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Rodent Models of Genetic Contributions to Motivation to Abuse Alcohol

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Abstract

The distinction between alcohol use (normative) and abuse (unfortunately common) implies dysregulation of motivation directed toward the drug. Genetic contributions to abuse risk are mediated through personality differences, other predispositions to drink excessively, and differences in sensitivity to the acute and chronic consequences of the drug. How to assess motivation in laboratory animals is not straightforward but risk factors for and consequences of alcohol abuse can be modeled with reasonable fidelity in laboratory rodents. Remarkably few rodent studies focus on the genetic contributions to alcohol's reinforcing value: almost all examine preferential drinking of unflavored alcohol over water. Such studies will likely never avoid the confounding role of taste preferences and most often yield intake levels insufficient to yield a pharmacologically significant blood alcohol level. Genotypes that avoid alcohol probably do so based on pre-ingestive sensory cues; however, post-ingestive consequences are also important. Thus, the quest for improved measures of reinforcing value continues. We have genetic differences aplenty, but still lack evidence that any genotype will readily self-administer alcohol to the devastating extent that many alcoholics will. Encouraging results that are emerging include improved behavioral methods for elevating alcohol intake and inferring alcohol reinforcement, as well as new genetic animal models. Several ingenious assays to index alcohol's motivational effects have been used extensively. Alcoholic drinking that attempts to prevent or to alleviate withdrawal symptoms has been modeled. Another characteristic of alcoholic drinking is its persistence despite abundant evidence to the drinker of the damaging effects of the excessive drinking on work, relationships, and/or health. Modeling such persistence in rodents has been uncommon to date. New genetic animal models include lines of mice selectively bred for chronic high drinking, and other bred for high binge-like drinking. We have a much more clear idea now about some important experiments remaining to be performed.

Introduction

The distinction between **alcohol** use, which is normative, and abuse, which is unfortunately common, implies dysregulation of motivation directed toward the drug. Genetic contributions to **individual differences** in the patterns and degree of human alcohol use and abuse account for about half of population variability (Goldman & Ducci, 2007). Genetic pathways to abuse risk are mediated through personality differences and other predispositions to drink excessively as well as through differences in sensitivity to the acute

and chronic consequences of the drug. Disentangling risk factors from consequences is difficult in human studies, which are usually not prospective: subjects are most often ascertained based upon a diagnosis of **alcohol dependence** or a **family history** thereof. Both risk factors for and consequences of **alcohol abuse** can be modeled with reasonable fidelity in laboratory rodents. **Rat** and **mouse** studies offer immense power to characterize genetic contributions to individual differences, as well as to manipulate the genome of the animals. Specific genes may be targeted for over- or under expression of their effector proteins. This review will discuss efforts to address the motivational aspects of individual differences surrounding alcohol use and abuse in rodent genetic **animal models**.

Not surprisingly, the biggest challenge remains relating human **motivation** (which can be inferred but also self-reported) in a convincing way to the underlying motivation for rodent behavior directed toward and resulting from alcohol. Despite the intrinsic difficulty, substantial progress has been made (Crabbe, 2012), and new approaches are appearing.

A little history

The willingness of laboratory rats to drink alcohol solutions was reported as early as 1926 by Curt Richter (Richter, 1926) who went on to develop what we now term the “two bottle preference test” to determine **taste** thresholds for various substances (e.g., salt, sugar, etc). He reported that when ethanol was offered by choice vs tap water, his rats apparently could not detect the taste of alcohol at concentrations below 1.8%. This was inferred from preference ratios (amount of alcohol ingested/total fluid ingested) that were approximately 50%. They preferred concentrations up to and including 4.8% over water, but rejected concentrations greater than 6% (Richter & Campbell, 1940). Data that there were genetic differences as well as individual differences in “alcohol appetite” were reported for both rat and mouse strains where the animals were also offered various diets. Interestingly, this study reported a **gene X environment interaction**, as “strain O” rats drank more alcohol than “strain H” rats when on diet “A”, but less than “strain H” rats when both were consuming diet “B” (Williams et al., 1949). Exactly what these genotypes actually were is anyone’s guess, as the now-standard genotypes were in considerable flux (although Williams may have used some early version of “dba” and “C3H” mice).

In 1948, Jorge Mardones began to create a rat line that preferred to drink 10% ethanol vs water and named it the University of Chile B (**UChB**) line; he also bred a **UChA** line for low ethanol preference (Mardones, 1951; Mardones, 1960). In **selective breeding**, mating animals with extreme values on the selected trait has the effect of capturing the allelic forms of the genes that influence that trait in the line. Each generation, their offspring show further enhancement of their **genetic predispositions** until all genetic variation affecting the trait (e.g., high preference drinking) is exhausted. Comparisons of high vs low selected lines then can reveal other differences in neurobiology and behavior between the lines, which are logically attributed to the influences of the same genes affecting the selected response.

Thus, before 1950, two of the principal approaches to the study of the genetics of preference drinking were already established – studies of natural strain variation and creation of a genetically modified rodent. I’ll return to the studies of selectively bred lines in a later

section. **Gene targeting (knockouts, transgenics)** was not developed until 1988. The ability to turn on or off the function of individual genes was first used to study alcohol responses in 1996. Many genes have since been targeted and studied for alcohol-related responses: about 80% of those mutants have been assessed for **preference** drinking. A systematic review of such studies appeared in 2003 (Cunningham & Phillips, 2003). Animals targeted for about 25 genes had been tested, and for 1/3, preference drinking significantly increased. Another 1/3 showed significantly *reduced* drinking, and the remainder had no significant effect on preference. A subsequent survey of 75 genes showed the same 1/3-1/3-1/3 pattern of outcomes (Crabbe et al., 2006). I stopped tracking new knockout/transgenic studies in 2008, by which time 86 genes still showed the same division of outcomes. Subsequent gene targeting studies have been compiled, and many more effects on preference drinking are documented in a more recent review (Bilbao, 2013). These organisms are extremely powerful tools, but a review of all the individual gene effects in the alcohol research literature is beyond the scope of this paper, as is consideration of the methods (e.g., tissue-specific, conditional knockouts).

The two-bottle preference test has proven to be extremely useful for genetic studies to assess motivation for alcohol (Crabbe et al., 2010). It is very easy to implement and can be conducted with high experimental throughput. We have an historical base of 60 years of rat data, and 50 of mouse. Consistent with the fidelity of genetic differences (see below), the heritability of alcohol preference is fairly substantial. Nonetheless, for assessing motivation for ethanol, this behavioral assay also has some limitations. It may be that an animal with a high alcohol preference score is experiencing more **reward** from ingesting alcohol than an animal with a low preference score. Alternatively, it may be experiencing *less* and is therefore increasing its intake in an effort to achieve greater reward. Low-preferring genotypes may be sensitive to alcohol's aversive effects. Another limitation is that although using this test as a screen for potential drug therapies is easily implemented, such studies have not been successful in discriminating therapeutically effective compounds from those that do not perform in human studies. This is largely because there are a great number of false-positive finding in the animal studies (Egli, 2005). And, as will be discussed below, few animals, even from highly preferring genotypes, are willing to ingest sufficient ethanol to become intoxicated (Crabbe, 2012). Nonetheless, the test remains by far the most common assay used to assess motivation for alcohol in rodents.

Recognizing the advantages of the mouse over other mammals for genetic studies (e.g., relatively small size and rapid generation time, the availability of many well-defined and replicable genotypes), Gerald McClearn was one of the pioneers of systematic studies of mouse **inbred strains**. Inbred strains are created by systematic brother-sister matings. Because each offspring inherits half its genetic composition (i.e., one of the two alleles it possesses for each gene) from each parent, half of each individual's genetic variability is shared by siblings. Thus, genetic variability is halved with each successive generation of sibling matings. After 20 generations, all same-sex mice of a given inbred strain are essentially genetic clones (Falconer & Mackay, 1996). Therefore, if one examines a trait in a number of mice from a number of strains and takes care to provide the same environmental conditions to all subjects, the individual differences in the trait can be partitioned into

among-strain-means (i.e., genetic) and within-strain (non-genetic, or environmental) sources. Strain mean differences that significantly exceed pooled within-strain variability in a simple analysis of variance are evidence of genetic influences on the trait.

At the 1968 Nebraska Symposium on Motivation (the year I entered graduate school), Professor McClearn presented a paper entitled “Genetics and Motivation of the Mouse” (McClearn, 1968). I consider this a *tour de force*: it is a paper I have returned to many times, usually to discover that my next, most interesting planned experiment had already been done! After explaining the process of inbreeding, he reviewed the state of the art with inbred strains regarding nearly the entire breadth of mouse behavior. He showed evidence of strain differences in aggression and dominance; sexual behavior; learning; hoarding and other responses to food; and activity. It is remarkable that he could cover this range in the first 10 pages of his manuscript—behavior genetics was a very new field. The bulk of his paper, however, dealt with the topic of this year’s Nebraska Symposium - alcohol studies, specifically the motivation to ingest alcohol solutions. McClearn in 1959 had shown that 5 inbred mouse strains differed significantly in their preference for 10% ethanol vs water (McClearn & Rodgers, 1959). Figure 2.1 from that paper is reproduced in adapted form here because it illustrates several findings that have been replicated many times, but are often overlooked. The figure showed data for individual mice from multiple strains, across days of two bottle preference testing for 10% alcohol vs water.

First, the strain differences are striking. C57BL mice showed nearly total alcohol preference, while DBA mice virtually completely avoided alcohol. Of the other 3 strains tested, A and BALB/c also avoided 10% alcohol, but C3H/2 showed, on average, intermediate preference scores. Second, for both strains that preferred alcohol, their preference grew over time. Third, there were fairly substantial individual differences *within* the preferring strains. One of the 4 C57 mice had only moderate preference, while the other 3 had nearly total preference. These differences between animals of exactly the same genotype must derive from non-genetic sources. A corollary of this point is that average scores for a genotype may be misleading. Two of the C3H mice had preference ratios of about 30–40% while for the other two the preference ratios were 10% or less. No animals of this strain actually scored very near the mean for the strain. Finally, among the other factors that influence tube choice, the roles of **learning** and perseverative behavior must be considered. Learning is inferred from the gradual development of preference across days of testing. The hatched vertical lines on Days 6–7 and 10–11 in the figure indicate when the position of the tube containing alcohol was reversed with that of the water tube. For all preferring animals, this resulted in a drop in preference scores for at least the following day, followed by recovery to a higher preference. Any investigator who has conducted preference studies knows that there are some animals that exhibit apparent, strong preference for drinking out of the tube on one side or the other of its cage, seemingly oblivious to its content. Fortunately, these are usually relatively rare, and with repeated position switches, the animals tend to find a characteristic preference ratio.

This experiment has been extended to survey multiple inbred mouse strains and has been performed in many laboratories since then: the results of these replications are quite striking. A comparison of multiple-strain data from four laboratories (15–28 strains per study) is

shown in Figure 2.2. Pairwise comparisons of data from surveys conducted between 1966 and 2006 yielded strain mean correlations ranging from 0.74 to 0.98, indicating the remarkable stability of strain differences in alcohol preference. These **correlations** reflected stability similar to that from 5 surveys of strain brain weight conducted between 1967 and 2000 (Wahlsten et al., 2006). While female mice are well-known to drink more alcohol than males, Rodgers' study (included in the historical comparison in Figure 2.2) tested both sexes from 19 strains and found that 97% of the variance in preference was accounted for by strain, with only 3% attributable to combined effects of sex, litter, and measurement error (Rodgers, 1966). In a later preference study of 22 inbred strains of both sexes offered 3 different alcohol concentrations serially, we found somewhat different results. There were overall strong main effects of strain and sex (favoring females), but there were also significant interactions of all types. The magnitude of sex differences ranged from marked to none across different strain-concentration combinations (Yoneyama et al., 2008) (n.b., it is these data, for 10% ethanol, pooled across males and females for each strain that are included in the Wahlsten comparison and identified as "Finn Lab" in Figure 2.2).

McClearn then discussed several other experiments (McClearn, 1968). Many of those experiments were designed to assess possible motivations for alcohol ingestion. He established taste thresholds for alcohol by offering multiple solutions of differing concentrations. He found that avoiding strains largely avoided all detectable concentrations, even when as low as 0.05% in the case of the DBA strain. Because this is far lower than could produce any discernible pharmacological effects, this suggests that avoidance in most such strains is strongly influenced by taste or odor, not necessarily by response to alcohol as a drug. He reported that adding **sucrose** to the ethanol solutions increased consumption, but did not appreciably affect the pattern of strain differences, which is consistent with taste avoidance. When we later conducted studies comparing the ethanol intake of 15 inbred strains for 3 concentrations of alcohol with or without the addition of saccharin, we found a somewhat different result. We found that some strains avoided higher concentrations of alcohol regardless of saccharin's presence. Other strains showed appreciably higher ethanol intake in saccharin, which we attributed to saccharin's ability to mask the odor or taste of alcohol. Finally, the remaining strains tended to avoid only the higher concentration of alcohol in saccharin but not water. Since they were ingesting much greater alcohol doses in saccharin, we concluded that they were avoiding the perceived pharmacological effects of alcohol (Belknap et al., 1993). Our subsequent study extended Belknap's findings to 22 strains and generally supported the earlier conclusions (Yoneyama et al., 2008).

Animals could be consuming alcohol for the calories it provides (Mardones, 1951; Richter, 1953). McClearn reported that establishing a state of food deprivation did not increase preference in avoiding strains. Forcing food-deprived mice to ingest alcohol solutions allowed mice to maintain body weight more effectively, indicating that alcohol could be used as a food. However, the pattern of most and least responsive strains was unrelated to their free-choice alcohol preference (McClearn, 1968). In a series of complex experiments serially offering C57BL mice choice between water and different concentrations of alcohol, McClearn tested several hypotheses about how preference might be related to concentration offered. All concentrations exceeded the taste threshold for this strain. A simplified

summary of the outcomes of these experiments is that amount (i.e. dose) of alcohol ingested increases monotonically and approximately logarithmically with the concentration offered. Thus, across 3, 6 and 12 % concentrations of ethanol, C57BL/6 mice showed twice-doubling intakes (McClearn, 1968). However, preference ratios show an inverted U function across increasing concentrations, with the peak preference ratio occurring at strain-specific concentrations. This is one reason we advocate reporting **consumption** (g EtOH/kg body weight) rather than preference ratios in preference experiments. Examination of our two later studies show increases in dose ingested by C57BL/6 in both studies roughly consistent with McClearn's data, but for many other strains, increases were less or even nonexistent for the highest concentration we studied, 10% (Belknap et al., 1993; Yoneyama et al., 2008). Thus, it cannot be concluded that inbred strains titrate a preferred dose of self-administered alcohol.

McClearn concluded with reports on several early studies attempting to relate rate of alcohol metabolism by **alcohol dehydrogenase (ADH)** and then **aldehyde dehydrogenase (ALDH)** to preference differences, mostly between the C57BL/6 and DBA/2 inbred strains. Because these and subsequent studies have failed to yield a consistent relationship between alcohol elimination and preference, they are not discussed here. It is certainly true, however, that alcohol metabolism is an important determinant of risk for alcohol abuse and dependence in humans. A high proportion of humans from East Asian gene pools (e.g., Han Chinese) possess a variant form of the ALDH gene that leads to slow elimination of acetaldehyde after drinking alcohol. Acetaldehyde produces several unpleasant effects including facial flushing, nausea, dizziness, and headaches. Individuals with the slow ALDH variant are at greatly reduced risk of developing alcohol dependence; polymorphisms in ADH also have protective effects, but they are much less marked (Goldman et al., 2005). These allelic variants have much more modest effects in Caucasian/European populations, mostly because they are much more rare in these populations (Liu et al., 2011). This strong genetic effect is a poster child for pharmacogenetics studies hoping to discover novel medications. One of the three therapeutic drugs for alcoholism approved for use in the USA is Antabuse® (disulfiram). Its mechanism of action is to inhibit ALDH activity, and individuals taking Antabuse experience the effects of acetaldehyde if they drink. Unfortunately, its use is limited by the fact that many alcoholics in **treatment** never are prescribed any of the three effective medications (Saxon & McCarty, 2005), and obtaining and monitoring compliance with addiction therapies remains a challenge (Barth & Malcolm, 2010). Nonetheless, **brain** acetaldehyde may play a role in individual differences in alcohol preference in rodents (Socaransky et al., 1984). Recent studies have infused **lentiviral vectors** into the **ventral tegmental area** to manipulate brain acetaldehyde levels bidirectionally in high-preferring UChB rats and report modulation of voluntary consumption of ethanol solutions (Karahanian et al., 2011). **Adenoviral vectors** administered intravenously to alcohol dependent UChB rats and designed to enhance ADH activity and inhibit ALDH activity led to 60% reductions in drinking (Rivera-Meza et al., 2012). Thus, the animal models may prove useful in discovering adaptations to the rather blunt tool offered by Antabuse for future treatments to alleviate alcoholic drinking.

Indices of motivation for alcohol

Assessing motivation in humans is possible by self report, though not without challenges (e.g., lying or self-delusion). How to assess motivation in laboratory animals is not straightforward, either theoretically or practically (Brown, 1961). Because rodents cannot speak to us about their motivational states, we must operationalize their motivation. I have discussed thus far only one such surrogate measure, their tendency to ingest alcohol when water is freely available, and the earliest studies nearly all employed this or some closely related assay. An excellent review in the 1968 Nebraska Symposium volume reviewed still-new data surrounding the physiological correlates of both specific (e.g. certain hypothalamic nuclei) and non-specific (e.g. reticular activation) drive states thought to accompany motivated behavior (Grossman, 1968). It is beyond the scope of this review to consider the theoretical aspects of motivation in depth. With respect to the dysregulated states associated with addictions including alcoholism, these include both postulated homeostatic disturbances and revaluations of alcohol's rewarding value, and thus invoke both **negative and positive reinforcement** to explain behavioral changes. A good example is Koob's "**allostatic dysregulation**" conceptualization, which postulates first an over-valuation of positively reinforcing effects of alcohol leading to regular over-indulgence. That increase in chronic excess drinking produces consequences that result in a lowering of a homeostatic set point, particularly with regard to mood and anxiety. Attempts to reestablish the original set point represent a negative reinforcement process termed "allostatic load" that sustains the deviant behavior (Koob, 2013). Koob's work (along with that of many others) strongly implicates neural **stress** pathways in drug dependence (George et al., 2012). [Interestingly, the same Nebraska Symposium volume also contained a review of then very new data linking stress hormones and behavioral conditioning and extinction (Levine, 1968)]. While there are numerous other theoretical frameworks for alcoholism from which to choose, all incorporate the notion of dysregulated motivation to consume alcohol [e.g. (Sommer & Spanagel, 2013; Stephens et al., 2010; West, 2006; Blane & Leonard, 1999)].

In addition to drinking, animals can manifest alcohol-directed behavior by performing work on operant schedules to gain access (Samson & Czachowski, 2002). One difficulty with standard **operant schedules** is that consumption of alcohol is allowed while, for example, animals are working on a **progressive ratio** schedule to gain additional access. This makes it difficult to distinguish appetitive (drug seeking) behavior from **consummatory behavior**, as well as having the problem of allowing potential pharmacological effects of the drug to affect responding later in the session. This problem has been circumvented by the use of a progressive ratio schedule which allows only a single reinforcing access to alcohol for each ratio (Samson et al., 2004). **Pavlovian conditioning** principles have also been used to assess the reinforcing value of an alcohol-induced **interoceptive state**. Mildly water-deprived rodents will generally learn to avoid a novel taste when administered intraperitoneal doses of psychoactive drugs including alcohol following each access period. Alcohol **conditioned taste aversion** studies have shown reliable strain differences in both inbred mouse and rat strains, as well as differential sensitivity in comparisons of some selectively bred lines. Nearly all of these studies have been reviewed elsewhere, along with taste conditioning studies using other drugs. This review also describes studies comparing targeted mutants

with controls for sensitivity to an alcohol conditioned taste aversion; it also lucidly delineates methodological considerations that can influence interpretation of genetic differences in taste conditioning studies (Cunningham et al., 2009). When alcohol injections are paired with exposure to one set of distinctive environmental cues and saline injections are paired with another set of cues, subsequent drug-free tests where both sets of cues are available often reveal a **conditioned place preference** for the alcohol-paired side. As with taste conditioning, genetic contributions to individual differences are pronounced and nearly all such studies (including those with mutants) have been reviewed (Cunningham & Phillips, 2003).

Methods other than preference drinking can also be used to assess **self-administration** of alcohol. For most drugs of abuse, the intravenous (iv) route of administration is feasible for rats, and is also used with some success in mice even though technically much more challenging. Alcohol is peculiar in that effective doses are in the g/kg range rather than mg/kg or ug/kg, so large volumes of dilute alcohol solutions would need to be administered iv, and high concentrations are apparently irritating. Nonetheless, alcohol is occasionally studied by iv self administration. Interestingly, both **C57BL/6J** and **DBA/2J** mice will readily self-administer alcohol iv (Grahame & Cunningham, 1997). Genetic studies with the iv route have been reviewed (Green & Grahame, 2008). After a state of **physical dependence** on alcohol is established, rats and mice will also administer alcohol by intragastric infusion (Fidler et al., 2006; Fidler et al., 2012) and mouse strains differ in their patterns of self-administration (Fidler et al., 2011). Finally, mice show a locomotor stimulant response to low to moderate doses of alcohol similar to that elicited by many other abused drugs. Because the **locomotor response**, and its tendency to grow with repeated drug administrations (called “**sensitization**”), are thought to model the **euphoria** experienced by drug users (Wise & Bozarth, 1987), sensitization to psychomotor stimulant drugs such as amphetamine and cocaine has been widely studied. Alcohol stimulation and sensitization have also shown marked influences of genetics on their magnitude [for review, see (Phillips, 1997)].

Thus, there are genetic differences apparent using any of several different methods to assess motivation for alcohol. There are abundant data for two-bottle preference, but generally sparse genetic data for the other available methods. Are these different rodent behavioral assays assessing the same trait? If so, genotypes showing enhanced motivation for alcohol with one assay should also do so in another. A thorough and thoughtful review of the genetic data concluded that two-bottle preference and operant oral self-administration studies yielded congruent findings (Green & Grahame, 2008). This conclusion was largely based on comparisons of the rat lines selected for high vs low preference; high preferers also tended to show greater operant self-administration. Detailed comparisons of several pairs of these rat lines under different operant conditions agreed that there appeared to be some genetic overlap between home-cage drinking and operant oral self-administration, but also pointed out that substantial differences in the patterns of self-administration suggested genetic divergence as well (Files et al., 1998; Samson et al., 1998). Genotypes sensitive to an alcohol conditioned taste aversion were less likely to self-administer alcohol than genotypes with weak conditioned taste aversion. This is a robust finding, as it was seen to distinguish

the **HAP** and **LAP** selected mouse lines (Chester et al., 2003; Grahame et al., 2001) as well as in some rat selected lines and, importantly, across multiple inbred mouse strains. These latter genotypes were not selected for either trait, but high preferers (Belknap et al., 1993) showed low sensitivity to an ethanol conditioned taste aversion (Broadbent et al., 2002) and vice versa. A positive, albeit weaker, positive relationship was seen between ethanol drinking and an ethanol conditioned place preference (Green & Grahame, 2008). The genetic data for other measures of motivation for ethanol are currently too sparse to support strong conclusions.

Alcohol use versus alcohol abuse

Given the array of behavioral and genetic assessment tools described above, which methods offer evidence for abusive self-administration of alcohol, or of pathological dysregulations of alcohol's reinforcing value? A pessimistic answer is, none of them (Crabbe, 2012). There are genetic differences aplenty, but we are still lacking evidence that any genotype will readily self-administer alcohol to the devastating extent that many alcoholics will. I have argued that part of this is due to the fixation of the field on the two bottle preference test. There are now six pairs of rat lines selectively bred for high vs low two-bottle preference for 10% ethanol offered continuously. A seventh line has also been selected for high preference. Three pairs of mouse lines have been bred for high vs low preference. With very minor variation, these have all been bred for the same trait. This offers the field of alcohol research a very powerful source for assessing convergent validity of the findings from the animal models employed to assess genetic differences. I recently surveyed reviews of the data from these lines in search of neurobiological and behavioral differences that differentiate high from low drinkers consistently across experiments (Crabbe, 2013); the results were sobering. All the high drinking lines typically drink modest amounts each day and achieve **blood alcohol levels (BALs)** that are most likely not intoxicating. While some animals will show escalations in drinking with very prolonged access, most have not been tested under these conditions. High drinking rat lines will work for access to alcohol. However, comparisons across multiple sets of selected rat lines suggest that operant responding for alcohol and voluntary preference drinking are genetically dissociable traits (Samson et al., 1998; Files et al., 1998; Leeman et al., 2010). We lack even basic information about the patterning of alcohol intake for most genotypes - do high preferers drink too much, too fast, or too much, too often? (Samson, 2000; Leeman et al., 2010). The size of drinking bouts may be one predictor of excessive drinkers. Non-human primates characterized as "gulpers" go on to develop patterns of very high voluntary drinking (Grant et al., 2008); however such studies to date cannot attribute this risk-associated phenotype specifically to genetics. The high drinking rat lines generally show patterns of larger **bout size** than the low preferring lines (Samson, 2000). Comparisons across these multiple rat lines have discussed their different characteristics (Ciccocioppo, 2013). Remarkably, a comprehensive review of neurobiological and pharmacological differences across the rat selected lines revealed no cases where all lines had been assessed for the same trait, and few where the same trait had been tested in 3 or more pairs of lines. In cases where 3 or 4 pairs of lines had been compared, the genetic results did not agree across lines. **Naltrexone** (another of the approved medications for alcoholism therapy) successfully reduced preference drinking in 4

pairs of lines (Bell et al., 2012). Lest I sound unduly pessimistic, in the remaining sections of this review I will highlight some of the more encouraging results that are emerging. These include improved behavioral methods for elevating genotype-specific levels of alcohol intake and inferring alcohol reinforcement, as well as new genetic animal models.

Alcohol Quantitative Trait Locus (QTL) studies

Most genetic studies discussed here thus far have offered evidence regarding *whether* genes predispose to different degrees of motivation for alcohol. Regardless of the motivational trait investigated, rodent studies suggest that genetic variation is important. To ascertain *how* genes can affect risk, it is useful to identify which of the 25,000 or more genes convey that risk. As mentioned earlier, many genes have been studied for such a putative role by simply being nominated as potential candidates due to their known biological function (e.g., dopamine or GABA receptor genes). However, starting in the 1990's it became possible to test a number of animals for an alcohol-related trait of interest and then survey their genomes, seeking associations between markers in the genome and trait scores. Markers of genetic variation (such as **single nucleotide polymorphisms**) have been mapped to specific locations in the genome of humans, rats and mice with steadily increasing resolution. Such studies amount, then, to **gene mapping**. They begin by identifying where systematic genetic variation in a rather large region of a particular rodent chromosome (a locus) is correlated with individual differences in the (quantitative) trait. Such studies are called QTL mapping studies.

As expected, most studies of alcohol-related QTL have focused on preference drinking. Early findings have been reviewed periodically (Belknap & Atkins, 2001; Cunningham & Phillips, 2003; Gill & Boyle, 2005; Fehr et al., 2005; Crabbe et al., 2010). Each QTL, identified by surveying multiple inbred strains or many animals from a hybrid cross of inbreds or selected lines, originally identifies a locus that harbors many genes, anywhere from a few to hundreds. Thus, further progress involves specialized tools that narrow the QTL interval, eliminating regions at both ends until only a few genes remain plausible candidates. Such studies have continued in both mice and rats (Bice et al., 2011; Bice et al., 2010; Drews et al., 2010; Izidio et al., 2011; Ehlers et al., 2010). Some QTL studies target ethanol conditioned taste aversion and ethanol conditioned place preference: these also have been reviewed (Cunningham & Phillips, 2003; Cunningham et al., 2006).

Most recently, the field has progressed to where not only variation in the sequence of base pairs can be assessed globally, but also the variation in levels of expression of genes. A good methodological review of this focuses on fine mapping of alcohol QTL for withdrawal severity (Milner & Buck, 2010). Knowing which genes are differentially expressed is conceivably of even more value for inferring function. The integration of gene sequence and expression data is now routinely applied, and this information is coupled by the use of informatics tools with information about the functional roles of the genes' proteins. This in turn is leading to a concentration on **networks** of genes whose expression is correlated (like groups of Facebook friends). This represents a profound shift in emphasis from individual genes to networks of potentially different genes that accomplish the same biochemical functions. Results of such "expression QTL" studies were reviewed recently (Crabbe et al.,

2010) and newer data continue to arise (Bjork et al., 2010; Mulligan et al., 2006; Iancu et al., 2013; Wang et al., 2012).

Withdrawal-associated drinking

One feature of human alcoholic drinking is that it is sometimes undertaken in attempts to prevent the occurrence of or to alleviate **withdrawal** symptoms. There are scales to assess withdrawal severity for humans (Penning et al., 2013), but there are few genetic studies of withdrawal *per se* (Gizer et al., 2011), and most focus on a single symptom such as seizures (Hughes, 2009) or blackouts (Nelson et al., 2004). Many recent studies have now documented increases in voluntary drinking in rats and mice during withdrawal from a period of forced physical dependence. The most common current method is to employ ethanol **vapor inhalation** to induce dependence. An excellent review of such studies discusses their history and identifies the most effective procedures for eliciting such escalations in intake (Becker, 2013). Becker points out that it is first necessary to establish ethanol as a positive reinforcer, and that the most effective procedures involve **chronic, intermittent ethanol vapor** exposure interspersed with the opportunity to self-administer alcohol. He postulates that this allows the animals to learn of the negatively reinforcing effects of drinking during withdrawal.

However, nearly all such studies thus far have been performed with a single mouse genotype (C57BL/6J), or have not compared multiple rat or mouse genotypes (exceptions with some selected lines of mice will be discussed below). Thus, these studies have for the most part not informed us about genetic contributions to withdrawal-related drinking; some exceptions based on deletions of single genes in circadian regulatory systems and glutamate pathways are reviewed elsewhere (Spanagel & Vengeliene, 2013). These studies also do not address the specific motivation for enhanced self-administration. Withdrawal has been shown to result in increases in **anxiety-like behavior** in rats and mice (Pandey et al., 2008; Valdez et al., 2002; Kliethermes et al., 2004), but none of the many extant studies have shown that the intent of the increased self-administration is to reduce the anxious state (admittedly, this is a high bar!). Becker points out that the elevated intake could result from **tolerance** to alcohol's aversive effects as well as enhanced reward value, and reviews several studies that demonstrate both these neural adaptations during withdrawal from chronic alcohol (Becker, 2013).

A promising approach has recently been renewed in Chris Cunningham's laboratory. Rats or mice are first made dependent by infusions of alcohol through an intragastric catheter and then learn to drink a flavored solution that delivers alcohol to the stomach. When they are subsequently offered a choice between the alcohol-delivering flavor and a different flavor that delivers water, they will maintain self administration by this route for several days (Fidler et al., 2006; Fidler et al., 2011). Even non-preferring DBA/2J mice will self-administer significant amounts of alcohol in this procedure, and they tend to ingest alcohol in large bouts, while C57BL/6J mice ingest in a pattern of small, steady "sips" (Fidler et al., 2011). Recent studies suggest that for DBA/2J mice, the motivation for self-administration in dependent mice is to reduce withdrawal, as access during withdrawal increased intake in a time-dependent manner (Cunningham et al., 2013). Pre-exposure in this paradigm increases

intragastric self-administration by increasing the number and not the size of bouts (Fidler et al., 2011). It seems likely that avoidance of the taste of alcohol is one of the keys to the success of this approach, as DBA/2J mice will generally avoid drinking any concentration of alcohol that they are able to detect (McClearn, 1968). However, at an earlier stage of the procedure when they are not dependent, the intragastric self-administration of DBA/2J mice (and HAP-2 mice as well) is low; this suggests that they also respond to some negative post-ingestional consequence of alcohol in addition to avoiding the taste (Fidler et al., 2011).

Persistence of self-administration despite negative consequences

Another characteristic of alcoholic drinking is its persistence despite abundant evidence to the drinker of the damaging effects of the excessive drinking on work, relationships, and/or health. Modeling such **persistence** in rodents has not been very common to date. Tom Gentry showed many years ago that C57BL/6J mice offered alcohol while treated with 4-methyl pyrazole, an alcohol dehydrogenase inhibitor, would continue to drink. These early studies revealed that the mice would self-administer lethal doses (Gentry, 1985). While this suggests that the motivation for alcohol is intense in this mouse strain, it does not really prove this, for the alcohol was available *ad libitum*. Thus, it is not clear how the animals would have ever learned to associate drinking the alcohol with the ensuing illness, even as it became more severe. Cunningham has also noted that access to intragastric alcohol must be limited during the initial phases of his procedure, lest the animals unwittingly overdose (C.L. Cunningham, personal communication).

Most recent research has attempted to model this feature of motivational shift by showing that after a period of relatively high levels of alcohol ingestion, the animals will then accept adulteration of the alcohol solution with a higher concentration of the bitter substance quinine than the concentration of an alcohol + quinine mixture accepted by naive animals [e.g., see (Lesscher et al., 2010)]. As discussed elsewhere, this may be a promising approach (Lesscher & Vanderschuren, 2012), but it does not eliminate the possibility that the experience of alcohol has not led to changes in the taste of the quinine + alcohol mixture, or a decrease in avoidance of quinine, rather than a devaluation of the rewarding effects of alcohol (Crabbe, 2012). Three recent studies appear to have at least in part avoided these alternative explanations. Hopf showed that after long term alcohol drinking, **quinine adulteration** could not reduce alcohol preference, but did affect either sucrose preference or water intake (Hopf et al., 2010). In another approach, rats were trained to work on an operant schedule to gain access to (and self-administer) alcohol (Vendruscolo et al., 2012). They were then made dependent on alcohol through daily cycles (14 hr on, 10 hr off) of chronic intermittent inhalation of alcohol vapor for one month. Animals then tested on a progressive ratio schedule during acute withdrawal from alcohol showed higher responding for alcohol than before dependence was induced, and the increase persisted for two weeks. They were then tested for responding during increasing doses of quinine adulteration, and were less sensitive than non-dependent/withdrawn rats to the effect of quinine to reduce responding. This study also reported that separate groups of animals were first offered quinine without alcohol to control for taste differences, and that dependent and non-dependent groups showed equivalent reduction in responding as compared with their baseline responding for alcohol. Furthermore, when alcohol was then added to the quinine-

only solutions, dependent/withdrawn rats showed *increases* in responding, while non-dependent rats showed further reductions (Vendruscolo et al., 2012). Thus, this study appears to have effectively controlled against taste-related effects.

Finally, a different approach has been taken using a **relapse** model (Marchant et al., 2013). Alcohol preferring **P** rats were given every other day access to 20% alcohol vs water for 12 access sessions, and then trained on a series of operant schedules to bar press for access in 2 hr sessions every other day. The tests were then moved to a different context. One group received **foot shock** with half the schedule-compliant lever presses in the new context - they also received access to alcohol (which they drank). Another group was given the same number of shocks non-contingently, and a third group received no shock. A fourth group was tested under extinction conditions. All animals were then tested under extinction conditions for relapse to alcohol-lever responding in both contexts every other day. Contingent foot shock and extinction training in the second context suppressed responding while non-contingent shock did not. When tested for relapse in the original context, both contingently-shocked and extinguished animals showed high responding for alcohol, while neither the non-contingent shock group nor the unshocked group did. The authors acknowledge that these animals were not administering alcohol to relieve withdrawal, because they were not consuming enough alcohol to become dependent. However, they suggest that to address this limitation, this context-dependency of relapse to alcohol self-administration could be tested in animals first made dependent (Marchant et al., 2013).

Although presented only in poster form, a recent study used methods similar to the Vendruscolo paper just discussed (Vendruscolo et al., 2012) to first train rats on an operant schedule to obtain alcohol (Shabani et al., 2012). Rats were then subjected to intermittent ethanol vapor for 6 weeks. Access to the alcohol schedule was returned during the 4th week, always during the period of 10 hours of acute withdrawal. Starting in the second inhalation week, rats were periodically subjected to extinction and then tested for cue-induced reinstatement. Some groups received contingent foot shocks during the reinstatement sessions. When no foot shocks were given, alcohol-paired cues consistently elevated responding for alcohol in both animals withdrawing from vapor and controls exposed only to air; the withdrawing rats responded at twice the rate as the controls. In rats given the intermediate shock level, alcohol cues suppressed reinstatement in controls, but not in the withdrawing rats. This suggests that the incentive value of alcohol was enhanced during periods of acute withdrawal from chronic intermittent vapor exposure (Shabani et al., 2012). These results are therefore consistent with the Marchant findings but extend them to dependent rats.

New selective breeding studies

As noted in an earlier section, the powerful technique of selective breeding has repeatedly been applied with rats to generate lines with relatively high two-bottle preference. A major limitation of these lines has been that when alcohol is freely available, they rarely drink enough to achieve intoxicating blood alcohol levels, unlike dependent humans. Two recent projects with mice have addressed this particular limitation with some success.

HAP/LAP mice

High (HAP) and Low (LAP) Alcohol Preferring mice were selectively bred in the 1990's for two bottle preference drinking of 10% alcohol vs water (Grahame et al., 1999). These lines were bred in replicate, with similar results in both replicates, and many other traits consistently covary with preference drinking [for review see (Green & Grahame, 2008)]. However, daily levels of drinking in the HAP lines is generally modestly elevated, as in the selected rat lines. To address this limitation, two of the high drinking HAP lines were intercrossed to produce a "crossed HAP" (cHAP) line which was then maintained under directional selection for high drinking (Oberlin et al., 2011). Presumably because each HAP line had captured somewhat different constellations of allelic variants increasing preference drinking, the intercross reintroduced some additional genetic variability, and the cHAP line drinks significantly more than any of the HAP lines. These animals reach blood alcohol levels exceeding 200 mg/dl (Matson & Grahame, 2011). Interestingly, the HAP and LAP mice from the original pairs of replicate selected lines (HAP-1 vs LAP-1 and HAP-2 vs LAP-2, respectively) were compared for their withdrawal-associated drinking. After establishing limited access preference drinking for four weeks, mice were exposed to two cycles of chronic intermittent access to ethanol vapor, each followed by a reassessment of drinking. Only the HAP-2 male (but not HAP-1 male) mice showed escalations in intake during withdrawal, while neither LAP-1 or LAP-2 males did so. Drinking was not elevated during withdrawal in females of either line (Lopez et al., 2011). C57BL/6J male mice showed significantly elevated drinking during withdrawal, as reported in many other studies from the Becker lab (Griffin, III et al., 2009). The cHAP line thus offers a promising new tool for exploring features of excessive alcohol self-administration.

HDID mice

In an attempt to achieve high blood alcohol levels following voluntary drinking in mice, we developed a procedure that takes advantage of rodents' tendency to engage in most of their ingestive behavior in the early hours of their circadian dark phase. We showed that C57BL/6J mice would ingest enough alcohol when offered a single bottle of a 20% solution to achieve intoxicating blood alcohol levels (> 100 mg/dl) within a 2 or 4 hr session starting 2 hr after lights go off in the colony room. The assay is known as "**drinking in the dark**" (**DID**) and has achieved fairly wide use (Rhodes et al., 2005). There are large inbred strain differences in both blood alcohol level attained, and in g/kg intake, and these correlate reasonably well with strain differences in intake in two bottle alcohol preference drinking (Rhodes et al., 2007; Crabbe et al., 2012d).

The existence of genetic contributions to individual differences led us to breed two lines of mice for High Drinking in the Dark (HDID-1 and HDID-2). We developed a two-day assay for drinking in the dark, comprising an initial access period of 2 hr followed the next day with a 4 hr period, after which we took a blood sample. We bred together mice with the highest blood alcohol levels and continued to breed selectively: we are currently breeding the 25th generation of HDID-1 mice and the 18th generation of HDID-2 mice. Blood levels increased slowly but steadily, and by the 11th generation exceeded the standard clinical levels for a binge (80 mg/dl). Figure 2.3 shows that most animals in both replicates now exceed 100 mg/dl BAL after their 4 hr drinking bout. These animals were intoxicated after

drinking as established by tests of motor performance (Crabbe et al., 2009). We have established a number of behavioral differences between HDID mice and their non-selected controls (Crabbe et al., 2012c; Crabbe et al., 2012a; Crabbe et al., 2012b). Interestingly, the HDID mice tend to drink in larger bouts than controls, a pattern that also characterizes alcoholic drinking (Barkley-Levenson & Crabbe, 2012).

When compared with control animals, HDID-1 mice showed lesser sensitivity to an ethanol-conditioned taste aversion than controls, which is perhaps a sign of a reduced role for alcohol's aversive effects in HDID-1 drinking. They did not differ from controls, however, in sensitivity to an ethanol conditioned place preference (Barkley-Levenson et al, submitted). We also compared HDID-1 and control mice for withdrawal-associated drinking, using the standard paradigm from the Becker lab (Griffin, III et al., 2009). Both HDID-1 and control mice showed significantly elevated alcohol drinking during withdrawal from a third cycle of chronic intermittent alcohol vapor exposure, but the genotypes showed equivalent degrees of **escalation**. This suggests that different genes affect binge-like drinking and its tendency to escalate during repeated withdrawal episodes (Crabbe et al., 2012d).

We are considering the possibility that HDID mice drink because they rapidly develop tolerance to the intoxicating effects of alcohol. Figure 2.4 shows the result of an experiment where forty naive HDID-1 mice from the 10th selected generation were familiarized with the balance beam, and then pretrained for two days. Each day they traversed the beam and the number of hind foot slip errors was counted. They averaged fewer than 1 foot slip on each pretraining day. Starting 24 hr later, mice were offered 20% ethanol for 4 hr using the DID procedure. Each day, they were immediately tested on the beam. On the first drinking day, mice averaged nearly 2 foot slips, showing significant impairment vs their own pre-drinking scores (paired $t = 5.32$, $p < .001$). Foot slip scores declined on DID Days 2 and 3, showing significant tolerance development (shown as D1 – D3, corrected for baseline). Intakes on the three days were fairly stable (5.27, 4.73, and 4.79 g/kg, respectively). The decline in foot slip errors between Days 2 and 3 is strong evidence for tolerance, because the animals' intakes were nearly identical on those days. However, we lack information about the timing and patterning of ingestion during each day, which could affect alcohol brain levels. Thus, it remains possible that the reduced behavioral effects reflect a metabolic adaptation.

The HDID **binge** model of drinking thus offers another promising approach to exploring genetic differences in motivation for alcohol and its dysregulation. The model used for selection does not offer a choice of fluids. Although HDID mice drink more alcohol than controls even when a water bottle is available, they also drink some water and therefore achieve lower blood alcohol levels than during then usual single-bottle test (Crabbe et al., 2009). The DID test has been used with mice and rats with various procedural adaptations. Most investigators are not focused on the absolute blood alcohol levels and therefore use a two-bottle choice variant. Many such studies of the physiology and pharmacology of DID have been reviewed; nearly all of these functional studies have employed C57BL/6J mice (Crabbe et al., 2011; Sprow & Thiele, 2012).

Summary and conclusions

In summary, there are remarkably few studies focused on the genetic contributions to alcohol's reinforcing values. Almost all such studies examine the two-bottle preference test. Despite the deficiencies I have raised in its interpretation, a rodent genotype's willingness to drink ethanol when water is freely available offers a reasonable aggregate estimate of alcohol's reinforcing value relative to other genotypes (Green & Grahame, 2008). As indicated above, however, preference drinking studies will likely never avoid the confounding role of taste preferences and most often yield intake levels not sufficient to yield a pharmacologically significant blood alcohol level. Thus, the quest for improved measures of reinforcing value continues.

Of the potential motivational factors considered by McClearn in his seminal review in this series, we can safely conclude that rodent alcohol drinking is not primarily directed at obtaining calories. The role of taste (and odor) remains a challenge. McClearn appears to have been correct that especially those genotypes that avoid alcohol are probably doing so based on pre-ingestive sensory cues; however, post-ingestive consequences are also important. Cunningham's intragastric model shows the role of both pre-ingestional and post-ingestional modulating factors for the best-known examples, the usually nearly absolutely alcohol avoiding DBA/2J and HAP-2 mice. Much subsequent data reinforces McClearn's earlier conclusion that C57BL/6J mice, at least, do not regulate their intake around a given self-administered dose of alcohol by adjusting their intake. This leaves us with the puzzle of why nearly all genotypes, even those directionally selectively bred for high voluntary intake for many generations, fail to self-administer intoxicating amounts of alcohol. Since McClearn's review, many ingenious assays to index alcohol's motivational effects have been used extensively, and new methods for inducing dependence have supplanted the older ones current in 1968. I have tried to identify promising areas where the power of genetics could be fruitfully harvested, and generally feel that we have a much more clear idea now about some important experiments remaining to be performed.

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Figure 2.1.

Daily preference ratio of consumed 10% alcohol solution/total fluid consumption. Data for 4 individual male mice per genotype are shown. Broken vertical lines between Days 6–7 and 10–11 indicate reversal of position of alcohol and water solutions. Adapted from Figure 1 in McClearn & Rodgers (1959), with permission.

Figure 2.2.

Correlations among means of inbred strains observed in different laboratories. The dashed line indicates what would be identical results for two laboratories, and the gray line is the best fit regression of data from the y axis to data on the x axis, plotted for the actual range of data. Numbers 1–30 correspond to inbred mouse strains. Effect sizes from the ANOVAs are shown for the strain main effect and strain-by-laboratory interaction as insets within each panel. The significance (P) of the interaction effect is also indicated. NS denotes an interaction not significant at $P \neq 0.05$. (A) Brain weight measured in the Edmonton laboratory versus four other laboratories. (B) Preference ratios for two bottle ethanol preference tests conducted in 4 laboratories. Adapted from Figure 1 in Wahlsten et al (2006), with permission.

Figure 2.3.

Percentage of subjects with blood alcohol levels greater than 100 mg/dl (1.00 mg/ml) at the end of 4 hr of drinking 20% ethanol on Day 2 of the Drinking in the Dark test. The horizontal bar indicates 50%. Approximately 50–120 mice were tested each generation, and data are collapsed on sex. Selection of the HDID-1 line was initiated 7 generations before the initiation of the HDID-2 line. For details of the selection procedures, see Crabbe et al (2009).

Figure 2.4.

Rapid tolerance to ingested alcohol in HDID-1 mice. Foot slip errors on the balance beam are shown for two pretraining days and three days when the animals were tested immediately after drinking 20% ethanol for 4 hr in the drinking in the dark test. Average dose of ethanol consumed is shown for each day. The bar indicates the difference between errors on Days 1 and 3, corrected for average baseline foot slips on pretraining days 1 and 2. Means " SE are shown.