. However, although this statistic provides compelling evidence for a genetic influence on alcoholism, it does not indicate the specific genes that increase or decrease risk of developing alcoholism.

The search for genes associated with a predisposition toward alcoholism began more than 25 years ago. One of the first concerted research efforts to map such genes, the Collaborative Studies on the Genetics of Alcoholism (COGA), was established in 1989. The COGA sample is derived from more than 100 nuclear families densely affected with alcoholism, for whom extensive genotypic as well as phenotypic information has been collected. To date, researchers have identified about 20 genes that contribute to the risk of alcoholism in this sample. Similar studies by investigators all over the world, in a range of different populations, have identified additional genetic variants that contribute to the vulnerability to alcoholism. These genes encode proteins involved in almost all of the major brain-signaling (i.e., neurotransmitter) systems, including the γ-aminobutyric acid (GABA), glutamate, serotonin, dopamine, and acetylcholine systems. Genes involved with alcohol metabolism, other signaling mechanisms (e.g., neuropeptide and neuroendocrine signaling), and cellular architecture also have been implicated (Edenberg and Foroud 2006; Kranzler and Edenberg 2010). Yet, although this work has identified some candidate genes, it is only the first step in gaining insight into the etiology of alcoholism. The next step is to understand how genetic variation alters brain function and to determine which genes are most important for alcoholism and, finally, its treatment.

The search for genes associated with a predisposition toward alcoholism began more than 25 years ago.

Understanding how the genotype of an organism is causally related to its phenotype is a fundamental goal of contemporary genetics. Over the last two decades, researchers have developed many innovative approaches to address this issue. Because of practical and ethical limitations associated with research in humans, the major thrust of this work has come from mechanistic studies in animal models of human diseases, particularly the use of genetically engineered animals. Indeed, animal models already have played a crucial role in understanding the genetic basis of alcoholism. The key approach used in these analyses is to manipulate genes in a controlled fashion in animal models in order to elucidate the genes' function(s) in alcohol-related phenotypes. This review highlights recent advances in determining gene function using animal models with relevance to alcohol research. The discussion focuses almost exclusively on mouse models, because numerous novel genetic tools have been accumulating that allow gene manipulation in these models. In addition, this review describes publicly and privately funded community efforts for large-scale genetic engineering and systematic phenotyping. Finally, a brief introduction to novel high-throughput genomic sequencing technologies (e.g., next-generation sequencing) is presented. These technologies have great potential for furthering understanding of how genotype is causally related to phenotype by providing the most comprehensive depiction of the genome, transcriptome, and epigenome ever attempted.

Conventional Genetic Strategies for Analyzing Gene Function

A widely used method to determine the function of a gene suspected of contributing to a certain trait (e.g., alcohol consumption) is to eliminate the gene from the organism under investigation (usually mice) through a method called homologous recombination. The resultant animal is called a null mutant or knockout for that gene. The investigator then determines what effect the absence of the gene (and the protein that it encodes) has on the trait being studied. Another conventional strategy for gene modification that uses the opposite approach to knockouts is called transgenesis. With this technique, a foreign gene (i.e., transgene) is introduced into a recipient organism's genome, resulting in a transgenic animal. The product of the transgene can be produced at higher-than-normal levels (i.e., overexpressed) or otherwise manipulated in the transgenic animal in order to study the gene's function. These approaches have been used extensively in alcohol research, and Crabbe and colleagues (2006) have published a comprehensive literature review covering the first 10 years (1996 to 2006) of studies using genetically engineered mice in this field. In addition, the Integrative Neuroscience Initiative on Alcoholism (INIA) West Consortium maintains a database containing historic and recent studies using genetically engineered mice in alcohol research.

Since 2007, numerous studies have used knockout mice to determine the

effects of specific genes on alcohol consumption, using a standard two-bottle choice procedure in which the animals can freely choose between a water bottle and an alcohol (i.e., ethanol)-containing bottle for drinking (see table 1). In these studies, the knockout animals showed increases and decreases in ethanol drinking, depending on the specific gene that had been deleted. For one of the genes studied—a gene encoding a molecule called the CB1 receptor—studies consistently found that the knockout mice showed reduced ethanol drinking (Hungund et al. 2003; Naassila et al. 2004; Poncelet et al. 2003; Thanos et al. 2005). The results of other studies measuring ethanol consumption in animals in which genes such as those encoding proteins called adiponectin receptor 2, agouti-related protein, neurokinin-1 receptor, PSD-95, and adenylyl cyclase type 5 had been knocked out have yet to be confirmed in independent studies. Nevertheless, these initial findings offer exciting new possibilities for expanding the knowledge of the functional roles of genes associated with alcohol-related traits. For example, in a recent study, a group of neuroimmune genes were examined for their effect on ethanol consumption using knockout mice (Blednov et al. 2011*a*) (table 1). Previous genomics data measuring gene expression had implicated these genes in the response to alcohol. The results of the knockout studies demonstrate that these genes have a role in regulating alcohol consumption, thereby providing functional evidence supporting the initial gene expression studies. Thus, knockout studies can play a critical role in confirming the findings of other genomic studies and uncovering hitherto unknown molecular targets of ethanol.

However, the conventional knockout approach is associated with inherent limitations (for a detailed review of these limitations and ways to circumvent some of them, see Gerlai 1996; Wolfer et al. 2002). Briefly, one of the main limitations of studying conventional knockouts is the issue of developmental compensation. Because the gene of interest is inactivated over the entire lifespan of the knockout animal, changes in gene expression (or another biological response) in another or similar system may occur to compensate for the deleted gene. This compensatory response may obscure the real effects of the knockout on the trait of interest, resulting in false-negative results. Alternatively, any observed effects may result from the compensatory response rather than the actual gene knockout, leading to a false-positive effect. Another issue is background strain effects—that is, the effect of the knockout may vary depending on the mouse strain in which the knockout animal was generated. Finally, passenger-gene effects may occur. This means that during the process of genetically engineering a knockout animal, unintended genetic material can be introduced into the organism along with the genetic material required to create the knockout. These so-called passenger genes also can result in false-negative and false-positive effects. The next section describes some of the strategies used to overcome these limitations.

Understanding Gene Function Through Conditional Knockout, Knockin, and Viral-Mediated Approaches

To overcome the limitations of conventional knockout studies, researchers have devised elegant and creative alternatives. Some of these strategies broadly can be classified as conditional strategies. The term "conditional" refers to the experimenter's ability to impose specific time and space constraints on when and where the knockout or mutant is generated in the organism. This is accomplished, for example, by engineering

Table 1 Examples of Knockout Mouse Studies Conducted Between 2007 and 2011 That Demonstrate the Effects of the Deletion of Various Genes on Ethanol Consumption

Knocked-Out Gene	Result*	Reference
CB1 receptor	↓ drinking	Vinod et al. 2008
δ Opioid receptor	↑ drinking	van Rijn and Whistler 2009
GABA A receptor α1	↓ drinking	June et al. 2007
PKCε	↓ drinking	Wallace et al. 2007
Adiponectin receptor 2	↓ drinking	Repunte-Canonigo et al. 2010
Agouti-related protein	↓ drinking	Navarro et al. 2009
Neurokinin-1 receptor	↓ drinking	Thorsell et al. 2010
PSD-95	↓ drinking	Camp et al. 2011
Adenylyl cyclase type 5	↑ drinking	Kim et al. 2011
Beta-2-microglobulin	↓ drinking	Blednov et al. 2011*a*
Cathepsin S	↓ drinking	Blednov et al. 2011*a*
Cathepsin F	↓ drinking	Blednov et al. 2011*a*
Interleukin 1 receptor antagonist	↓ drinking	Blednov et al. 2011*a*
CD14 molecule	↓ drinking	Blednov et al. 2011*a*
Interleukin 6	↓ drinking	Blednov et al. 2011*a*

*Ethanol consumption was measured in a two-bottle choice (ethanol vs. water) paradigm

NOTE: For a complete catalogue of studies (1996–2006) on the use of genetically engineered mice in alcohol research see Crabbe et al. 2006.

engineering genetic elements that can be activated (i.e., induced) at a specific time by exposing the animal to a certain chemical agent. Another strategy is to engineer the knockout so that the gene only is deleted when a certain enzyme is present in the cell. By using enzymes that are expressed only in certain cells or tissues, the effects of the gene knockout also only would be limited to those cells or tissues.

Other approaches are using viruses to modify gene expression only in certain tissues. For example, viruses can be used to deliver inhibitory genetic material (referred to as RNA interference [RNAi]) directly to the brain, thereby allowing investigators to selectively reduce or "knockdown" the expression of target genes in specific brain areas. Conversely, viral-mediated approaches can help to overexpress a gene in a specified region.

Finally, as an alternative to eliminating an entire gene from an organism, researchers can introduce mutations into the gene that only change one or several amino acids in the protein that is encoded from the gene and determine the effect of this slight modification on function. This strategy is known as the knockin approach. Recently developed techniques even allow investigators to precisely control the timing and location of the expression of the knockin gene in the organism, resulting in a conditional knockin approach (Skvorak et al. 2006). One example of such a knockin approach, which will be discussed in more detail below, is a mutation in one of the genes encoding a component of the receptor for the neurotransmitter GABA. This modified variant of the $GABA_A$ receptor subunit no longer responds to alcohol but retains its function as a GABA receptor. Use of this gene variant has allowed investigators to define the role of specific GABA receptors in the behavioral actions of alcohol without completely deleting the receptors (Blednov et al. 2011*b*; Harris et al. 2011; Werner et al. 2006). In particular, this approach demonstrated that alcohol acts on the α2 subunit of the $GABA_A$ receptor to produce its activating and aversive behavioral effects (Blednov et al. 2011*b*). This result is intriguing because the gene encoding this subunit previously has been identified as a strong candidate gene for alcohol dependence in humans (Enoch 2008).

Alcohol researchers are just beginning to systematically apply these newer genetic-engineering approaches to their work, and the following paragraphs will illustrate a few recent examples, along with examples from related fields. Studies using the conventional knockout strategy found that global knockout of the gene encoding a brain enzyme called protein kinase C epsilon (PKCε) reduced alcohol self-administration as well as signs of alcohol withdrawal (Hodge et al. 1999; Olive et al. 2000). However, the specific brain region that mediated this effect was unknown, and effects of developmental compensation could not be ruled out conclusively. To address these issues, Lesscher and colleagues (2009) applied a conditional knockout strategy using viral vectors containing RNAi that were delivered directly into a brain region called the amygdala, thereby preventing expression PKCε in that region. Genetic knockdown of PKCε with these viral vectors significantly reduced alcohol consumption (Lesscher et al. 2009), indicating that PKCε expression in the amygdala is important for ethanol consumption in mice. This strategy enabled the investigators to not only rule out the issues of developmental compensation but also to determine the brain region where the PKCε gene exerted its effect.

Using a slightly different conditional knockout strategy, Brigman and colleagues (2010) examined the role of a glutamate receptor subunit in synaptic plasticity and learning, two phenomena that are critically involved in alcoholism. To generate the conditional glutamate receptor knockout animal, the researchers engineered a gene encoding the glutamate receptor subunit NR2B that would be removed only in the presence of a specific enzyme called Cre recombinase. Expression of the Cre recombinase, in turn, was restricted to the cortex and hippocampus by using a genetic element (i.e., promoter) called the CaMKII promoter that only is active in these brain tissues. As a result, deletion of the *NR2B* gene would be limited to those brain regions in which the CaMKII promoter was active. The *NR2B* conditional knockout mice showed significant impairments in a form of synaptic plasticity called long-term depression, altered morphology of nerve cells (i.e., neurons), and deficits in a learning task (Brigman et al. 2010). Because the *NR2B* gene knockout was restricted to the hippocampus and cortex, these brain regions obviously were crucial to the function of the *NR2B* gene. In addition, these analyses also partially controlled for development-specific factors of the genetic knockout because the CaMKII-driven expression of Cre recombinase occurs late in postnatal development. Thus, this approach eliminates any confounding effects that could be associated with the absence of the *NR2B* gene during earlier developmental stages.

The knockin strategy to understand gene function also has shown promise. The brain's GABA-mediated (i.e., GABAergic) signaling system has been implicated in alcohol's actions on the brain and also in mediating a genetic predisposition toward alcoholism (Enoch 2008). A recent study (Blednov et al. 2011*b*) used the knockin strategy to genetically modify the α2 subunit of the $GABA_A$ receptor at just two amino acids. Mice carrying this α2 mutant still responded to GABA but failed to show enhanced GABA activity (i.e., potentiation) in response to alcohol. Behaviorally, α2 mutant mice failed to show alcohol-induced conditioned taste aversion and motor stimulation as well as displayed altered alcohol intake and preference. Therefore, using this knockin strategy, the researchers were able to support the α2 subunit's role in specific actions of alcohol (Blednov et al. 2011*b*). Additional studies in these mice ruled out major developmental compensation effects of the mutated subunit (Harris et al. 2011), further confirm-

Community Resources for High-Throughput Genetic Engineering

As the above examples indicate, genetic-engineering techniques hold tremendous power for dissecting the role of specific genes in alcohol-related phenotypes. The generation of genetically modified animals, however, requires a lot of time and resources, which can prevent investigators from creating needed mutant animals. Several community-wide resources have been developed to help facilitate the use of genetically engineered animal models for studying human diseases (table 2). This section briefly describes some of these resources.

The Knockout Mouse Project (KOMP)

Both publicly and privately funded resources are available that aim to facilitate the use of genetically engineered animals to model human disease and understand gene function. The sequencing of several mouse genomes, including that of the widely used C57BL/6 strain, motivated the development of a resource to elucidate gene function. In 2003, a conference at the Banbury Center at the Cold Spring Harbor Laboratory discussed mouse genomics and genetic engineering, leading to an agreement to begin construction of a collection of mouse knockout mutants for every gene in the mouse genome (Austin et al. 2004). The strategy was to first generate null and conditional-ready knockout mutants in embryonic stem (ES) cells, using both gene-targeting and gene-trapping methodology. Next, mice would be generated from these ES cells to characterize the effects of the mutants at multiple levels of analysis, including gene expression and behavioral analyses. Another workshop, convened in Bethesda, Maryland, by the National Institutes of Health (NIH) in 2005, endorsed the proposals from the Banbury conference. This meeting launched a trans-NIH initiative called the Knockout Mouse Project (KOMP) As of May 2012, the KOMP now contains approximately 8500 targeted null mutations in the C57BL/6 mouse strain (http://www.nih.gov/science/models/mouse/knockout/). Several other similar domestic and international efforts also are being coordinated with the KOMP. These include the European Conditional Mouse Mutagenesis Program (EuCOMM) and the North American Conditional Mouse Mutagenesis Program (NorCOMM). Approximately 9000 knockout targeted alleles are available from these consortia. All three of

Table 2 Community-Wide Resources for Genetically Engineered Mouse Models

Resource	Description	Website
Knockout Mouse Project (KOMP)	NIH initiative with the aim of generating mouse knockouts in ES cells for every gene	www.nih.gov/science/models/mouse/knockout/
International Knockout Mouse Consortium (IKMC)	Consortium that coordinates international effort to produce mouse null mutants in ES cells for every gene	www.knockoutmouse.org
Knockout Mouse Project Phase 2 (KOMP^2)[1]	Project aimed at producing knockout mice from ES cells and conducting broad-based phenotyping on them; knockout mice can be obtained at a central repository by individual investigators for use in their own laboratories	www.komp.org
International Gene Trap Consortium (IGTC)	Consortium that coordinates the international effort to use gene-trap technology for generating knockout mouse lines	www.genetrap.org
Cre-driver network	NIH initiative to produce Cre-driver mouse lines for conditional mouse knockout studies	www.credrivermice.org
Gene Expression Nervous System Atlas (GENSAT)	Project aimed at cataloguing gene expression patterns in the mouse central nervous system as well as providing a collection of Cre mouse lines.	www.gensat.org

[1] The KOMP phase 2 (KOMP^2) has just been initiated and resources generated by this project will be available in the near future.

these community efforts are coordinated under the International Knockout Mouse Consortium (IKMC) (http://www.knockoutmouse.org/).

Knockout Mouse Project Phase 2 (KOMP^2)

As the community-wide efforts to generate an extensive collection of null and conditional-ready mouse mutants are nearing the completion of their first phase, they are gearing up to begin generating mice for phenotyping. For example, the KOMP already has generated over 400 null mutant mouse lines and, at the current production rates, is set to produce a total of over 800. Storage of ES cells and production of mice are coordinated at a central repository, which is essential for ensuring that investigators rapidly can obtain animals and reagents for their research (http://www.komp.org/). The logistics of the phenotyping phase of the KOMP (i.e., the KOMP phase 2 [KOMP^2]) were formalized at a workshop convened by the NIH in October 2009. The main recommendation was to initiate a coordinated high-throughput phenotyping effort for knockout mice generated by the KOMP, EuCOMM, and NorCOMM. The first phase of phenotyping was proposed to be broad based and conducted by a centralized phenotyping center. In addition, the gene list from the IKMC would be prioritized with the aim of discovering new phenotypes rather than confirming already-known knockout phenotypes. It was envisioned that the broad-based phenotyping program would draw on examples from other phenotyping efforts across Europe, such as the EmpressSlim model at MRC Harwell. This primary screen would include such phenotypes as measures of body weight, locomotion, pain sensitivity (i.e., nociception), and various immunological measures. Of particular interest to the alcohol research community, neurobehavioral measures also would be included in this stage. The broad-based phenotyping then would be followed by more specialized phenotyping by individual laboratories, which also would allow the alcohol research community to benefit greatly from this resource. For example, an alcohol researcher interested in gene X could obtain broad-based phenotyping data from the central repository for the knockout mouse generated for gene X, which would provide basic information on many baseline measures. The investigator then could design experiments in his or her own laboratory to test the knockout mouse for gene X on more specific measures, such as alcohol intake, withdrawal severity, and alcohol-induced motor stimulation. It is clear that this resource will provide an efficient means for alcohol researchers to discover novel genes associated with alcohol dependence.

Tang and colleagues (2010) recently published the results of such a systematic phenotyping approach using a large-scale mouse knockout library to elucidate gene function. In a tour de force, the researchers generated a mouse knockout library of 472 proteins that are either secreted by the cells or span the cell membrane (i.e., transmembrane proteins). The investigators reasoned that the genes encoding these proteins would be ideal knockout candidates, because the proteins are easily accessible therapeutic targets and understanding their function would be beneficial for drug development. The researchers performed systematic, broad-based phenotyping that encompassed several therapeutic areas, including embryonic development, metabolism, the immune system, the nervous system, and the cardiovascular system. Almost 90 percent of the knockout mutants (i.e., 419 mutants) showed an observable phenotype across various organ systems studied. Specifically, approximately 30 percent (i.e., 150 mutants) exhibited an observable phenotype in just one organ system, whereas approximately 60 percent exhibited a phenotype in two or more systems. This latter finding highlights the importance of pleiotropy—that is, the fact that a single gene can have more than one function so that a single mutation can give rise to multiple phenotypes. However, it is important to note that the mutants generated in this study all were conventional knockouts, and the caveats discussed above apply. Thus, although the approach is impressive for its large-scale effort in systematic phenotyping, it is difficult to draw clear conclusions from it because of potential developmental compensation on the phenotypes displayed by any particular knockout in this study. Community-wide efforts are under way to address some of these limitations. For example, large-scale projects to examine tissue- and cell-type– specific knockout animals are discussed below. Nonetheless, the study by Tang and colleagues (2010) is a seminal account demonstrating the utility of community-wide efforts to understand gene function using large-scale mouse knockout technology and systematic phenotyping.

International Gene Trap Consortium: An Alternative Approach

As mentioned above, the community-wide effort to generate knockout ES cells for every gene in the mouse includes both the more common gene-targeting approach and gene-trapping technology. The International Gene Trap Consortium (IGTC) (www.genetrap.org) uses gene-trap technology for high-throughput mutagenesis to produce null mutants in mouse ES cells (Nord et al. 2006). It uses small DNA pieces (i.e., vectors) that simultaneously disrupt the target gene at the point of insertion and report the level of expression of the disrupted gene. Thus, gene trapping can produce gene variants (i.e., alleles) that entirely lose their function as well as a variety of other experimental alleles if newer gene-trap vectors are used that allow for modification of expression after the insertion. The IGTC oversees a repository of all publicly available gene-trap cell lines, which are freely available to investigators. Initially, there was some skepticism regarding the percentage of gene traps that could produce true null mutants and the fraction of the genome that ultimately can be covered by gene-trap mutations. The first attempts using this approach estimated a mutational

coverage of approximately 60 percent of the mouse genome (Skarnes et al. 2004; Zambrowicz et al. 2003). More recent attempts, however, have shown more than 90 percent mutational coverage of the mouse genome (Gragerov et al. 2007). Thus, both gene-targeting and gene-trapping approaches are proving to be efficient high-throughput means of generating a community-wide resource of genetically engineered mice for studies of human disease.

Cre-Driver Mouse Project

Another gene-targeting system that has provided investigators with extraordinary control of experiments to determine gene function in living organisms is called the Cre/loxP system, which already was alluded to earlier in this article. It allows for inducible and conditional gene targeting in the mouse by engineering the bacterial gene that encodes Cre recombinase into a mouse. Expression of the Cre recombinase then can be spatially restricted by fusing the gene with a cell- or tissue-specific promoter. Cre recombinase recognizes and cuts a short bacterial DNA segment called a loxP site. These sites can be genetically engineered into a separate mouse line (referred to as a floxed line or "conditional ready" line), in which the loxP sequences are placed strategically around a critical genomic region containing a gene or gene segment of interest. When animals from the Cre-line are crossed with animals from the floxed line, the gene or gene segment of interest is excised in a specific cell type or tissue, depending on the promoter used to control the Cre gene, and researchers can study the resulting effects.

As mentioned earlier, this system has been used to elucidate the function of a glutamate receptor subunit by using a cell-type–specific promoter to drive Cre recombinase expression in a restricted brain area. In addition, the Cre/loxP system has been used in several community-wide efforts to analyze gene function. One such key community-wide project is the Cre-driver network established by the NIH Neuroscience Blueprint initiative (http://www.credrivermice.org/index). This project was motivated by a major bottleneck in the process of establishing Cre/loxP lines—that is, a lack of efficient animal lines in which the Cre gene is under the control of different promoters (i.e., Cre-driver lines) resulting in differential patterns of spatial expression, particularly in the brain. Establishing these lines is particularly challenging because of the marked differences in gene expression among various brain regions (Sandberg et al. 2000), and the Cre-driver network was spawned by the research community's need for a resource of Cre-driver lines that can be used for spatial/temporal and/or inducible knockout studies. To this end, the NIH Blueprint for Neuroscience Research funded three centers in the United States to generate genetically modified C57BL/6 mice expressing Cre recombinase in the nervous system. The resources generated from these projects will be made freely available to the neuroscience community. To date, more than 200 novel Cre-driver lines have been constructed, and many investigators are expected to use this resource as more Cre-lines are produced. Similar Cre-driver-line projects that are funded by private sources also are becoming available to the research community. For example, the Allen Institute for Brain Science reported on a robust and high-throughput Cre-reporting and characterization system for the whole mouse brain (Madisen et al. 2010).

One of the challenges inherent in using the Cre/loxP system for conditional gene modification is to verify the actual pattern of deletion of the gene of interest. This problem partially can be resolved by genetically engineering the mice so that they also express an easily measurable reporter gene (e.g., β-galactosidase or a fluorescent probe such as green fluorescent protein [GFP]) that is activated after Cre-mediated excision of a transcriptional stop signal. The expression of the reporter gene then can be visualized in the brain as a measure of the deletion pattern of a Cre-expressing line. These Cre reporter lines also are useful for mapping neuronal circuitry, imaging, and tracking cell populations in the intact organism. However, although reporter lines are useful as a first approximation of the pattern of Cre-mediated recombination, not all "floxed" reporter genes and target genes yield the same result. For example, the pattern of Cre-mediated recombination sometimes is specific to the floxed gene, and cautious interpretation of reporter line results therefore is warranted. Nevertheless, the Allen Institute for Brain Science is continuing to produce and characterize novel Cre reporter lines and making this data public through an online database (http://transgenicmouse.alleninstitute.org). This resource undoubtedly will enable investigators to determine the usefulness of various Cre-driver lines for cell-type–specific genetic manipulations.

The Gene Expression Nervous System Atlas

The Gene Expression Nervous System Atlas (GENSAT) is another remarkable project that aims to catalogue gene-expression patterns of the developing and adult central nervous system in the mouse (http://www.gensat.org/index.html). This is accomplished by using a fluorescent reporter (such as GFP) to replace the coding region of a gene of interest in a bacterial artificial chromosome (BAC) that also carries the regulatory regions (e.g., promoter) required for the gene's expression in the brain. These BAC constructs are injected into mouse eggs, and transgenic animals are generated. Using fluorescence microscopy, investigators then can visualize where the reporter gene is expressed, which reflects the natural expression of the gene of interest. To date, over 500 genes have been analyzed using this approach. This atlas of brain gene expression has significant implications for understanding the great variety of neuronal cell types (Gong et al. 2003). In addition to being a community resource for cataloging cell-type–specific gene expression in

the brain, GENSAT has targeted Cre recombinase to specific neuronal populations using the BAC approach (Gong et al. 2007), thereby allowing investigators to use the lines for genetic manipulations, such as producing inducible or conditional knockout mice.

High-Throughput Novel Genomic Sequencing

Although substantial progress has been made in identifying some of the genes associated with the risk for alcoholism, much of the genetic variation that contributes to alcoholism has yet to be identified because of the heterogeneous nature of the disease. However, the advent of novel high-throughput genomic-sequencing technologies that have become available within the last decade likely will accelerate this progress. These new sequencing technologies are termed "next-generation sequencing," to distinguish them from the conventional sequencing technologies developed in the 1970s. The greatest improvements in these new technologies are massive increases in speed and an exponential drop in cost to sequence. Thus, next-generation sequencing machines can read up to 250 billion DNA building blocks (i.e., bases) per week, compared with approximately 25,000 per week using conventional sequencing. In addition, the price per base for sequencing has dropped approximately 100,000-fold over the last decade, which makes next-generation sequencing a realistic application for all areas of biology, including sequencing of large cohorts of humans and other experimental organisms. This new approach also has the power to rapidly uncover variation in non–protein-coding regions of the genome (e.g., regulatory regions or microRNAs) and to characterize all isoforms of a particular gene by detecting alternatively spliced variants.[1] Another application of this new technology will be the complete depiction of the transcriptome and epigenome, which will have important implications for understanding how the genome functions in normal and pathophysiological conditions such as alcoholism. Creating comprehensive whole-genome maps that contain detailed information on genomic, epigenomic, and transcriptomic variation associated with alcohol dependence would greatly advance the alcohol research field.

With the advances in sequencing technologies and the resulting acceleration of data generation, the rate-limiting step of fully realizing the potential of this information now has become data analysis and bioinformatics, and it is quite clear that novel analytical approaches must be developed for meaningful data interpretation. An obvious way to address this issue would be to use a systems-based approach to interpret genomic data, including novel methods to analyze and detect gene–gene interactions (i.e., epistasis). In addition, because complete genomic information (including noncoding regulatory regions) will be at hand, novel methods to understand gene regulation are essential.

Finally, another important variable in determining vulnerability to alcohol dependence is the environment. Environmental factors can contribute as much as one-half of the total risk for developing alcoholism. However, this has not been studied systematically in relationship to gene-by-environment interactions, at least in part because of an incomplete knowledge of the genome. Next-generation sequencing, with its massive output of genomic data, likely will change this scenario by providing a foundation on which the effects of environmental perturbations can be assessed on a grand scale.

An Exciting Future for Alcohol Genetics

The postgenomic era has seen the development of global efforts to understand the function of the genome. Over the last 10 years, international research consortia have been created to tackle this enormous task, and this model is proving to be efficient for high-throughput science. In particular, concerted efforts to knock out every gene in the mouse genome are succeeding because of the use of focused resource centers. The alcohol research community is just beginning to use these resources and stands to benefit greatly from them. For example, with the availability of knockout lines for every gene, it will be possible to define the genes responsible for specific actions of alcohol. In addition, readily available conventional and conditional knockout animals will advance quantitative trait locus mapping studies. In particular, conditional knockout studies will become more abundant in the alcohol research community, allowing investigators to avoid some of the major interpretative difficulties associated with the conventional knockout studies that have dominated in the last 15 years.

Another exciting possibility is the use of new animal genetic-engineering techniques to reproduce specific genetic changes seen in human alcoholics. For example, as described above, detailed genetic sequencing of both DNA and RNA (i.e., the genome and transcriptome) from many humans now is feasible, owing to the rapidly decreasing cost of next-generation DNA sequencing. This will lead to the discovery of changes in gene sequence or gene expression that are candidates for differences in the development of alcohol dependence. These same genomic changes then can be introduced into mice or other animal models by knockin, transgenic, or other approaches (see figure). This approach already has shown promise in the alcohol field. For example, one of several variants (i.e., polymorphism) of the gene encoding the μ-opioid receptor is associated with enhanced subjective responses to alcohol in humans and differentially affects treatment response to naltrexone (Ray and Hutchinson 2007). To directly deter-

[1] During gene expression, a "copy" of the gene, called the messenger RNA (mRNA) first is generated, which then is modified (i.e., spliced) to eliminate all sequences that do not encode the final protein product. For many genes, however, more than one splicing pattern exists, resulting in several variants, or isoforms, of the gene.

subjective responses to alcohol in humans and differentially affects treatment response to naltrexone (Ray and Hutchinson 2007). To directly determine the functional consequences of this polymorphism, a knockin mouse was generated that harbors the human allele. The results indicate that the knockin mouse shows a greater brain dopamine response after alcohol challenge, possibly providing a mechanism by which the human variant of the μ-opioid receptor affects drinking (Ramchandani et al. 2010). Additional characterization of this "humanized" mouse model surely will provide important information about the functional consequences of this polymorphism on alcohol behaviors.

Besides generating mice with human-specific polymorphisms in known genes via the knockin approach, genetic engineering could be used to manipulate the vast array of noncoding regions of the genome that are copied into mRNA (i.e., are transcribed) but do not encode a specific protein. For example, recently discovered large noncoding RNAs (lncRNAs) are known to have a critical role in maintaining the state of the DNA–protein complex (i.e., chromatin) that makes up the chromosomes. Chromatin states influence gene expression on a fundamental level (Khalil et al. 2009). Although it has not been attempted yet, genetically manipulating these lncRNAs in an animal model could uncover significant functional roles in alcohol-related behaviors.

Finally, the human and mouse genomes are estimated to contain approximately 25,000 genes. However, the number of alternative forms of these known genes (i.e., alternatively spliced variants) may be about 10 times this amount, creating substantial genomic diversity with unknown function. Genetic manipulation of the roughly 200,000 alternatively spliced gene variants has not been explored systematically. This area also holds tremendous potential for discovering novel relationships between genotype and phenotype by generating genetically engineered animal models with alternatively spliced gene variants. Given

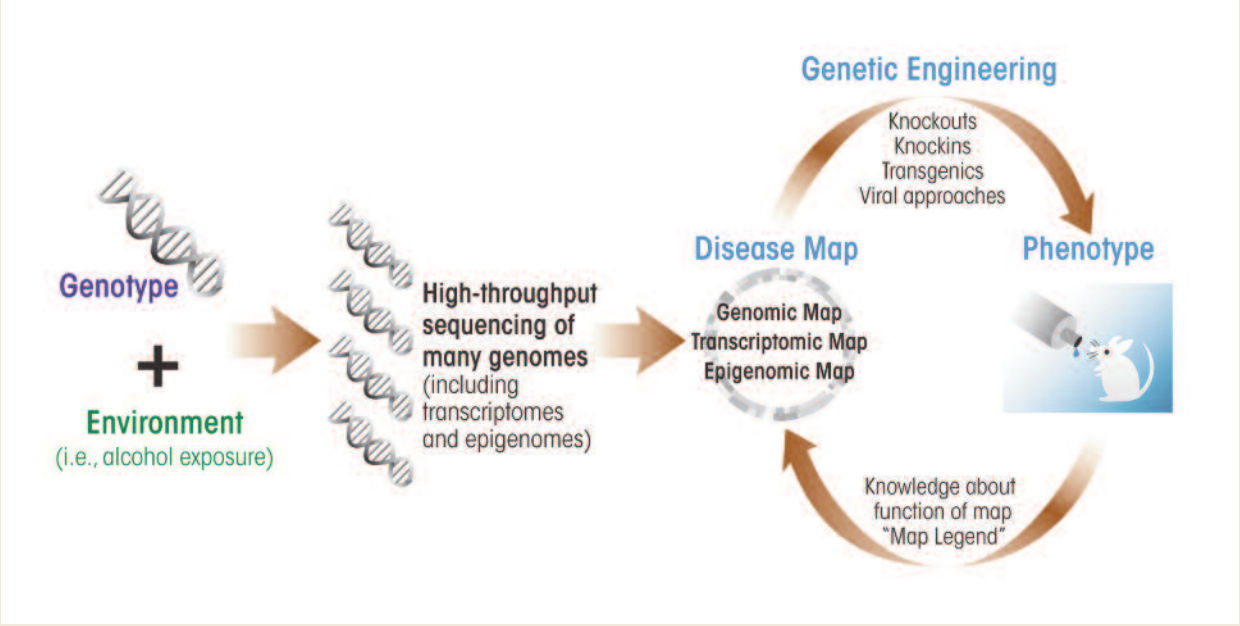


Figure Exploring the relationship between genotype and phenotype by using high-throughput sequencing and genetically engineered animal models. Novel high-throughput "next-generation sequencing" technology can be used together with new genetic engineering technology to understand gene function in alcoholism. Compared with traditional sequencing, "next-generation sequencing" allows researchers to efficiently and cost-effectively obtain large amounts of genomic data (e.g., from large cohorts of humans with and without disease) to detect all the genomic, epigenomic, and transcriptomic variation associated with the disease, creating comprehensive "disease maps." In a next step, functional information can be attached to these disease maps that defines how the various components of the map (i.e., individual genes) act and interact, for example, using genetically engineered animal models. Genomic variations associated with human diseases can be engineered into rodent models (or other experimental organisms) and detailed phenotypic analyses can be performed, further refining disease maps with functional annotation.

understanding the genetic basis of alcoholism are on the horizon. ■

Financial Disclosure

The authors declare that they have no competing financial interests.

References

Austin, C.P.; Battey, J.F.; Bradley, A.; et al. The knockout mouse project. *Nature Genetics* 36(9):921–924, 2004. PMID: 15340423

Blednov, Y.A.; Ponomarev, I.; Geil, C.; et al. Neuroimmune regulation of alcohol consumption: Behavioral validation of genes obtained from genomic studies. *Addiction Biology,* 2011*a* (in press) PMID: 21309947

Blednov, Y.A.; Borghese, C.M.; McCracken, M.L.; et al. Loss of ethanol conditioned taste aversion and motor stimulation in knockin mice with ethanol-insensitive alpha2-containing GABA(A) receptors. *Journal of Pharmacology and Experimental Therapeutics* 336(1):145–154, 2011*b.* PMID: 20876231

Brigman, J.L.; Wright, T.; Talani, G.; et al. Loss of GluN2B-containing NMDA receptors in CA1 hippocampus and cortex impairs long-term depression, reduces dendritic spine density, and disrupts learning. *Journal of Neuroscience* 30(13):4590–4600, 2010. PMID: 20357110

Camp, M.C.; Feyder, M.; Ihne, J.; et al. A novel role for PSD-95 in mediating ethanol intoxication, drinking and place preference. *Addiction Biology* 16(3):428–439, 2011. PMID: 21309945

Crabbe, J.C.; Phillips, T.J.; Harris, R.A.; et al. Alcohol- related genes: Contributions from studies with genetically engineered mice. *Addiction Biology* 11(3–4):195–269, 2006. PMID: 16961758

Edenberg, H.J., and Foroud, T. The genetics of alcoholism: Identifying specific genes through family studies. *Addiction Biology* 11(3–4):386– 396, 2006. PMID: 16961766

Enoch, M.A. The role of GABA(A) receptors in the development of alcoholism. *Pharmacology, Biochemistry, and Behavior* 90(1):95–104, 2008. PMID: 18440057

Gerlai, R. Gene-targeting studies of mammalian behavior: Is it the mutation or the background genotype? *Trends in Neurosciences* 19(5):177– 181, 1996. PMID: 8723200

Gong, S.; Doughty, M.; Harbaugh, C.R.; et al. Targeting Cre recombinase to specific neuron populations with bacterial artificial chromosome constructs. *Journal of Neuroscience* 27(37): 9817–9823, 2007. PMID: 17855595

Gong, S.; Zheng, C.; Doughty, M.L.; et al. A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. *Nature* 425(6961):917–925, 2003. PMID: 14586460

Goodwin, D.W.; Schulsinger, F.; Hermansen, L.; et al. Alcohol problems in adoptees raised apart from alcoholic biological parents. *Archives of General Psychiatry* 28(2):238–243, 1973. PMID: 4684290

Gragerov, A.; Horie, K.; Pavlova, M.; et al. Large-scale, saturating insertional mutagenesis of the mouse genome. *Proceedings of the National Academy of Sciences of the United States of America* 104(36): 14406–14411, 2007. PMID: 17720809

Harris, R.A.; Osterndorff-Kahanek, E.; Ponomarev, I.; et al. Testing the silence of mutations: Transcriptomic and behavioral studies of GABA(A) receptor $\alpha 1$ and $\alpha 2$ subunit knock-in mice. *Neuroscience Letters* 488(1):31–35, 2011. PMID: 21056629

Hodge, C.W.; Mehmert, K.K.; Kelley, S.P.; et al. Supersensitivity to allosteric GABA(A) receptor modulators and alcohol in mice lacking PKCepsilon. *Nature Neuroscience* 2(11): 997–1002, 1999. PMID: 10526339

Hungund, B.L.; Szakall, I.; Adam, A.; et al. Cannabinoid CB1 receptor knockout mice exhibit markedly reduced voluntary alcohol consumption and lack alcohol-induced dopamine release in the nucleus accumbens. *Journal of Neurochemistry* 84(4):698– 704, 2003. PMID: 12562514

June, H.L., Sr.; Foster, K.L.; Eiler, W.J., 2nd; et al. Dopamine and benzodiazepine-dependent mechanisms regulate the EtOH-enhanced locomotor stimulation in the GABAA alpha1 subunit null mutant mice. *Neuropsychopharmacology* 32(1):137–152, 2007. PMID: 16710315

Khalil, A.M.; Guttman, M.; Huarte, M.; et al. Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. *Proceedings of the National Academy of Sciences of the United States of America* 106(28):11667–11672, 2009. PMID: 19571010

Kim, K.S.; Kim, H.; Baek, I.S.; et al. Mice lacking adenylyl cyclase type 5 (AC5) show increased ethanol consumption and reduced ethanol sensitivity. *Psychopharmacology (Berl)* 215(2):391– 398, 2011. PMID: 21193983

Kranzler, H.R., and Edenberg, H.J. Pharmacogenetics of alcohol and alcohol dependence treatment. *Current Pharmaceutical Design* 16(19):2141– 2148, 2010. PMID: 20482509

Lesscher, H.M.; Wallace, M.J.; Zeng, L.; et al. Amygdala protein kinase C epsilon controls alcohol consumption. *Genes, Brain, and Behavior* 8(5):493–499, 2009. PMID: 192243450

Madisen, L.; Zwingman, T.A.; Sunkin, S.M.; et al. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nature Neuroscience* 13(1):133–140, 2010. PMID: 20023653

Naassila, M.; Pierrefiche, O.; Ledent, C.; and Daoust, M. Decreased alcohol self-administration and increased alcohol sensitivity and withdrawal in CB1 receptor knockout mice. *Neuropharmacology* 46(2):243–253, 2004. PMID: 14680762

Navarro, M.; Cubero, I.; Ko, L.; and Thiele, T.E. Deletion of agouti-related protein blunts ethanol self-administration and binge-like drinking in mice. *Genes, Brain, and Behavior* 8(4):450–458, 2009. PMID: 19566712

Nord, A.S.; Chang, P.J.; Conklin, B.R.; et al. The International Gene Trap Consortium Website: A portal to all publicly available gene trap cell lines in mouse. *Nucleic Acids Research* 34(Suppl. 1):D642–D648, 2006. PMID: 16381950

Olive, M.F.; Mehmert, K.K.; Messing, R.O.; and Hodge, C.W. Reduced operant ethanol self-administration and in vivo mesolimbic dopamine responses to ethanol in PKCepsilon-deficient mice. *European Journal of Neuroscience* 12(11):4131–4140, 2000. PMID: 11069609

Poncelet, M.; Maruani, J.; Calassi, R.; and Soubrié, P. Overeating, alcohol and sucrose consumption decrease in CB1 receptor deleted mice. *Neuroscience Letters* 343(3):216–218, 2003. PMID: 12770700

Ramchandani, V.A.; Umhau, J.; Pavon, F.J.; et al. A genetic determinant of the striatal dopamine response to alcohol in men. *Molecular Psychiatry,* 16(8):809–817, 2011. PMID: 20479755

Repunte-Canonigo, V.; Berton, F.; Cottone, P.; et al. A potential role for adiponectin receptor 2 (AdipoR2) in the regulation of alcohol intake. *Brain Research* 1339:11–17, 2010. PMID: 20380822

Ray, L.A., and Hutchison, K.E. Effects of naltrexone on alcohol sensitivity and genetic moderators of medication response: A double-blind placebo-controlled study. *Archives of General Psychiatry* 64(9):1069–1077, 2007. PMID: 17768272

Sandberg, R.; Yasuda, R.; Pankratz, D.G.; et al. Regional and strain-specific gene expression mapping in the adult mouse brain. *Proceedings of the National Academy of Sciences of the United States of America* 97(20):11038–11043, 2000. PMID: 11005875

Skarnes, W.C.; von Melchner, H.; Wurst, W.; et al. A public gene trap resource for mouse functional genomics. *Nature Genetics* 36(6):543–544, 2004. PMID: 15167922

Skvorak, K.; Vissel, B.; and Homanics, G.E. Production of conditional point mutant knockin mice. *Genesis* 44(7):345–353, 2006. PMID: 16832820

Tang, T.; Li, L.; Tang, J.; et al. A mouse knockout library for secreted and transmembrane proteins. *Nature Biotechnology* 28(7):749–755, 2010. PMID: 20562862

Thanos, P.K.; Dimitrakakis, E.S.; Rice, O.; et al. Ethanol self-administration and ethanol conditioned place preference are reduced in mice lacking cannabinoid CB1 receptors. *Behavioral Brain Research* 164(2):206–213, 2005. PMID: 16140402

Thorsell, A.; Schank, J.R.; Singley, E.; et al. Neurokinin-1 receptors (NK1R:s), alcohol consumption, and alcohol reward in mice. *Psychopharmacology (Berlin)* 209(1):103–111, 2010. PMID: 20112009

van Rijn, R.M., and Whistler, J.L. The delta(1) opioid receptor is a heterodimer that opposes the actions of the delta(2) receptor on alcohol intake. *Biological Psychiatry* 66(8):777–784, 2009. PMID: 19576572

Wallace, M.J.; Newton, P.M.; Oyasu, M.; et al. Acute functional tolerance to ethanol mediated by protein kinase C epsilon. *Neuropsychopharmacology* 32(1):127–136, 2007. PMID: 16541084

Werner, D.F.; Blednov, Y.A.; Ariwodola, O.J.; et al. Knockin mice with ethanol-insensitive alpha1-containing gamma-aminobutyric acid type A receptors display selective alterations in behavioral responses to ethanol. *Journal of Pharmacology and Experimental Therapeutics* 319(1):219–227, 2006. PMID: 16785315

Wolfer, D.P.; Crusio, W.E.; and Lipp, H.P. Knockout mice: Simple solutions to the problems of genetic background and flanking genes. *Trends in Neuroscience* 25(7):336–340, 2002. PMID : 12079755

Zambrowicz, B.P.; Abuin, A.; Ramirez-Solis, R.; et al. Wnk1 kinase deficiency lowers blood pressure in mice: A gene-trap screen to identify potential targets for therapeutic intervention. *Proceedings of the National Academy of Sciences of the United States of America* 100(24):14109–14114, 2003. PMID: 14610273

Identifying Genetic Variation for Alcohol Dependence

Arpana Agrawal, Ph.D., and Laura J. Bierut, M.D.

Arpana Agrawal, Ph.D., *is an assistant professor and* **Laura J. Bierut, M.D.,** *is a professor in the Department of Psychiatry, Washington University School of Medicine, St. Louis, Missouri.*

Researchers are using various strategies to identify the genes that may be associated with alcoholism. The initial efforts primarily relied on candidate gene and linkage studies; more recently, however, modern advances in genotyping have resulted in widespread use of genome-wide association studies for alcohol dependence. The key findings of the earlier studies were that variations (i.e., polymorphisms) in the DNA sequences of the genes encoding alcohol dehydrogenase 1B (i.e., the ADH1B gene), aldehyde dehydrogenase 2 (i.e., the ALDH2 gene), and other alcohol-metabolizing enzymes mediate the risk for alcoholism; moreover, these polymorphisms also have an impact on the risk of alcohol-related cancers, such as esophageal cancer. In addition, a gene encoding one of the receptors for the neurotransmitter γ-aminobutyric acid (GABA) known as GABRA2 seems to have a role in the development of alcohol dependence. Genome-wide association studies now offer a host of emerging opportunities, as well as challenges, for discovering the genetic etiology of alcohol dependence and for unveiling new treatment strategies. **KEY WORDS: Alcoholism; alcohol dependence; alcohol-metabolizing genes; genetic factors; genetic mapping; genome-wide association studies; candidate gene studies; genetic variants; alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH); alcohol-related cancers; esophageal cancer; γ-aminobutyric acid (GABA); DNA**

Over the last decade, three large-scale projects have catalyzed a revolution in genetic technologies and studies. First, the Human Genome Project laid the foundation for modern genetic studies of disease by determining the basic sequence of the 3 billion building blocks (i.e., base pairs) that make up the human genome and by identifying the approximately 25,000 genes included in this sequence (www.ornl.gov/sci/techresources/human_genome/home.shtml). A key component of the Human Genome Project was its collaborative nature, with participating researchers sharing their data within 24 hours of generation. A draft sequence of the human genome was released in 2000 with much fanfare, and the project was completed in 2003 with the release of the final sequence data. Second, the International HapMap Project shortly followed behind as a multicountry effort to identify and catalog common genetic similarities and differences across populations (http://hapmap.ncbi.nlm.nih.gov/). This project built on the knowledge that 99.5 percent of the DNA sequence in humans is identical amongst individuals, and that it is only the remaining 0.5 percent that contribute to the development of diseases and differences in traits. The HapMap project mapped these common differences in the genome and, in 2007, published the human haplotype map with over 3 million identified human genetic variants. Finally, since 2008, the 1000 Genomes Project seeks to discover and more finely catalogue genetic variation, particularly those variants that occur at lower frequencies in different populations (www.1000genomes.org). In addition, this project expands the study of human populations around the world to capture more genetic diversity. The information developed from these three projects has transformed the field of genetics and led to genome-wide association studies (GWASs), which aim to identify regions of the genome that are associated with diseases.

GWASs analyze the presence of hundreds of thousands, or even millions, of polymorphisms across a person's genome. The goal of GWASs is to identify those variants that occur more frequently in people with an illness (e.g., alcoholism) than in those without the disease. Standard GWASs testing now is available, which can query over a million variants that differ in only a single DNA building block (i.e., single nucleotide polymorphisms [SNPs]), indexing variations in the human genome and thereby providing relatively easy access to an individual's genetic makeup (i.e., genotype). Because the cost of genetic testing has decreased with standardization and mass production, GWASs are designed as large-scale studies to examine genetic variation in thousands to tens of thousands of people.

These types of studies already have uncovered thousands of genetic variants that alter the risk of developing many complex diseases, including type 2 diabetes, Crohn's disease, and Parkinson's disease (Hindorff et al. 2009). Recently, GWASs also have been applied to the study of alcohol dependence, resulting in the discovery of additional genes that join the existing candidate gene literature for alcohol dependence. This review will place GWASs in the context of the history of the genetic examination of alcohol dependence.

Role of Alcohol-Metabolizing Genes

Alcohol dependence was one of the first disorders to be associated with a genetic contribution. In 1972, facial flushing and decreased tolerance after alcohol exposure was observed in subjects of Asian ancestry (Wolff 1972)—a response that is associated with characteristic alterations in alcohol metabolism. Upon ingestion and absorption into the blood stream, alcohol first is converted to acetaldehyde in the liver in a process catalyzed by the enzyme alcohol dehydrogenase (ADH). Acetaldehyde is a highly toxic cancer-inducing substance (i.e., carcinogen) that normally is converted rapidly to acetate, a less toxic form. This reaction is mediated by the mitochondrial enzyme aldehyde dehydrogenase (ALDH). In this metabolic chain of events, two basic mechanisms can result in the accumulation of acetaldehyde in the body—faster metabolism of alcohol to acetaldehyde, which is related to increased ADH activity, and/or slower metabolism of acetaldehyde to acetate, which is caused by decreased ALDH activity. The excessive production and accumulation of acetaldehyde then results in the flushing response, which may be accompanied by lightheadedness, nausea, accelerated heart rate, and headaches. Because of the unpleasantness of this reaction, people experiencing flushing typically drink little or no alcohol.

Two key functional polymorphisms, both of which are common in Asian populations, have been implicated in the flushing response to alcohol and consequently identified as protective influences on alcohol consumption and dependence. In the *ADH1B* gene,[1] a polymorphism called rs1229984 (also referred to as Arg48His), which differs from the normal, or wild-type, DNA sequence in a single nucleotide, results in an amino acid change at position 48 in the β subunit of alcohol dehydrogenase from arginine to histidine (Edenberg 2007). The gene variant (i.e., allele) that encodes histidine in place of arginine at amino acid 48 is called *ADH1B*2*; the resulting enzyme leads to accelerated oxidation of alcohol to acetaldehyde and, consequently, increased acetaldehyde accumulation after alcohol consumption. It has been linked to reduced alcohol consumption and a reduced risk of alcoholism. For example, in a recent meta-analysis of published studies, Li and colleagues (2011) reported that the association between rs1229984 and alcoholism is highly significant ($P < 10^{-30}$) in Asian populations. Although the rs1229984 variant is common in populations of Asian descent, it is found infrequently (i.e., in less than 5 percent of individuals) in populations of European or African descent. Bierut and colleagues (2012) recently genotyped and determined the influence of this variant in three large European and African American case–control studies. The results indicated a strong protective effect of rs1229984 for alcohol dependence ($P = 6.6 \times 10^{-10}$). The allele also was strongly associated with lower alcohol consumption, defined as the maximum number of drinks consumed in a 24-hour period ($P = 3 \times 10^{-13}$). Thus, although rs1229984 is rare among European and African populations, at an individual level the effect of this *ADH1B* variant on amount of alcohol consumed and on the risk of developing alcohol dependence is the same regardless of ethnicity.

Likewise, a variant called rs671 in the *ALDH2* gene results in an amino acid change from glutamic acid to lysine at position 504 (Glu504Lys) in the ALDH enzyme. The allele containing this variant, called *ALDH2*2*, is associated with substantially reduced ALDH activity. As a result of this inactivation, acetaldehyde accumulates after alcohol consumption and a flushing response occurs, which in turn leads to a reduced likelihood of alcoholism. For instance, Thomasson and colleagues (1991) found that only 12 percent of Chinese men with alcoholism were *ALDH2*2* carriers (i.e., had one or two copies of the allele), compared with 48 percent of nonalcoholic men. Although this variant is common in Asian populations, it rarely occurs in populations of European and African ancestry.

In addition to the widely replicated associations between the rs1229984 and rs671 with alcoholism risk, certain variants in other genes encoding alcohol-metabolizing enzymes, such as *ADH1C*, *ADH4*, and *ADH5*, also have been linked to alcohol dependence in other populations. Alterations in the *ADH1C* gene, which encodes the γ subunit of alcohol dehydrogenase, can change alcohol metabolism by the encoded protein and have been associated with alcoholism, although these effects are not as pronounced as those noted with *ADH1B**2 (Edenberg 2007). For instance, the *ADH1C* variants rs698 and rs1693482 also alter the amino acid sequence of the encoded protein and occur with high frequency in European American populations (minor allele ≈ 47 percent). Likewise, variants in *ADH4* and *ADH5* sporadically have been linked to alcoholism. For example, Macgregor and colleagues (2009) reported associations between SNPs in these genes and alcohol dependence symptoms as well as quantity, frequency, and maximum drinks, whereas two other independent studies reported highly significant associations between *ADH4* variants and alcohol dependence (Edenberg et al. 2006;

Luo et al. 2006). The study by Mcgregor and colleagues (2009) also is among the few studies of non-Asian populations that demonstrate an association between *ADH* and *ALDH2* polymorphisms and problem drinking.

In summary, genetic variations in many of the genes encoding alcohol-metabolizing enzymes contribute to differences in alcohol intake and, thus, the risk for development of alcohol dependence. The frequency of these genetic variants differs dramatically across human populations of Asian, African, and European ancestry. Some of these genetic effects on alcohol metabolism are strongly correlated with differences in the risk of developing alcohol dependence, whereas others have a more modest effect.

Candidate Gene Studies

Other early genetic studies of alcohol dependence relied on candidate gene association studies and genome-wide linkage studies. Candidate gene studies leveraged the earliest identified genetic variations in specific genes by examining one gene, and often one variant, at a time to determine whether the variant was associated with alcoholism. These experiments identified hundreds of genes as potentially contributing to alcoholism. For example, in addition to the genes encoding alcohol-metabolizing enzymes, genes involved in brain signaling (i.e., neurotransmitter) systems, such as the dopaminergic, cholinergic, and serotonergic systems, have been nominated for their association with alcohol dependence. In contrast to the alcohol-metabolizing genes, however, these other candidate gene associations have not yet been validated in the modern large-scale genetic studies. This lack of convergence of candidate gene studies and GWASs potentially reflects a large number of false-positive findings from the previous candidate gene findings. Another potential explanation is that extensive genetic heterogeneity exists, meaning that multiple genes each modestly contribute to the development of alcohol dependence and that the samples collected to date, although they were obtained from thousands of subjects, are not yet sufficient in size to detect variation in these regions.

Genome-Wide Linkage Studies

Genome-wide linkage studies, which examine whether large genetic regions distributed across the entire genome are cotransmitted along with a disease within families, were the first genetic studies to query the entire genome with hundreds or thousands of genetic markers. These studies had two main characteristics: they were family based, and the marker coverage across the genome was modest. Although these linkage studies implicated multiple regions of the genome as being involved in the development of alcoholism, the most consistent findings have pointed to two regions on chromosome 4, with peaks at chromosome locations 4p13–4p12 and on 4q21–4q23.[2] The 4q21–4q23 region covers the cluster of alcohol dehydrogenase genes that includes, among others, *ADH1B*, supporting the candidate gene findings that alcohol-metabolizing genes are associated with alcohol dependence. The chromosome 4p13 region also contains a cluster of genes encoding the α-subunit of the receptor for the inhibitory neurotransmitter γ-aminobutyric acid (GABA), suggesting that polymorphisms in this receptor also may be involved in the development of alcohol dependence. This cluster of GABA receptor genes is of particular interest because of their putative biological relevance to alcohol-related behaviors, as discussed in the following section.

Genetic variations in many of the genes encoding alcohol-metabolizing enzymes contribute to differences in alcohol intake and, thus, the risk for development of alcohol dependence.

Alcohol Use Disorders and GABRA2

Several SNPs in a gene called *GABRA2*, which encodes the α2-subunit of the GABA receptor, have been associated with alcohol dependence. The first study to implicate *GABRA2* in the etiology of alcoholism was conducted by Edenberg and colleagues (2004), who found an association between multiple SNPs (in moderate to high correlation with each other) and alcohol dependence. These results initially were replicated by multiple independent efforts (Drgon et al. 2006; Fehr et al. 2003; Lappalainen et al. 2005; Soyka et al. 2008); however, other investigators could not replicate the findings (e.g., Lind et al. 2008). In contrast to the variants in the alcohol-metabolizing genes, the *GABRA2* variants that are associated with alcohol dependence do not change the amino acid sequence in the encoded protein but instead likely alter the regulation of *GABRA2* protein production.

Because the genetic variants do not change the protein structure of the GABA-α2 subunit, the associations between these genetic variants and alcoholism are less obvious than those for the alcohol-metabolizing genes. Recent laboratory research has focused on the specific functional aspects of *GABRA2* in the etiology of alcoholism. *GABRA2* encodes a subtype of one of five subunits that form the commonly occurring $GABA_A$ ionotropic receptor. Binding of GABA to this receptor results, among several outcomes, in sedation and relief of anxiety (i.e., anxiolysis). This anxiolytic effect is more closely

related to the α2-subunits than to other subunits (e.g., Dixon et al. 2008). Most α2-containing GABA receptors also are key binding sites for benzodiazepines, whereas receptors containing other subunits only show sensitivity to ethanol. The genetic variants in the *GABRA2* gene do not result in functional changes in the receptor, and, in fact, little is known about how the variants that are related to the development of alcohol dependence influence *GABRA2* activity. Some investigators (Haughey et al. 2008) demonstrated that a variant called rs279858 resulted in changes in the levels of an intermediary molecule formed during the conversion of the genetic information encoded in the gene into a protein product (i.e., in mRNA levels). Kareken and colleagues (2010) found variations in the brain's response[3] to preferred alcoholic drink aromas in a study comparing people who carried two copies (i.e., were homozygotes) or only one copy (i.e., were heterozygotes) of the "risk" allele for a variant called rs279871. Finally, Villafuerte and colleagues (2012) reported that two *GABRA2* variants called rs279826 and rs279858, which previously had been implicated in the etiology of alcoholism, not only correlated with impulsiveness but also with activity in a brain region called the insula cortex during monetary reward and loss. Thus, in experiments assessing responses during reward anticipation and loss, study participants from alcoholic families with the haplotype that is associated with increased alcoholism vulnerability showed significantly higher activation in the left insula than did participants with a different haplotype. However, much more research is needed to understand the biology underlying these genetic associations with *GABRA2*.

Animal models also have begun to address these functional aspects of *GABRA2*, as follows:

- Work with frog eggs (i.e., *Xenopus* oocytes) has demonstrated that the α2 subunits are involved in regulating GABA currents upon ethanol exposure (Hurley et al. 2009).
- Studies of mice that had been genetically modified to produce or not produce the GABA-α2 subunit (i.e., knock-in and knock-out mice) demonstrated the important role of this subunit in anxiolysis (Boehm et al. 2004; Dixon et al. 2008). Additional studies with these animals provided evidence for the involvement of the α2 subunits in the hypnotic effects of ethanol when administered together with the benzodiazepine diazepam (Tauber et al. 2003).
- In analyses comparing alcohol-preferring (P) and non-preferring (NP) rats (McBride and Li 1998), Liu and colleagues (2011) reported that P rats show elevated levels of GABA-A subunits, including the α2 subunits, in a brain region called the central nucleus of the amygdala. Intriguingly, when molecules that specifically could inhibit the *GABRA2* gene (i.e., α2 silent RNA [siRNA]) were introduced into the central amygdala, even the P animals no longer demonstrated binge drinking for a short period of time, and this effect correlated with a significant reduction of α2 levels. This effect did not occur when siRNA for another receptor subunit (i.e., α1 siRNAs) was used or when α2 siRNA was introduced in other control regions of the brain.

Thus, much research has examined the role of the α-subunit of the GABA receptor, and this protein clearly plays a central role in alcohol-mediated anxiolysis and other effects of alcohol use. Nevertheless, it remains important to more clearly connect genetic variations found to be associated with alcoholism risk in humans with basic biologic function. Functional studies using animal models, postmortem brain tissue, and novel methodologies that examine gene expression in the context of neuronal connectivity in alcoholics and nonalcoholics are among a few methods that may serve to further elucidate the function of candidate variants identified in human gene-association studies.

GWASs

GWASs represent the most recent paradigm change for gene discovery. The allure of GWASs is that they allow for interrogation of hundreds of thousands to over 1 million SNPs across the human genome at relatively modest cost. Thus, GWASs can potentially lead to the identification of variants of modest effect size that may not be recognized in candidate gene studies for their biological significance in alcoholism. The principal disadvantage is the fairly severe multiple-testing burden (i.e., the need to account for the possibility that when 1 million tests are conducted, some positive results will be obtained due to chance alone) imposed by GWASs, which results in the requirement for statistical significance to be denoted by *P* values of 5×10^{-8} and lower. This represents a very high significance level for validation; consequently, many SNPs that truly are associated with alcoholism risk may not yet have been recognized because they have not yet surpassed this threshold.

Four GWASs of alcohol dependence and two of alcohol consumption now have been completed in populations of European ancestry. In the first of these, Treutlein and colleagues (2009) found several SNPs with *P* values in the range of 10^{-6}; upon pooling with a replication sample, two of these SNPs, rs7590720 and rs1344694, showed statistical significance in the combined male-only sample of 1,460 alcohol-dependent subject and 2,332 community control subjects. These SNPs are located in a region of the long arm of chromosome 2 where prior linkage studies have identified increased allele-sharing for

alcoholism. The gene closest to the association signal, *PECR* (which encodes the enzyme peroxisomal trans-2-enoyl-CoA reductase) is involved in the metabolism of fatty acids, particularly during deprivation when energy expenditure transitions from carbohydrates to fatty acids. In addition, the investigators noted that a SNP called rs1614972 in the *ADH1C* gene was strongly associated with alcohol dependence ($P = 1.4 \times 10^{-4}$) even though it did not meet strict standards for genome-wide multiple testing. Two subsequent studies have identified a SNP in *PKNOX2* (rs10893366, $P = 1.9 \times 10^{-7}$) and a cluster of SNPs on chromosome 11 ($P < 10^{-5}$) that also are associated with alcohol dependence but did not meet the level of statistical significance (Bierut et al. 2010; Edenberg et al. 2010). A fourth GWASs of quantitative indices of excessive alcohol consumption recently was completed in a large cohort of Australian families, in which no genetic variant met the standards for genome-wide significance (Heath et al. 2011). Across the four studies, there was no evidence for replication of any genetic signals, raising the possibility that the identified signals were false positives or that true signals could not be detected as a result of the (by GWASs standards) relatively small sample sizes (i.e., several thousand subjects).

Although GWASs of subjects of European descent have struggled to identify and replicate SNPs associated with alcohol dependence, a recent GWASs of a sample of 1,721 Korean men reported associations with *P* values of 10^{-58} and lower for SNPs in moderate to high linkage disequilibrium[4] with SNPs in the *ALDH2* gene, including rs671 (Baik et al. 2011). In this study, carriers of the protective allele of the SNP rs11066280 in the gene *C12orf51*, which frequently occurs together with rs671 in *ALDH2* (the SNP that inactivates *ALDH2*), had a 13-fold increased risk for alcohol flushing and a corresponding 5-fold decreased likelihood of developing alcohol problems consistent with alcohol dependence. Because this variant is rare in subjects of European and African descent, this strong finding is not seen, nor expected, in non-Asian populations.

In addition to studying alcohol dependence, a strategy to increase the power of GWASs has been to examine alcohol consumption as a quantitative variable. A recent meta-analysis of alcohol consumption by Schumann and colleagues (2011) used results from an initial sample of 26,316 population-based subjects and a follow-up sample of 21,185 European American subjects to identify a SNP called rs6943555 in the autism susceptibility candidate 2 (*AUTS2*) gene that was associated with alcohol consumption (in grams/day/kilogram of body weight) at $P < 5 \times 10^{-9}$. Specifically, people who carried the minor allele of rs6943555 showed 5.5 percent lower alcohol consumption than people who carried the major allele; however, secondary analyses did not reveal any association with alcohol dependence. Follow-up studies showed changes in mRNA expression that correlated with the rs6943555 genotype; moreover, the gene itself was expressed differently in low-alcohol– versus high-alcohol–preferring rats. Likewise, Heath and colleagues (2011) examined a quantitative factor score created from items assessing quantity and frequency of consumption, frequency of intoxication, and maximum drinks in a single 24-hour period, all reported for the 12-month period when the participants indicated that they drank the heaviest. The results implicated variants in the *TMEM108* gene, which encodes a transmembrane protein of unknown function ($P = 1.2 \times 10^{-7}$). These two initial studies of alcohol consumption implicate different genomic regions, and their findings have not converged. With larger sample sizes, it will become clearer whether these results represent true findings.

The health effects of alcohol consumption remain a public health priority that needs to be studied from all angles, including improving prevention and treatment, while also examining basic biological underpinnings.

Genetic Studies of Alcoholism in the Context of Other Diseases

Alcohol is consumed by an overwhelming majority of the U.S. population, and 55 percent of the world's population has consumed alcohol (World Health Organization [WHO] 2011). Per capita consumption varies greatly by country. For example, the most recent figures from the WHO (2011) indicate that among people ages 15 and older, per capita annual alcohol consumption was 11 liters in Russia and 8.5 liters in the United States, with much lower consumption levels reported in parts of Asia and northern Africa. Although modest alcohol consumption is associated with health (e.g., cardiovascular) benefits, according to the WHO, 2.5 million people die each year from the harmful effects of alcohol (http://www.who.int/substance_abuse/facts/en/). In the United States, nearly 80,000 people die annually from the short- and long-term consequences of alcohol use (Mokdad et al. 2004). Excessive alcohol consumption can lead to alcohol dependence, which affects 12.5 percent of people in the United States across their lifetime (Hasin et al. 2007). Thus, the health effects of alcohol consumption remain a public health priority that needs to be studied from all angles, including improving prevention and treatment, while also examining basic biological underpinnings.

Alcohol consumption increases the likelihood of several other disorders. For example, population-based studies

[4] The term linkage disequilibrium means that some combinations of alleles or genetic markers in a population occur more often or less often than would be expected from a random formation of haplotypes based on the alleles' frequencies.

have demonstrated that esophageal cancer is linked to alcohol consumption as a result of the tissue's exposure to ethanol and its metabolite acetaldehyde. Consequently, functional variants in *ADH1B* and *ALDH2* that affect alcohol clearance and acetaldehyde accumulation also play a role in the development of esophageal cancer. The effects of these variants on cancer are strongly moderated by accompanying alcohol exposure. For example, people who carry the *ADH1B*2* allele that results in more rapid ethanol conversion to acetaldehyde, experience flushing and, hence, have reduced likelihood of alcohol intake, protecting them from esophageal cancer. Conversely, people who carry the *ADH1B**1 variant, which results in slower alcohol metabolism, are at increased risk for esophageal cancer, arguably because of extended tissue exposure to ethanol. Likewise, having the *ALDH2**2 allele, which inactivates aldehyde dehydrogenase, has protective effects on both alcohol intake and esophageal cancer. However, people who carry only one copy of *ALDH2**2 often continue to drink, which leads to acetaldehyde accumulation and consequently increases the risk for esophageal cancer (Brooks et al. 2009).

Because both protective gene variants are more common in Asian populations, several studies have demonstrated the influence of *ADH1B**2 and *ALDH2**2 on esophageal cancer in these populations (Ding et al. 2010; Hashibe et al. 2008). A meta-analysis of published studies on Asian populations (Yang et al. 2010) further confirmed that the potentially large effect of these gene variants on esophageal cancer was exacerbated by alcohol exposure.

A recent GWASs of cancers of the upper aerodigestive tract (UADT) (i.e., cancers of the oral cavity, pharynx, larynx, and esophagus) identified SNP rs1229984 in *ADH1B* as a risk variant for esophageal cancer in samples with European ancestry (McKay et al. 2011). This variant is present in as many as 70 to 80 percent of people in Asian populations (Han et al. 2007; Kosoy et al. 2009) but is found in less than 5 percent of European populations. Nevertheless, it is by far the strongest genetic variant that alters the risk for the development of UADT cancer, even after accounting for alcohol consumption. Variants in other alcohol-metabolizing genes also have been implicated in the development of esophageal cancer.

In several of these studies, the effects of *ADH1B* and *ALDH2* variants predominantly were noted in those who drink alcohol. For instance, even in people with two copies of the *ALDH2**2 allele, which is highly protective for alcohol intake, the few drinkers are at a profoundly elevated risk for esophageal cancer (Yang et al. 2010). It might be hypothesized that this finding reflects a genotype–environment correlation where *ALDH2* and *ADH1B* variants influence the likelihood of continued alcohol (and acetaldehyde) exposure, which, in turn, serves as the environmental risk factor for the development of esophageal cancer, either as a result of prolonged ethanol exposure or of the carcinogenic effects of acetaldehyde. An alternative explanation is that of a genotype x environment interaction—that is, people who carry the *ALDH2* and *ADH1B* genotypes that protect against alcohol intake might be exquisitely sensitive to the joint effects of genotype and alcohol consumption on their vulnerability to esophageal cancer.

Genes to Function to Treatment

Genetic association studies only are a first step in understanding the biology underlying alcohol dependence. An association represents a correlation with a tested variant (and all the untested correlated variants). These groups of associated SNPs often span many genes on a chromosome, and once a genetic association is confirmed the task is to investigate how these variants and the genes they are located in contribute to the biological mechanisms underlying disease development. The ultimate goal of understanding these biological mechanisms is to gain new insights into potential treatment approaches.

To some extent, clinicians already are exploiting the understanding of genetic variation in the development of alcoholism in their treatment strategies. For example, the variation in enzymatic pathways of alcohol metabolism found in the population is mimicked in the pharmacologic treatment of alcohol dependence using the medication disulfiram, which inhibits ALDH and is a pharmacologic treatment similar to the natural variation related to rs671 and decreased ALDH activity in Asians. If alcohol is consumed after taking disulfiram, acetaldehyde levels increase, causing flushing, nausea, vomiting, and headache in the patient, which produces an aversive response to continued alcohol use.

Future Directions

Researchers long have known that alcohol dependence clusters in families. During the past 50 years, adoption and twin studies definitively have demonstrated that this clustering is related to genetic influences. Twin studies have estimated that 30 to 60 percent of the variance in alcohol dependence is attributable to the effects of genetic variants segregating in families (Dick and Bierut 2006). Researchers now are entering a new phase in genetic studies where they are beginning to unlock the genetic code that leads to disease.

> ***"Ebrii gignunt ebrios" (One drunkard begets another)***
>
> ***—attributed by Richard Burton to Plutarch***

Although the GWASs approach has been successful for many illnesses, much of the genetic variation underlying the development of alcohol dependence remains undiscovered. Thus, although the heritability of alcohol

dependence approaches 50 percent, the explained genetic variance to date is less than 1 percent, which raises the question "Where is the missing genetic variance?" Three main explanations have been given for the missing variance in the genetic risk for alcohol dependence: (1) many genes of small effect may be involved that do not yet surpass the stringent threshold for genome-wide significance; (2) rare variations—that is, SNPs that only occur in less than 1 percent of the population—exist that are not included on standard chips and hence not captured by GWASs genotyping; and (3) other mechanisms, including the interplay of genes and environments, may contribute that are not detected in current analyses.

A key component for the future success of genetic studies is the creation of large-scale genetic research consortia that permit the study of large numbers of comprehensively assessed subjects. Collaboration is necessary for these new genetic studies, and resources are shared with the scientific community through the Database of Genotypes and Phenotypes (dbGaP), which allows investigators to test new hypotheses about the genetic contributions to alcohol dependence (www.ncbi.nlm.nih.gov/sites/entrez?db=gap). This collaborative research is necessary because of the large sample sizes—tens of thousands or more—needed to study genetic effects. The sharing of genetic information has been a corner stone of the Human Genome Project, the International HapMap Project, and the 1000 Genomes Project, and the collaborative aspect must continue if researchers are to gain a better understanding of disease.

The future of genetic studies potentially may help people understand their personal risks and potential treatments for disease. The strongest genetic contributors to alcohol dependence to date are related to the pharmacologic responses to alcohol represented by variations in alcohol-metabolizing genes. This knowledge represents the first level of genetic understanding, and researchers now are poised to move to the next level with the identification of additional genes that contribute to alcoholism. Several challenges remain, however, as this field moves forward. First, investigators must increase the diversity of the populations under study. Varying allele frequencies across populations mean that important genetic contributors in one population may not be seen in another, with the classic example being the variation in the alcohol-metabolizing genes that contributes to alcohol dependence. Increasing diversity in the populations under study will allow researchers to leverage these differences to refine association signals and also to increase the potential discovery of genetic variants. A second challenge is to integrate the findings from candidate gene studies and GWASs. The results from these two methods only have converged modestly, primarily with the alcohol-metabolizing genes, and the discrepancies should be resolved so that the high false-negative rate of GWASs can be balanced with the high false-positive results in candidate gene studies. Finally, the environment will always play an important role in the development of alcoholism by providing the exposure to and availability of alcohol. Therefore, it will be essential to integrate genetic predispositions with environmental exposures in order to better prevent and treat alcohol dependence, one of the Nation's most devastating illnesses. ■

Acknowledgements

This work was supported by National Institutes of Health (NIH) grants U10–AA–008401 and K02–DA–021237 to Laura Bierut. Arpana Agrawal receives funds from NIH grants (R01–DA–23668 and R03–DA–25886) and from ABMRF/Foundation for Alcohol Research.

Financial Disclosure

Dr. Bierut is an inventor on the patent "Markers for Addiction" (US 20070–258898) covering the use of certain SNPs in determining the diagnosis, prognosis, and treatment of addiction. Dr. Bierut served as a consultant for Pfizer in 2008. No other potential conflicts of interest relevant to this article were reported.

References

Baik, I.; Cho, N.H.; Kim, S.H.; et al. Genome-wide association studies identify genetic loci related to alcohol consumption in Korean men. *American Journal of Clinical Nutrition* 93(4):809–816, 2011. PMID: 21270382

Bierut, L.J.; Agrawal, A.; Bucholz, K.K.; et al. A genome-wide association study of alcohol dependence. *Proceedings of the National Academy of Sciences of the United States of America* 107(11):5082–5087, 2010. PMID: 20202923

Bierut, L.J.; Goate, A.M.; Breslau, N.; et al. *ADH1B* is associated with alcohol dependence and alcohol consumption in populations of European and African ancestry. *Molecular Psychiatry,* 17:455–450, 2012. PMID: 21968928

Boehm, S.L. 2nd; Ponomarev, I.; Jennings, A.W.; et al. gamma-Aminobutyric acid A receptor subunit mutant mice: New perspectives on alcohol actions. *Biochemical Pharmacology* 68(8):1581–1602, 2004. PMID: 15451402

Brooks, P.J.; Enoch, M.A.; Goldman, D.; et al. The alcohol flushing response: An unrecognized risk factor for esophageal cancer from alcohol consumption. *PLoS Medicine* 6(3):e50, 2009. PMID: 19320537

Dick, D.M., and Bierut, L.J. The genetics of alcohol dependence. *Current Psychiatry Reports* 8(2):151–157, 2006. PMID: 16539893

Ding, J.H.; Li, S.P.; Cao, H.X.; et al. Alcohol dehydrogenase-2 and aldehyde dehydrogenase-2 genotypes, alcohol drinking and the risk for esophageal cancer in a Chinese population. *Journal of Human Genetics* 55(2):97–102, 2010. PMID: 20010786

Dixon, C.I.; Rosahl, T.W.; and Stephens, D.N. Targeted deletion of the GABRA2 gene encoding alpha2-subunits of GABA(A) receptors facilitates performance of a conditioned emotional response, and abolishes anxiolytic effects of benzodiazepines and barbiturates. *Pharmacology, Biochemistry, and Behavior* 90(1):1–8, 2008. PMID: 18313124

Drgon, T.; D'Addario, C.; and Uhl, G.R. Linkage disequilibrium, haplotype and association studies of a chromosome 4 GABA receptor gene cluster: Candidate gene variants for addictions. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics* 141B(8): 854–860, 2006. PMID: 16894595

Edenberg, H.J. The genetics of alcohol metabolism: Role of alcohol dehydrogenase and aldehyde dehydrogenase variants. *Alcohol Research & Health* 30(1):5–13, 2007. PMID: 17718394

Edenberg, H.J.; Dick, D.M.; Xuei, X.; et al. Variations in GABRA2, encoding the alpha 2 subunit of the GABA(A) receptor, are associated with alcohol dependence and

with brain oscillations. *American Journal of Human Genetics* 74(4):705–714, 2004. PMID: 15024690

Edenberg, H.J.; Koller, D.L.; Xuei, X.; et al. Genome-wide association study of alcohol dependence implicates a region on chromosome 11. *Alcoholism: Clinical and Experimental Research* 34(5):840–852, 2010. PMID: 20201924

Edenberg, H.J.; Xuei, X.; Chen, H.J.; et al. Association of alcohol dehydrogenase genes with alcohol dependence: A comprehensive analysis. *Human Molecular Genetics* 15(9):1539–1549, 2006. PMID: 16571603

Fehr, C.; Rademacher, B.L.; and Buck, K.J. Evaluation of the glutamate decarboxylase genes Gad1 and Gad2 as candidate genes for acute ethanol withdrawal severity in mice. *Progress in Neuropsychopharmacology & Biological Psychiatry* 27(3):467–472, 2003. PMID: 12691782

Han, Y.; Gu, S.; Oota, H.; et al. Evidence of positive selection on a class I ADH locus. American Journal of Human Genetics 80(3):441–456, 2007. PMID: 17273965

Hashibe, M.; McKay, J.D.; Curado, M .P.; et al. Multiple ADH genes are associated with upper aerodigestive cancers. *Nature Genetics* 40(6):707–709, 2008. PMID: 18500343

Hasin, D.S.; Stinson, F.S.; Ogburn, E.; et al. Prevalence, correlates, disability, and comorbidity of DSM-IV alcohol abuse and dependence in the United States: Results from the National Epidemiologic Survey on Alcohol and Related Conditions. *Archives of General Psychiatry* 64(7):830–842, 2007. PMID: 17606817

Haughey, H.M.; Ray, L.A.; Finan, P.; et al. Human gamma-aminobutyric acid A receptor alpha2 gene moderates the acute effects of alcohol and brain mRNA expression. *Genes, Brain, and Behavior* 7(4):447–454, 2008. PMID: 18005236

Heath, A.C.; Whitfield, J.B.; Martin, N.G.; et al. A quantitative-trait genome-wide association study of alcoholism risk in the community: Findings and implications. *Biological Psychiatry* 70(6):513–518, 2011. PMID: 21529783

Hindorff, L.A.; Sethupathy, P.; Junkins, H.A.; et al. Potential etiologic and functional implications of genome-wide association loci for human diseases and traits. *Proceedings of the National Academy of Sciences of the United States of America* 106(23):9362–9367, 2009. PMID: 19474294

Hurley, J.H.; Ballard, C.J.; and Edenberg, H.J. Altering the relative abundance of GABA A receptor subunits changes GABA- and ethanol-responses in Xenopus oocytes. *Alcoholism: Clinical and Experimental Research* 33(6):1089–1096, 2009. PMID: 19382902

Kareken, D.A.; Liang, T.; Wetherill, L.; et al. A polymorphism in GABRA2 is associated with the medial frontal response to alcohol cues in an fMRI study. *Alcoholism: Clinical and Experimental Research* 34(12):2169–2178, 2010. PMID: 20698837

Kosoy, R.; Nassir, R.; Tian, C.; et al. Ancestry informative marker sets for determining continental origin and admixture proportions in common populations in America. *Human Mutation* 30(1):69–78, 2009. PMID: 18683858

Lappalainen, J.; Krupitsky, E.; Remizov, M.; et al. Association between alcoholism and gamma-amino butyric acid alpha2 receptor subtype in a Russian population. *Alcoholism: Clinical and Experimental Research* 29:493–498, 2005. PMID: 15834213

Li, D.; Zhao, H.; and Gelernter, J. Strong association of the alcohol dehydrogenase 1B gene (ADH1B) with alcohol dependence and alcohol-induced medical diseases. *Biological Psychiatry* 70(6):504–512, 2011. PMID: 21497796

Lind, P.A.; Macgregor, S.; Agrawal, A.; et al. The role of GABRA2 in alcohol dependence, smoking, and illicit drug use in an Australian population sample. *Alcoholism: Clinical and Experimental Research* 32(10):1721–1731, 2008. PMID: 18727688

Liu, J.; Yang, A.R.; Kelly, T.; et al. Binge alcohol drinking is associated with GABAA alpha2-regulated Toll-like receptor 4 (TLR4) expression in the central amygdala. *Proceedings of the National Academy of Sciences of the United States of America* 108(11):4465–4470, 2011. PMID: 21368176

Luo, X.; Kranzler, H.R.; Zuo, L.; et al. ADH4 gene variation is associated with alcohol dependence and drug dependence in European Americans: Results from HWD tests and case-control association studies. *Neuropsychopharmacology* 31(5):1085–1095. 2006. PMID: 16237392

Macgregor, S.; Lind, P.A.; Bucholz, K.K.; et al. Associations of ADH and ALDH2 gene variation with self report alcohol reactions, consumption and dependence: An integrated analysis. *Human Molecular Genetics* 18(3):580–593, 2009. PMID: 18996923

McBride, W.J., and Li, T.K. Animal models of alcoholism: Neurobiology of high alcohol-drinking behavior in rodents. *Critical Reviews in Neurobiology* 12(4):339–369, 1998. PMID: 10348615

McKay, J.D.; Truong, T.; Gaborieau, V.; et al. A genome-wide association study of upper aerodigestive tract cancers conducted within the INHANCE Consortium. *PLoS Genetics* 7(3):e1001333, 2011. PMID: 21437268

Mokdad, A.H.; Marks, J.S.; Stroup, D.F.; and Gerberding, J.L. Actual causes of death in the United States, 2000. *JAMA: Journal of the American Medical Association* 291(10):1238–1245, 2004. PMID: 15010446

Schumann, G.; Coin, L.J.; Lourdusamy, A.; et al. Genome-wide association and genetic functional studies identify autism susceptibility candidate 2 gene (AUTS2) in the regulation of alcohol consumption. *Proceedings of the National Academy of Sciences of the United States of America* 108(17):7119–7124, 2011. PMID: 21471458

Soyka, M.; Preuss, U.W.; Hesselbrock, V.; et al. GABA-A2 receptor subunit gene (GABRA2) polymorphisms and risk for alcohol dependence. *Journal of Psychiatric Research* 42(3):184–191, 2008. PMID: 17207817

Tauber, M.; Calame-Droz, E.; Prut, L.; et al. alpha2-gamma-Aminobutyric acid (GABA)A receptors are the molecular substrates mediating precipitation of narcosis but not of sedation by the combined use of diazepam and alcohol in vivo. *European Journal of Neuroscience* 18(9):2599–2604, 2003. PMID: 14622161

Thomasson, H.R.; Edenberg, H.J.; Crabb, D.W.; et al. Alcohol and aldehyde dehydrogenase genotypes and alcoholism in Chinese men. *American Journal of Human Genetics* 48(4):677–681, 1991. PMID: 2014795

Treutlein, J.; Cichon, S.; Ridinger, M.; et al. Genome-wide association study of alcohol dependence. *Archives of General Psychiatry* 66(7):773–784, 2009. PMID: 19581569

Villafuerte, S.; Heitzeg, M.M.; Foley, S.; et al. Impulsiveness and insula activation during reward anticipation are associated with genetic variants in GABRA2 in a family sample enriched for alcoholism. *Molecular Psychiatry,* 17(5):511–519, 2012. PMID: 21483437

Wolff, P.H. Ethnic differences in alcohol sensitivity. *Science* 175(20):449–450, 1972. PMID: 5007912

World Health Organization. *Global Status Report on Alcohol and Health.* Geneva, Switzerland: WHO Press, 2011.

Yang, S.J.; Yokoyama, A.; Yokoyama, T.; et al. Relationship between genetic polymorphisms of ALDH2 and ADH1B and esophageal cancer risk: A meta-analysis. *World Journal of Gastroenterology* 16(33):4210–4220, 2010. PMID: 20806441

Genetics Primer

Genetics is the study of genes—the heritable information that contains the codes for proteins and other molecules which form and maintain an organism's structure and function. In most organisms, these genes are found in strands of deoxyribonucleic acid (DNA) molecules. The specific structure of the DNA (described below) ensures that the genetic information can be passed from one generation to the next, while allowing for some reorganization that results in new variations and, ultimately, evolution.

Although nearly all cells in an organism have the same set of DNA (i.e., genome), the genomes vary among organisms, ensuring that (with few exceptions) each individual is unique. The degree of this variation is a measure of how closely related two organisms are. Thus, the differences among the genomes will be smaller among members of a family than among two completely unrelated persons, and those between related species (e.g., humans and chimpanzees) will be smaller than those between more diverse species (e.g., humans and flies).

Higher organisms are made up of various tissues and organs composed of cells with a range of functions, such as nerve, blood, or muscle cells. Yet all these cells contain the same genome. To achieve the necessary variation in cell structure and function, some DNA portions are "active," or expressed, in certain cells and at particular developmental stages, leading to the production of different end products. In addition, the environment, to some extent, can influence gene expression, resulting in changes in how the organism functions in, and adapts to, its environment.

What Is DNA?

DNA is a large complex molecule constructed from building blocks called nucleotides, each of which consists of a sugar molecule (deoxyribose) attached to an organic base. There are four organic bases and, accordingly, four different nucleotides called adenosine, cytosine, guanosine, and thymidine, generally referred to by their initials A, C, G, and T. In the cell, the nucleotides are arranged as long strings, with two strings interacting at the organic bases to form a double helix. Moreover, because of the chemical structures of these bases, their interactions are highly specific, so that T nucleotides in one strand only can interact with A nucleotides on the other strand and C nucleotides only can interact with G nucleotides. As a result, the two strands are said to be complementary. This feature is the basis for the ability of the DNA to be duplicated faithfully (at least for the most part) when cells divide so that all cells in an organism carry the same DNA sequence, which also can be passed on to the next generation. However, some variations or errors (i.e., mutations) can occur during this duplication, which lead to the variations that ensure the diversity of individuals within one species and also across species.

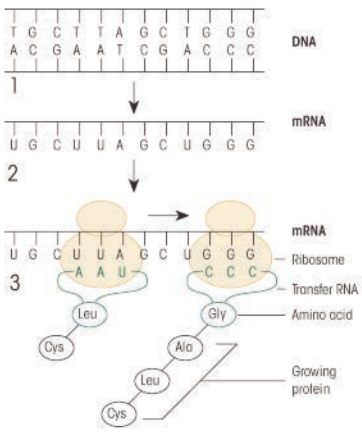
How Is Genetic Information Converted Into Proteins?

The genetic information is encoded in the order of the nucleotides. A gene is a particular set of these nucleotides that serves as the blueprint for a specific protein. But how does the cell read this building instruction? When a protein is needed in the cell, the DNA double helix at the corresponding gene briefly splits into single strands. This allows certain proteins that mediate specific chemical reactions (i.e., enzymes) to copy the appropriate DNA strand by bringing in new nucleotides complementary to those in the strand (see figure). This process is called transcription. However, these new nucleotides contain a different sugar (i.e., ribose) and instead of the T nucleotides use a fifth nucleotide called uracil (U). The resulting new strand, which is made up of ribose-containing A, C, G, and U nucleotides, is called a ribonucleic acid (RNA). The RNA is released from the DNA (which then "zips" back up with its complementary strand) and is processed further into a messenger RNA (mRNA) that moves as a single strand out of the nucleus into the cytoplasm. There, other enzymes can bind to the mRNA and bring protein building blocks (i.e., amino acids) together to form chains. This process is known as translation (see figure). The order in which the amino acids are assembled is determined by the sequence of nucleotides in the mRNA, with blocks of three mRNA nucleotides representing one amino acid. The sequential steps of transcription and translation—from DNA through the intermediate mRNA to protein—are the process by which genes are expressed.

Variations Among Genes

Variations among genes, known as polymorphisms, lead to the production of different gene products (i.e., proteins) and underlie the unique characteristics of each individual. In general, any given gene is quite similar to the same gene in another organism—in other words, the nucleotide sequence is conserved. For example, the genes that code for the alcohol-metabolizing enzyme alcohol dehy-

The conversion of genetic information into protein.



(1) The nucleotide sequence (gene) of one of the two DNA strands is copied (transcribed) into messenger RNA (mRNA).

(2) The mRNA moves from the cell's nucleus into the cytoplasm.

(3) Ribosomes move along the mRNA, recruiting carrier molecules (transfer RNA's, or tRNA's) that each carry a specific amino acid. The amino acids are linked to form the protein.

Figure Steps 1–3a show the way in which the information on a DNA strand is transcribed into mRNA and then translated into proteins.

SOURCE: Hiller-Sturmhöfel, S.; Bowers, B.J.; and Wehner, J.M. Genetic engineering in animal models. *Alcohol Health & Research World* 19(3):206–213, 1995.

drogenase are of the same size and base sequence in most individuals. However, small differences in the base sequence, affecting as little as one nucleotide, can result in a different protein. In some cases, these modifications still allow the gene to produce a functional protein but with slight variations that may affect its function. Other changes in the nucleotide sequence, however, may result in a nonfunctional or incomplete protein. The ability to conserve the sequences coding for important enzymes clearly is important to cells and organisms.

One important aspect of current genetics research is the identification of groups within populations that carry polymorphisms in various genes, resulting in gene variants called alleles. Identifying these polymorphisms can help scientists to better understand how the functions of these genes and their encoded proteins differ and how they relate to certain diseases or other characteristics. For example, do some people produce a form of alcohol dehydrogenase that is more (or less) efficient than other people? And does this influence their risk of developing alcoholism?

To better understand how genes relate to diseases and other characteristics, the National Institutes of Health created the Human Genome Project, which set out to map the human genome by sequencing the entire DNA sequence found in human cells. (Similar mapping efforts also have been conducted for many other organisms.) This project determined not only that the DNA sequence is highly conserved among humans (about 99.5 percent is the same in all humans) but also that numerous combinations of genetic variants exist that are transmitted together and which are known as haplotypes.

To identify such variations in populations with large numbers of samples, researchers are using genetic probes for specific genes with known sequences. These probes typically are short DNA or RNA sequences complementary to a distinctive portion of the gene of interest. This probe then is marked with, for example, a fluorescent tag so that it can be detected if it interacts with the corresponding complementary DNA sequence in a sample.

This idea of using probes to identify differences in DNA sequences can be expanded into broad-scale studies analyzing numerous gene variants across a number of organisms. These genome-wide association studies (GWAS) allow researchers to identify large numbers of variants and to relate them to different outcomes—for example, those associated with different diseases, such as alcoholism.

Identifying candidate genes of particular significance, either through individual probes or large-scale methods such as GWAS, then allows more detailed study of the particular characteristics and expressions of those genes and their role in disease.

Assessing the Genetic Risk for Alcohol Use Disorders

Tatiana Foroud, Ph.D., and Tamara J. Phillips, Ph.D.

Tatiana Foroud, Ph.D., *is a professor in the Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, Indiana.*

Tamara J. Phillips, Ph.D., *is a professor in the Department of Behavioral Neuroscience, Oregon Health & Science University, and a senior research career scientist at the Portland Veterans Affairs Medical Center, Portland, Oregon.*

The past two decades have witnessed a revolution in the field of genetics which has led to a rapid evolution in the tools and techniques available for mapping genes that contribute to genetically complex disorders such as alcohol dependence. Research in humans and in animal models of human disease has provided important new information. Among the most commonly applied approaches used in human studies are family studies, case–control studies, and genome-wide association studies. Animal models have been aimed at identifying genetic regions or individual genes involved in different aspects of alcoholism, using such approaches as quantitative trait locus analysis, genome sequencing, knockout animals, and other sophisticated molecular genetic techniques. All of these approaches have led to the identification of several genes that seem to influence the risk for alcohol dependence, which are being further analyzed. Newer studies, however, also are attempting to look at the genetic basis of alcoholism at the level of the entire genome, moving beyond the study of individual genes toward analyses of gene interactions and gene networks in the development of this devastating disease. **KEY WORDS: Alcohol dependence; alcoholism; genetics; genetic basis of alcoholism; genetic mapping; gene interactions; genetic technology; gene knockout technology; human studies; animal models**

According to the World Health Organization (http://www.who.int/substance_abuse/facts/alcohol/en/index.html), each year alcohol causes 2.5 million (3.8 percent of total) deaths and 69.4 million (4.5 percent of total) disability-adjusted life-years (DALYs) lost to disease worldwide. Alcohol dependence (alcoholism) also is a major health problem in the United States, affecting 4 to 5 percent of the population at any given time (Grant et al. 2004); its lifetime prevalence is 12.5 percent (Hasin et al. 2007). Initially, it was unclear whether environmental factors, genetic factors, or both contributed to the risk for alcohol dependence. Early studies clearly demonstrated that genes have a role in the risk for alcohol dependence; however, it also is clear that a substantial portion of the risk for alcoholism is not genetically determined and may result from other factors, such as the environment in which a person is raised or peer influences. In addition, gene–environment interactions exist that modify alcoholism risk (for more information, see the accompanying article by Dick and Kendler, pp. 318–324).

Ever since it has become clear that genetic factors influence the risk for alcoholism, researchers have sought to identify the genes involved. However, the complex nature of alcohol dependence and related disorders has slowed progress in identifying these genes. Thus, existing data suggest that each individual genetic element has only a small influence and that it will be necessary to identify the relevant gene networks to gain a greater understanding of the contribution of genetics to alcohol abuse and dependence (for more information on genetic and molecular networks of risk, see the article by Wolen and Miles, pp. 306–317).

Historically, two major approaches have been used to determine the magnitude of the overall genetic contribution to alcohol dependence in specific populations. The first approach was to compare the similarity (i.e., concordance) for alcohol dependence among identical (i.e., monozygotic) and fraternal (i.e., dizygotic) twins—that is, these studies assessed whether if one twin had alcohol dependence the other twin did so as well. If the risk for alcoholism, at least in part, results from genetic factors, one would expect monozygotic twins, who have identical genomes, to have a higher concordance rate for alcohol dependence than dizygotic twins, who on average only share one-half of their genomes. Studies indeed have shown higher concordance rates among monozygotic twins, confirming the presence of a genetic component in the risk for alcoholism. The second approach involved family studies to

estimate the overall similarity among family members sharing differing proportions of their genome (e.g., comparing sons with fathers or grandfathers). Together, these family and twin studies provided convergent evidence that genetic factors account for 50 to 60 percent of the total variance in the risk for alcohol dependence (Heath et al. 1997; McGue 1999).

On the basis of these findings, the next step was to identify specific genes that could influence the risk for alcoholism. Over the past three decades, new developments have made it possible to search for specific genes that influence the risk for alcohol dependence, both in human populations and in animal models. This article summarizes some of these approaches used in human populations and in studies of animal models. It also describes newer approaches aimed at analyzing the genetic basis of alcoholism at the level of the entire genome, thus moving beyond analyses of the roles of individual genes in the development of this devastating disease.

Identifying Genes Contributing to the Risk for Alcoholism

Approaches in Human Populations

In human studies, several strategies have been used to search for the genes that influence complex traits such as alcohol dependence, which are influenced by multiple genes with smaller effects rather than by one or more genes with larger effect sizes (Edenberg and Foroud 2006; also see the article by Agrawal and Bierut, pp. 274–282). One approach, often termed linkage analysis, involves studying families with multiple members who have alcohol dependence. This approach is based on the hypothesis that genes might have a greater effect in these families than in families with only a single alcoholic member. To perform this type of study, researchers recruited hundreds of families having two or more alcoholic members (see figure 1A) and analyzed DNA samples from both alcoholic and nonalcoholic family members at approximately 400 different positions within the human genome for sequence differences. The data then were examined to determine whether alcohol-dependent individuals within families shared common gene variants (i.e., alleles). Finally, the investigators reviewed the data across all families in a study to determine whether individuals with alcoholism seemed to have inherited particular parts of the genome. Those portions of the genome that seem to be shared are called quantitative trait loci (QTLs) and are hypothesized to include genes that contribute to the risk for alcoholism. The QTLs can be quite large, often covering 10 or 20 million base pairs that may include hundreds or even thousands of genes, of which the right one or ones (because more than one in the region could contribute) would need to be identified. Although this approach was quite challenging, investigators were able to locate several genome regions that are thought to include genes that contribute to the risk for alcohol dependence. However, conclusively identifying the relevant gene(s) from the many within each large region has proven to be more difficult than anticipated.

The second approach, called a case–control study, often has been used to examine the role of a single gene in complex disorders such as alcoholism. This strategy involves comparing two groups of individuals: people with alcohol dependence and control subjects who are not alcoholic, without regard to the participants' family histories (see figure 1B). In this type of study, investigators analyze the distribution of sequence variants within or near a gene suspected to be involved in alcoholism in the two groups, using statistical methods to compare the frequencies either of different alleles or of the resulting genotypes between the two groups. If a certain allele contributes to the risk for alcohol dependence, one would expect the allele and/or genotype to occur more frequently among the alcohol-dependent case subjects than among the nonalcoholic control subjects. Case–control studies often have been performed on small numbers of alcoholics and control subjects, limiting their statistical power. Moreover, many results from these studies could not be replicated, although this inability may be caused by population differences in genetic risk. The most robust result to emerge from these studies was the demonstration that the genes involved in alcohol metabolism—that is, genes encoding the alcohol dehydrogenase and aldehyde dehydrogenase enzymes—play important roles in the risk for alcoholism (for more information, see the article by Hurley and Edenberg, pp. 339–344). In addition, such analyses have implicated several gene pathways that encode brain-signaling molecules (i.e., neurotransmitters) and the molecules that mediate the actions of opioids (i.e., opioid receptors) (for more information, see the article by Borghese and Harris, pp. 345–354), as well as genes in the neuroendocrine and neuroimmune system (see the article by Crews, pp. 355–361) and genes regulating circadian rhythms (see the article by Sarkar, pp. 362–366).

With the rapid advances in molecular genetic technology, it now is possible to test the entire genome rather than focus on individual genes suspected to play a role (i.e., candidate genes) or use genetic variants spaced at wide intervals throughout the genome. Although these new approaches do not test all 3 billion nucleotides that make up the human DNA sequence, they can test a few thousand (or in some cases a million or more) different positions within the genome (Stranger et al. 2010). This type of study, which is called a genome-wide association study (GWASs) (Manolio 2010), allows a comprehensive test of association across the genome, often while comparing case and control subjects. GWASs have been used for many different diseases, with varying success. Several studies have now applied this approach to begin to tackle the genetics of alcohol dependence (see the article by Edenberg, pp. 336–338). However, the statistical

power of GWASs is a significant hurdle. Thus, very large samples are needed because most genetic variants only have small effects, and many tests need to be performed when analyzing hundreds of thousands or a million of the genetic variants known as single nucleotide polymorphisms (SNPs). In addition, the frequency of the influential alleles in a population has an impact on the sample size that is needed to detect their influence. Furthermore, it is likely that the role that a specific allele plays in the risk for alcoholism may differ among individuals, even if they all seem to have the same disorder. This can be thought of as akin to differences among patients in response to different blood pressure medications: Although the patients all have high blood pressure, the genetic makeup may determine which medication will be most effective for a given patient.

Approaches in Animals

Animal models of traits related to human alcohol use disorders can provide pertinent information about the human condition. The usefulness of this information depends on the validity of the animal model, and there is great interest in the level of consilience between the human and laboratory animal findings (for more information, see the article by Barkley-Levenson and Crabbe, pp. 325–335).

As in human studies, approaches aimed at identifying QTLs have been used in animal studies of alcohol-related traits, such as alcohol consumption and sensitivity to alcohol; however, the nature of the genetic models used varies somewhat from that used in human studies. Most of the data have come from studies performed in mice, although rats have been used as well, and a recent study has compared results for rats and humans (Tabakoff et al. 2009). In one commonly used strategy, two or more strains of mice that are known to differ with regard to the alcohol-related trait under investigation are mated to each other and their offspring (called the F1 generation) then are interbred to create

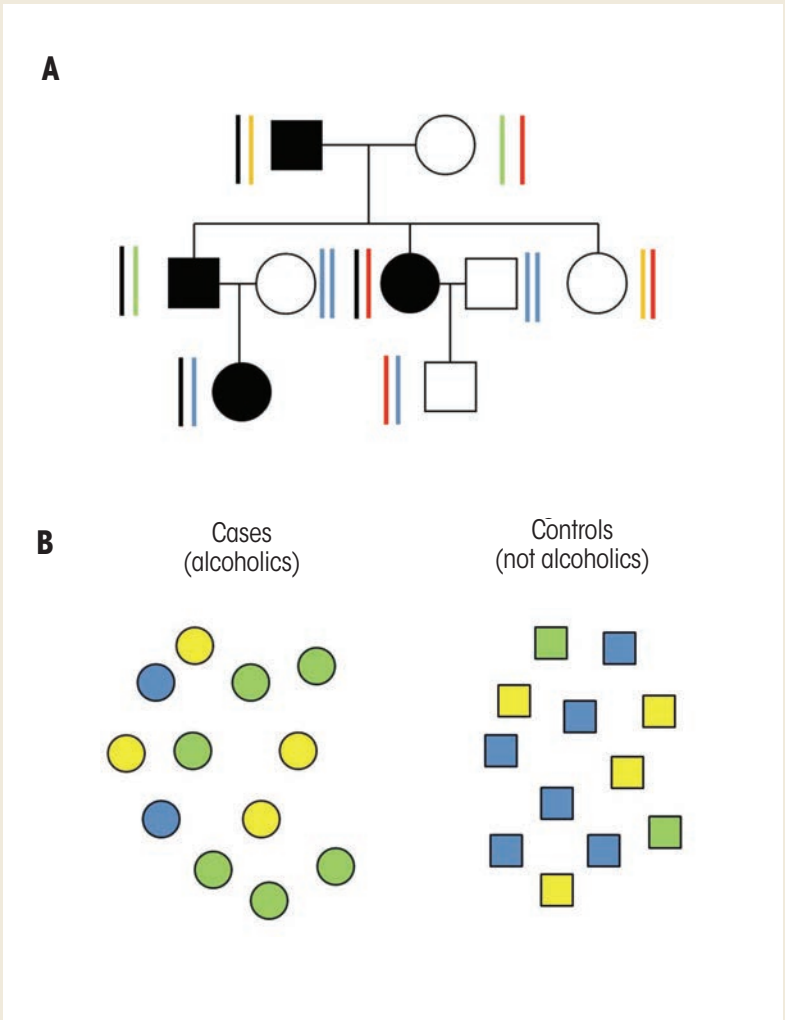


Figure Strategies to identify genes contributing to alcohol dependence. **A)** Family study. In this figure, the squares represent males; the circles, females. The individuals with lines connecting their symbols produced children together and the lines down from that pair of individuals depict their offspring. Fully shaded symbols indicate individuals in the family who are alcohol dependent. The bars beside each symbol represent a region in the genome. Each individual has two copies of this region (one inherited from their mother and one from their father). The black bar carries a version of the gene (i.e., an allele) with a variation in its sequence that increases the risk of alcoholism. Notice that in this family, all four alcoholic individuals carry one copy of the allele that increases the alcoholism risk. The individuals who are not alcohol dependent do not carry this allele. If this pattern is repeated across many families, then there is likely to be a gene that influences the risk for alcoholism in this part of the genome. **B)** Case–control study. The three colors represent the three possible genetic makeups (i.e., genotypes) at the marker. The cases have more individuals with the green genotype and fewer with the blue genotype, whereas the controls have more individuals with the blue genotype and fewer with the green, suggesting that the green genotype is associated with an increased risk for alcohol dependence.

a population (the F2 generation) in which the individual animals possess genes from each of the originating strains in different proportions. The F2 animals then are scored for the trait studied (e.g., amount of alcohol ingested), and samples of their DNA are analyzed (i.e., genotyped) to identify genetic differences that correspond to differences in the alcohol trait. Technological advances have reduced the labor and cost associated with genotyping, making it possible to handle larger numbers of samples and to reduce genotyping intervals. Then, QTL analyses are performed largely as described above for the human studies.

This approach has identified many regions that contain QTLs; however, determining which gene(s) in the region has a significant impact has been difficult and requires the use of additional methods. One such method is finer mapping using animal populations generated by applying other breeding strategies (for reviews of such strategies, see Milner and Buck 2010; Palmer and Phillips 2002). This approach has identified several genes for which strong evidence supports their role in alcohol-related traits (for more information, see the article by Buck et al., pp. 367–374). Currently, second-generation DNA-sequencing technologies, often called next-generation sequencing, are enhancing both sequencing efficiency and the detection of genetic differences (Metzker 2010), and third-generation sequencing is emerging (Schadt et al. 2010).

Perhaps the most popular strategy for examining the influence of single candidate genes has been the use of single-gene mutant, or knockout, mice. For this approach, genetic engineering is used to generate a mutant gene that no longer can express the protein it normally produces, and that nonfunctional (knockout) gene is inserted into the genome of test animals. Mice possessing the mutated gene then are compared with nonmutant mice for differences in alcohol-related traits. Between 1996 and 2006, this approach was used to study approximately 100 genes for their effects on alcohol-related traits (Crabbe et al. 2006), and more genes have been studied since. It is important to realize, however, that although a difference identified between knockout and normal mice provides evidence for a role of the gene studied, this cannot be considered definitive because many interpretational issues are associated with this genetic tool. Other, more refined, gene-manipulation strategies that do not entirely eliminate the activity of a gene can provide additional evidence for the influence of a given gene. These strategies include approaches such as conditional inactivation or rescue, in which gene activity is reduced or eliminated only under certain conditions that can be controlled by the researcher (e.g., Choi et al. 2002) and RNA interference, in which reduced gene expression occurs only in a small region of the brain (e.g., Lesscher et al. 2009; Rewal et al. 2009).

Animal models also can be useful in research directed toward understanding the health consequences of alcohol consumption, including consequences in different organs. Much of this research is aimed at exploring how alcohol affects the brain and how the brain influences different individual responses to alcohol that may contribute to the development of alcohol dependence. One strategy that can be used in studies of both the brain and other tissues is called whole genome expression profiling, which examines the activity (i.e., expression) of thousands of genes located throughout the genome (Rockman and Kruglyak 2006). Particularly in studies of the brain, however, this approach can be applied more readily to animals than to humans, because a sample of the tissue to be studied is needed. This tissue sample is used to extract a type of genetic material called messenger RNA (mRNA) that is generated during the process of gene expression. The mRNA then is added to a microarray—that is, a membrane or slide on which known gene sequences have been placed that can interact with complementary RNA sequences from the brain sample. The amount of mRNA interacting with the gene sequences on the microarray then can be measured, giving an indication of the level of gene expression for each gene studied. A more recent modification of this method called RNA-seq (because it involves determination of the mRNA building blocks, or RNA sequence) has allowed researchers to obtain even more detailed information on gene expression (Ozsolak and Milos 2011).

Whole-genome expression profiling can be used in different ways. For example, studying gene-expression differences between animals that are sensitive or resistant to a given alcohol effect can provide evidence for genes that influence sensitivity to that alcohol effect. Comparisons between alcohol-exposed and non–alcohol-exposed animals provide information about alcohol's interactions with genes and gene expression.

Gene-expression studies also have been performed with human samples, using postmortem brain tissue from alcohol-dependent individuals and nondependent control subjects (e.g., Kryger and Wilce 2010). However, factors such as quality of the sample and incomplete history of the subject from which the tissue was obtained complicate interpretation. In animals, these factors can be better controlled. Finally, in addition to whole genomes being examined for expression differences using RNA, DNA microarrays have been developed for global examination of genomic variation (Gresham et al. 2008).

Moving Beyond Studying One Gene at a Time

Using the approaches described here, researchers now have identified some genes that are thought to influence the risk for alcoholism in humans or to contribute to alcohol-related traits in animal models. However, it is clear that the genetics of alcoholism and alcohol-related traits are complex and will include not only the effect of individual

continued on p. 272

continued from p. 269

genes but also the interaction of genes with each other, a phenomenon termed epistasis. Identification of these interactions is only just beginning and is complicated by the fact that such analyses often require large numbers of subjects or animals. However, this area likely will garner much research interest in the future.

For now, the more immediate goal is to use the information currently available about the genes important in alcohol dependence in an integrated way to identify how these genes might work together and to help researchers identify additional genes that may be involved (for more information, see the article by Buck et al., pp. 367–374). One approach in this direction has been to study the genes already identified and determine whether they might work together in known biological pathways or networks. Such methods initially were used with gene-expression data, but newer statistical methods now permit pathway analysis also to be applied to GWASs data (Holmans 2010). When evidence for a coordinated network of interactions is identified, other genes known from previous research to be members of that network immediately can be considered as candidate genes to be tested for their potential role in alcohol-related traits.

Researchers also are developing and testing more complex models. For example, an approach called genetical genomics simultaneously takes into consideration both the gene-expression and gene-sequence (QTL) information to narrow the set of possibly involved genes down to a smaller set based on convergent results (Farris et al. 2010). More recently, it also has become clear that some inherited characteristics cannot be attributed to specific gene sequences but must be related to epigenetic effects—heritable modifications that are not based on differences in DNA sequence. The mechanisms underlying these epigenetic effects are being carefully scrutinized (for more information, see the article by Starkman et al., pp. 293–305).

It is worth noting that all these network studies have become possible because of a growing understanding of the mouse and human genomes. In the coming years, genetic discoveries likely will grow along with further technological advances. And we can hope that all of these developments will someday allow researchers and clinicians to identify those individuals who are at greatest risk for developing alcoholism. A related significant goal of current alcohol-related genetic research is to identify interventions that might decrease the risk for this devastating disease in those most likely to be affected. Finally, researchers are striving to better understand the genetic basis of differences in the effects of alcohol between people who are susceptible to alcoholism and those who are resistant to it, so that better treatment medications can be developed. ■

Financial Disclosure

The authors declare that they have no competing financial interests.

References

Choi, D.S.; Wang, D.; Dadgar, J.; et al. Conditional rescue of protein kinase C ε regulates ethanol preference and hypnotic sensitivity in adult mice. *Journal of Neuroscience* 22(22):9905–9911, 2002. PMID: 12427847

Crabbe, J.C.; Phillips, T.J.; Harris, R.A.; et al. Alcohol-related genes: Contributions from studies with genetically engineered mice. *Addiction Biology* 11(3–4):195–269, 2006. PMID: 16961758

Edenberg, H.J., and Foroud, T. The genetics of alcoholism: Identifying specific genes through family studies. *Addiction Biology* 11(3–4):386–396, 2006. PMID: 16961766

Farris, S.P.; Wolen, A.R.; and Miles, M.F. Using expression genetics to study the neurobiology of ethanol and alcoholism. *International Review of Neurobiology* 91:95–128, 2010. PMID: 20813241

Foroud, T.; Edenberg, H.J.; Goate, A.; et al. Alcoholism susceptibility loci: Confirmation studies in a replicate sample and further mapping. *Alcoholism: Clinical and Experimental Research* 24(7):933–945, 2000. PMID: 10923994

Grant, B.F.; Dawson, D.A.; Stinson, F.S.; et al. The 12-month prevalence and trends in DSM-IV alcohol abuse and dependence: United States, 1991–1992 and 2001–2002. *Drug and Alcohol Dependence* 74(3):223–234, 2004. PMID: 15194200

Gresham, D.; Dunham, M.J.; and Botstein, D. Comparing whole genomes using DNA microarrays. *Nature Reviews. Genetics* 9(4):291–302, 2008. PMID: 18347592

Hasin, D.S.; Stinson, F.S.; Ogburn, E.; and Grant, B.F. Prevalence, correlates, disability, and comorbidity of DSM-IV alcohol abuse and dependence in the United States. *Archives of Genetic Psychiatry* 64(7):830–842, 2007. PMID: 17606817

Heath, A.C.; Bucholz, K.K.; Madden, P.A.; et al. Genetic and environmental contributions to alcohol dependence risk in a national twin sample: Consistency of findings in women and men. *Psychological Medicine* 27(6):1381–1396, 1997. PMID: 9403910

Holmans, P. Statistical methods for pathway analysis of genome-wide data for association with complex genetic traits. *Advances in Genetics* 72:141–179, 2010. PMID: 21029852

Kryger, R., and Wilce, P.A. The effects of alcoholism on the human basolateral amygdala. *Neuroscience* 167(2): 361–371, 2010. PMID: 20153402

Lesscher, H.M.; Wallace, M.J.; Zheng, L.; et al. Amygdala protein kinase C epsilon controls alcohol consumption. *Genes, Brain, and Behavior* 8(5):493–499, 2009. PMID: 19243450

Manolio, T.A. Genomewide association studies and assessment of the risk of disease. *New England Journal of Medicine* 363(2):166–176, 2010. PMID: 20647212

McGue, M. The behavioral genetics of alcoholism. *Current Directions in Psychological Science* 8:109–115, 1999.

Metzker, M.L. Sequencing technologies—the next generation. *Nature Reviews. Genetics* 11(1):31–46, 2010. PMID: 19997069

Milner, L.C., and Buck, K.J. Identifying quantitative trait loci (QTLs) and genes (QTGs) for alcohol-related phenotypes in mice. *International Review of Neurobiology* 91:173–204, 2010. PMID: 20813243

Ozsolak, F., and Milos, P.M. RNA sequencing: Advances, challenges and opportunities. *Nature Reviews. Genetics* 12(2):87–98, 2011. PMID: 21191423

Palmer, A.A., and Phillips, T.J. Quantitative trait locus (QTL) mapping in mice. In: Liu, Y., and Lovinger, D.M., Eds. *Methods in Alcohol-Related Neuroscience Research.* Boca Raton, FL: CRC Press LLC, 2002, pp. 1–30.

Rewal, M.; Jurd, R.; Gill, T.M.; et al. $\alpha 4$-containing $GABA_A$ receptors in the nucleus accumbens mediate moderate intake of alcohol. *Journal of Neuroscience* 29(2):543–549, 2009. PMID: 19144854

Rockman, M.V., and Kruglyak, L. Genetics of global gene expression. *Nature Reviews. Genetics* 7(11):862–872, 2006. PMID: 17047685

Schadt, E.E.; Turner, S.; and Kasarskis, A. A window into third-generation sequencing. *Human Molecular Genetics* 19(R2):R227–R240, 2010. PMID: 20858600

Stranger, B.E.; Stahl, E.A.; and Raj, T. Progress and promise of genome-wide association studies for human complex trait genetics. *Genetics* 187(2):367–383, 2011. PMID: 21115973

Tabakoff, B.; Saba, L.; Printz, M.; et al. Genetical genomic determinants of alcohol consumption in rats and humans. *BMC Biology* 7:70, 2009. PMID: 19874574

A Watershed Year for an Update on the Genetics of Alcoholism

Robert W. Williams, Ph.D., and Antonio Noronha, Ph.D.

Robert W. Williams, Ph.D.,
Center for Integrative and Translational Genomics, The University of Tennessee Health Science Center, Memphis, Tennessee.

Antonio Noronha, Ph.D.,
Division of Neuroscience and Behavior, National Institute on Alcohol Abuse and Alcoholism, Bethesda, Maryland.

It is easy to think of genetics as the study of genes, but given our current knowledge of genetics, this definition is now considered inadequate. Genetics is the study of differences among individuals—even between identical twins. We know that some differences between individuals are linked to variations in DNA sequence (i.e., the genome), but most differences actually are caused by complex interactions between our genetic endowment and the many environments to which we are exposed, by choice and fate.

The genetics underlying the body's responses to alcohol are a superb example of the complexity of the issues related to the study of genetics. The role of the environment—including familial and social setting, age, and exposure—is obvious. Yet a complementary role of genes was doubted for many decades, and it was not put on a firm scientific foundation until the 1970s and 1980s. This coincided roughly with the creation of the National Institute on Alcohol Abuse and Alcoholism (NIAAA) by Congressional act on New Year's Eve, 1970.

From its inception, NIAAA has been at the vanguard of research in genetics using an array of powerful methods and resources. The 13 insightful reviews in this issue highlight the cutting edge of molecular and statistical genetic analysis.

Now, moving forward, we can consider 2012 a watershed year in genetics and genomics. Two short years ago, fewer than 10 humans had had their genomes fully sequenced. Today more than 10,000 individuals have been sequenced—a rate of increase that puts Moore's Law[1] to shame. Recent advances in sequencing technology have broken through the cost barrier. Ten years from now, many of us will have been sequenced and even resequenced multiple times as part of our routine medical record.

It is easy to overpromise solutions, but we can be optimistic that finding effective cures to diseases such as alcoholism will not be limited by technology. The next challenge will be to determine how best to use this technology to devise rational and individualized approaches to prevent, intervene in, and cure complex disorders such as alcohol abuse and alcoholism. Read the great reviews here to see how far we have come in the last decade, and definitely stay tuned for the next decade!

[1] Moore's Law predicts the doubling of the number of transistors on a computer chip every 18 months to 2 years. It is based on an observation made in 1965 by Gordon Moore, Intel co-founder, about how the number of transistors in integrated circuits had increased since the invention of that device and his suggestion that the increase would continue into the future.