Published in final edited form as:

Alcohol Clin Exp Res. 2013 December ; 37(12): 2019–2028. doi:10.1111/acer.12188.

Genetic Studies of Acute Tolerance, Rapid Tolerance, and Drinking in the Dark in the LXS Recombinant Inbred Strains

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Abstract

Background—We hypothesized that rapid tolerance (one-day tolerance) for the duration of the loss of righting reflex (“sleep time”; ST) was mediated by an increase in AFT. We also hypothesized that increased AFT would correspond to increased drinking. These questions were addressed using the LXS RI panel.

Methods—Mice were given a pretreatment dose of either saline or 5 g/kg alcohol on day one. On day two, mice were tested for ST (4.1 g/kg) using a method with which it is possible to accurately assess AFT. Genetic correlation analysis was conducted among the ST-related variables and also with “drinking in the dark” (DID) which was previously measured by Saba et al. (2011).

Results—Saline pretreated mice showed a continuous distribution of ST ranging from ~40 minutes to over 3 hours. Out of the 43 strains tested, 9 showed significantly decreased ST after alcohol pretreatment while in 3 strains, ST was significantly increased. AFT scores ranged from 0 to over 200 mg% in the saline group and in the alcohol group, 8 strains showed a significant increase in AFT and 2 strains showed significant decrease in AFT. In the saline group, AFT was significantly correlated to ST ($r = -0.47$), but not in the alcohol group ($r = -0.22$). DID was significantly correlated to only AFT in the alcohol pretreated group ($r = 0.64$).

Conclusions—The results suggest that AFT is an important component of the overall ST response, but that the alcohol pretreatment-induced change in AFT does not contribute to rapid ST tolerance. The significant correlation between DID and AFT in the alcohol group suggests that AFT may be a more relevant predictor of drinking behavior than the static measurement of ST. Moreover, pre-exposure to alcohol seems to change AFT in a way that makes it an even stronger predictor of drinking behavior.

Keywords

Ethanol; genetics; mouse; alcoholism

Introduction

Twin studies and other areas of research have identified genetics as an important factor in alcohol use disorders (AUDs) accounting for as much as 50% or more of the phenotypic variance (Dick and Bierut, 2006). A clear understanding of the molecular basis for these genetic effects, however, remains elusive. One well described genetic effect on AUDs is innate sensitivity to alcohol. Schuckit (1980) observed that individuals who were “family history positive” (FHP) for alcoholism compared to those who were “family history negative” (FHN) were reliably less sensitive to an acute alcohol challenge, a phenomenon that Schuckit termed “low level of response” (LR). The LR effect has been noted for a variety of physiological, cognitive, and behavioral measures, including the alcohol-induced loss of motor control as well as subjective feelings of intoxication (Newlin and Thomson, 1990; Quinn and Fromme, 2011).

While the FHP/FHN design does not exclude the possibility of non-heritable explanations for alcohol sensitivity differences, it has been clearly demonstrated that acute sensitivity does have a heritable component in humans (Heath et al., 1999). Retrospective analyses indicated that the lower sensitivity of the at-risk groups was evident prior to the onset of regular drinking behavior suggesting that the effect was probably not related to chronic tolerance (Schuckit et al., 2007). These observations led to the hypothesis that genetically-determined LR is a predisposing risk factor in the etiology of alcoholism (Schuckit, 1980). Importantly, LR was found to be a reliable predictor of future drinking problems and it is thought to be a valid and useful construct for investigating the genetics of alcoholism risk (Quinn and Fromme, 2011).

Newlin and Thomson (1990) noted some conflicting results in the LR literature and argued that the discrepancies were due, in part, to the development of greater acute functional tolerance (AFT) in FHP individuals on the descending limb of the alcohol distribution curve. The concept of AFT is first credited to Mellanby (1919) who observed that dogs that had been administered alcohol were more ataxic on the ascending limb than they were at the same blood ethanol concentration (BEC) on the descending limb indicating that they had developed tolerance within that single exposure. It subsequently has been shown that AFT occurs in humans as well as in rodents and also that it has a well-described genetic component (Bennett et al., 1993; Erwin and Deitrich, 1996; Ponomarev and Crabbe, 2004; Radcliffe et al., 2006). Thus, the FHN/FHP difference in LR and its association to AUDs may be due in part to genetic differences in AFT.

AFT tends to dissipate fairly soon after alcohol has been cleared (Erwin et al., 2000), but more enduring forms of tolerance are just beginning to appear. So-called rapid tolerance (RT) is evident up to at least 24 hours after a single administration of alcohol (Crabbe et al., 1979) while chronic tolerance is associated with continuous or multiple dosings over an extended period lasting from days to years (Kalant et al., 1971; Melchior and Tabakoff, 1984). RT may represent the initial stages of chronic tolerance, but the relationship between AFT, rapid, and chronic tolerance has not been completely resolved (Kalant et al., 1971; Khanna et al., 1991; Radcliffe et al., 2006; Rustay and Crabbe, 2004; Tabakoff et al., 1982; Wu et al., 2001).

The analysis of drinking behavior in rodents has been an important tool for investigating the underlying neurobiology of human AUDs. The most common test for genetic effects on drinking behavior has been the two-bottle choice, unlimited access method primarily because it is easy to perform and can be of relatively short duration (Crabbe et al., 2010b). Recently, the limited access “drinking in the dark” method (DID) has been introduced to overcome a major weakness of the unlimited access method which is that rodents tend to not achieve physiologically relevant BECs (Crabbe et al., 2010b; Rhodes et al., 2005), although there are two exceptions: C57BL/6 × FVB/NJ F1 mice (Blednov et al., 2005) and the selected High Alcohol Preferring (HAP) mice (Matson and Grahame, 2011).

The Inbred Long and Short Sleep mouse strains (ILS, ISS) were selectively bred for extreme differences in the duration of the loss of the righting reflex (“sleep time”; ST), an acute response to a hypnotic dose of alcohol, and have been widely used to putatively study genetic effects on LR (McClearn and Kakihana, 1973). The ST assay is unique in that in addition to providing the duration of the response, one can also measure sensitivity at the loss of function (BEC at loss) and at the regain of function (BEC at regain) allowing for an assessment of AFT similar to the Mellanby (1919) approach. However, ST AFT typically has not been considered in studies of the ILS and ISS and rarely in any study that used ST as a measure of acute alcohol sensitivity. This is because it is technically difficult to obtain an accurate estimate of AFT for ST. Ponomarev and Crabbe (2002) developed a variation of the traditional ST test with which it is possible to measure AFT more accurately. Using this method, it was shown that a large portion of the enormous ILS/ISS difference in ST sensitivity was mediated by AFT (Radcliffe et al., 2006). RT was also assessed in this study, defined as the difference in LORR duration between groups of mice that had received a pretreatment of either a saline or alcohol 24 hours prior to the ST test. A pronounced genetic effect on RT was observed; i.e., 24 hours after a single dose of alcohol, ST was significantly reduced in the ILS, but not in the ISS. (Radcliffe et al., 2006). It was hypothesized that the observed ILS RT was mediated by a change in the rate or magnitude of the development of AFT.

We have begun to explore the genetics of AFT, RT, and drinking behavior with the use of the LXS recombinant inbred mouse strain panel (RI). The LXS panel was created from pairs of ILS/ISS-derived F₂ offspring that were subsequently bred through brother-sister matings for more than 20 generations resulting in a panel of inbred strains, each of which contains a random assortment of alleles from the progenitor ILS and ISS (Williams et al., 2004).. The strains were given either saline or alcohol on day one of a two day test. On the second day, all of the mice were tested for ST AFT using the modified method of Ponomarev and Crabbe (2002). RT was also determined as described above. Genetic correlation analyses were used to gain insight into the relationships between the two forms of tolerance and between tolerance and drinking behavior as assessed using a slightly modified version of the DID method of alcohol self-administration (Saba et al., 2011).

Materials and Methods

Animals

LXS RI breeders were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in-house in the UCAMC vivarium, a pathogen-free facility. Offspring were weaned and sex-separated at 21 days of age. All experiments were conducted with males that were group-housed in standard housing with aspen chip bedding (Harlan Laboratories, IN) and containing from 2 to 5 mice per cage; the mice were 58 to 99 days of age at the time of testing (mean = 85.2 $\pm$ 0.2). Forty-four strains were tested with an average n of 10.1 ($\pm$ 0.4) and a range of 4 to 17 per strain. The mice were maintained in a constant temperature (22 to 23°C), humidity (20–24%), and light (14L/10D) environment. The procedures described in this report have been established to ensure the absolute highest level of humane care and use of the animals, and have been reviewed and approved by the UCAMC IACUC.

Loss of Righting Reflex

Mice were weighed and administered a single intraperitoneal (i.p.) injection of vehicle (normal saline) or alcohol (95% ethanol, PHARMCO-AAPER, CT; 5 g/kg, 16% w/v in saline) on day one; this is referred to as the pretreatment dose. The pretreatment dose of 5 g/kg was selected because it was found to be not overtly toxic and it elicited a robust rapid tolerance compared to other doses (Radcliffe et al., 2005). The mice were not behaviorally tested on day 1; they were administered the pretreatment and placed back into their home cage with their original cage-mates. The loss of righting reflex (LORR) was assessed in both groups 24 h following the pretreatment as described below. The pretreatment procedure was conducted in this way to minimize associative learning that might contribute to tolerance, although it was probably not possible to completely eliminate this effect.

Acute alcohol sensitivity was measured using a modified version of the traditional ST test to better estimate parameters of AFT (Ponomarev and Crabbe, 2002; 2004; Radcliffe et al., 2006). Body weights from day 1 were used to calculate the test dose (4.1 g/kg, ip). The mice were not weighed on the day 2 test day to avoid potential associative learning effects as discussed above. Immediately after alcohol injection, the mouse was placed in a small, closed Plexiglas cylinder that was rotated 90° every 2–3 s. Loss of righting was defined as the time at which the mouse remained supine for at least 5 s; a retro-orbital blood sample was drawn at this point for determination of BEC at the loss of righting reflex (BEC1; initial sensitivity). The animals were tested for recovery of LORR every 3–6 min thereafter. A second blood sample was drawn (BEC2) when the animal was able to right itself within a 5 s period after being placed in a supine position or could not be placed on their backs after 8 successive 90° turns of the cylinder. Duration of LORR was defined as the elapsed time between the loss and regain of the righting reflex; this is referred to as “sleep time” (ST) in the remainder of the text. An increase in BEC2 from BEC1 was interpreted as development of AFT which was quantitatively expressed as the difference between BEC2 and BEC1. Mice that had not regained the righting reflex by 180 mins were given a ST score of 180 and a blood sample for determination of BEC2 was taken at that time. BEC values were determined by spectrophotometry with the use of a reliable enzyme assay (Lundquist, 1959). Rapid tolerance for ST was defined as the difference between mean duration in the alcohol

group and mean duration in the saline group resulting in a single value for each strain (ST_RT). In tables 2 through 4 and in the remainder of the text, the pretreatment group is designated with either “_sal” (saline) or “_Et” (alcohol) following the variable name.

Drinking in the Dark

The drinking in the dark study (DID) was previously published; details can be found in Saba et al. (2011) and LXS strain means can be found at [GeneNetwork.org](http://GenNetwork.org) (record ID # 10162).

Data Analysis

Measures of ST, BEC1, BEC2, and AFT were investigated for main effects of strain, pretreatment, and interaction using a strain-by-pretreatment two-way factorial ANOVA. After a preliminary analysis, one outlier strain was removed (strain 100). Effect size is represented as ω^2 and was calculated as: $(SS - df * MS_{\text{error}}) / (SS_{\text{total}} + MS_{\text{error}})$. Simple effects analysis was conducted for ST and AFT to determine if there was an effect of treatment for each individual strain (one-way ANOVA). The pairwise comparisons were not corrected. One-sample t-tests were used to determine if a strain's AFT was greater than zero; i.e., if it had developed significant AFT. The Pearson product-moment was used with strain means to test for significant genetic correlations (r). The correlation analysis was corrected for multiple comparisons using Bonferroni.

Results

Table 1 shows the results of a two-way ANOVA (strain-by-pretreatment) for the four measures taken during the ST test. There was a significant strain effect for all of the measures and significant pretreatment and interaction effects for all of the measures except BEC1. Note that the strain effect size was large for ST and BEC2 and somewhat less for BEC1 and BEC2. The pretreatment effect size was negligible for all measures.

The distribution of strain means for ST 24 hours after saline (ST_sal) or 5 g/kg alcohol (ST_Et) is shown in figures 1-a and 1-b, respectively (arranged in order of ST_sal); ST rapid tolerance (ST_RT; difference between mean ST_ET and mean ST_sal) is shown in 1-c. The significant interaction for ST suggests that the strains did not all respond equally to the alcohol pretreatment. Indeed, post hoc analysis indicated that nine strains showed rapid tolerance (ST significantly decreased; asterisks in figure 1-c) while three of the strains showed sensitization (significant increase in ST; plus signs in fig. 1-c); the rest of the strains did not change.

The distribution of strain means for AFT 24 hours after saline (AFT_sal) or 5 g/kg alcohol (AFT_Et) is shown in figures 2-a and 2-b, respectively (arranged in order of AFT_sal). The majority of the strains developed AFT as indicated by the finding that their AFT values were significantly greater than zero (27 and 32 out of 43 for the saline and alcohol pretreatment groups, respectively; one-sample T-test). As with ST, the significant interaction for AFT indicated that all strains did not respond equally to the alcohol pretreatment. Post hoc analysis indicated that eight strains showed significantly increased AFT after alcohol (asterisks in fig 2-b) while two strains showed a significant decrease in AFT (plus signs in fig. 2-b). Note that four of the nine strains that showed rapid tolerance for ST also had a

significant increase in AFT after alcohol pretreatment and there was one strain that had a sensitized ST response and decreased AFT.

Genetic correlation analysis was conducted to determine the extent of shared genetic influence among the LORR responses (table 2). A key correlation is that between ST and BEC2 which, as expected, was negative and highly significant in both the saline and alcohol pretreatment groups (figure 3). The fact that the correlations were very high suggests that the ST was primarily driven by neuronal sensitivity rather than metabolic effects.

We had postulated that rapid ST tolerance is driven by the alcohol pretreatment-induced change in AFT (Radcliffe et al., 2006). As shown in table 3 and figure 4, ST was significantly correlated to AFT only in the saline pretreatment group. In addition, ST_RT was not significantly correlated to AFT_S and the correlation to AFT_E was nearly zero (table 3). These observations suggest that the alcohol pretreatment-induced change in AFT was not responsible for rapid ST tolerance in the LXS strains.

Previous testing of drinking in the dark (DID) by Saba et al. (2011) made it possible to test the hypothesis that AFT and rapid ST tolerance are genetically related to alcohol drinking behavior in the LXS RI panel. Table 4 shows the Pearson's product-moment coefficients for correlations between drinking in the dark (DID; g/kg/2 hrs) and the eight LORR-related measurements. DID was significantly correlated to only AFT in the alcohol pretreatment group; the correlation to AFT in the saline group, although reasonably high, did not reach significance after correcting for multiple tests. Note that the correlation between DID and ST_RT was non-significant at an r value of only 0.10. Scatterplots for DID vs. ST or AFT are shown in figures 5 and 6, respectively.

Discussion

Schuckit (1980) postulated that a genetically-mediated low sensitivity to acute alcohol is an important risk factor for the development of alcohol use disorders. This hypothesis has been supported by an abundance of human studies from Schuckit's lab and others (Quinn and Fromme, 2011). However, in genetic studies using model organisms, the association between drinking behavior and ST as the measure of acute sensitivity has been inconsistent (Crabbe et al., 2006). One reason for this, particularly in genetically engineered models in which only a single gene is being examined, is that it is unlikely that all of the genes that confer acute sensitivity are involved in drinking behavior and vice versa. Thus, the nature of the relationship is at least partly dependent on the specific genetic architecture of the population being examined. Another important reason, as argued by Newlin and Thomson (1990), is that the dynamic process of AFT may be more relevant than a single static endpoint, such as "sleep time". Finally, prior to the introduction of the DID method, mouse drinking behavior typically was and continues to be assessed primarily using the unlimited access, two-bottle choice method. Other than a few exceptions (Matson and Grahame, 2011; Blednov et al., 2005), the vast majority of mice that have been tested with the unlimited access method generally never reach BEC's that produce substantial physiological or behavioral effects (Rhodes et al., 2005). In contrast, with the DID procedure, some mouse strains do achieve relatively high BEC's which is thought to mimic "binge" drinking making

it generally more relevant to the human condition than the unlimited access method and perhaps more effective at distinguishing genotypes that drink for the intoxicating effects of alcohol rather than for some other reason (Crabbe et al., 2010b; Rhodes et al., 2005; 2007). Note that the LXS DID data were generated using a slightly modified version of the original procedure that did not include the measurement of BEC; however, it has been shown that DID intake and BEC are genetically correlated suggesting that the highest drinking strains may have had similar BEC values to the prototypical heavy drinking strain C57BL/6 which drank less than several of the LXS (Crabbe et al; 2009; Rhodes et al., 2007; Saba et al., 2011).

Perhaps the most important finding of this study is the highly significant genetic correlation between AFT_Et and DID ($r = 0.64$). None of the single static measures of LORR (BEC1, ST, and BEC2) were found to be significantly correlated to DID, although they were non-zero and ST and BEC2 were in the expected direction: decreased sensitivity was associated with increased drinking. Yet, among all of the LORR-related measures, AFT_sal and AFT_Et showed the two highest correlations with DID and only AFT_Et was statistically significant. Both correlations were in the predicted direction: higher drinking strains tended to be those with higher AFT. Notably, the alcohol pretreatment activated (or inhibited) some unknown mechanism that changed AFT in such a way that it was more predictive of DID behavior than in saline-pretreated mice. A similar result was seen in the UChA and UChB rat lines, selected for low and high drinking, respectively, in a two-bottle choice, unlimited access procedure. The UChB line initially showed greater development of acute tolerance in the tilting plane test and increased acute tolerance as well as increased drinking after prior treatment with alcohol (Tampier and Quintanilla, 2002). Replicate lines of mice selected for high BEC in the DID procedure showed increased tolerance to alcohol-induced hypothermia after three days of administration (males only; females showed the opposite (Crabbe et al., 2012a). An early study of the High and Low Alcohol Preferring mouse lines (HAP, LAP), selected using the standard two-bottle choice unlimited access method, showed no difference in AFT using the stationary dowel test (Grahame et al., 2000); however, a later experiment using a modified version of the test that was more closely aligned with the Mellanby (1919) approach found that HAP mice were more sensitive on the ascending limb of alcohol distribution and developed greater AFT during the early part of the test than the LAP (Fritz et al., 2013). Similarly, mice selected for High and Low AFT on the stationary dowel (HAFT, LAFT) were found to differ in their drinking behavior (unlimited two-bottle choice) with the HAFT drinking more than the LAFT (Erwin et al., 2000). These results with selected lines are generally consistent with the current study, although the DID line results are mixed. Rhodes et al. (2007) measured DID in a panel of 12 inbred mouse strains and tested for genetic correlations with 609 previously measured traits. Using fairly stringent statistical criteria, correlations with only 8 other traits were reported as significant and none of these were related to LORR or to any measure of alcohol tolerance. This study, however, was statistically limited by the small number of strains and the large number of traits; it is not even known if LORR or tolerance was included among the tested traits. Regardless, one must exercise caution in interpreting these studies in the context of the current results since the tasks and the doses are in some cases quite different and may be engaging different neuronal systems (Erwin et al., 2000).

In a previous study investigating rapid tolerance in the ILS and ISS using the same LORR procedure as in the current study, we observed that the ILS showed rapid ST tolerance and a significant increase in AFT after alcohol pretreatment, whereas there was no effect of alcohol pretreatment on either measure in the ISS (Radcliffe et al., 2006). We had postulated that the increased AFT in the ILS was the underlying basis of its rapid ST tolerance; however, the current results do not support this hypothesis. AFT and ST were significantly correlated within the saline pretreatment group, but not within the alcohol group. In addition, rapid tolerance did not significantly correlate to AFT in either the saline or alcohol groups, with the correlation between AFT_Et and ST_RT being nearly zero. These results suggest that AFT is indeed an important component of variance in ST; however, the alcohol pretreatment apparently caused independent alterations in AFT and ST suggesting a dissociation between acute and rapid tolerance, at least in the LXS RI strains. An advantage of the current design is that the same basic behavior was being studied for both AFT and RT. This is also a disadvantage in that the current results may not generalize well for other alcohol-related behaviors.

The relationship between acute and rapid or chronic tolerance (rapid and chronic tolerance are often considered to be manifestations of the same phenomenon; Khanna et al., 1991; Rustay and Crabbe, 2004) has been the subject of numerous studies, but its exact nature remains unclear. Several lines of evidence point to differences in the two forms of tolerance (Kalant, 1998); for example, blockade of the type1 cannabinoid receptor inhibits rapid tolerance, but does not affect acute tolerance (Lemos et al., 2007), and results of experiments from Spear's lab suggest that the developmental profiles of acute and rapid tolerance are different (Broadwater et al., 2011). In addition, genetic studies using the HAFT and LAFT selected mouse lines suggest a difference between acute and chronic tolerance. The lines did not differ in their development of chronic tolerance (Rustay et al., 2001) and, in a separate study, they showed a similar increase in AFT after a chronic alcohol regimen (Wu et al., 2001). On the other hand, the two main neurotransmitter systems that are affected by alcohol – NMDA and GABA – have been shown to mediate both acute and longer forms of tolerance. Results from pharmacological manipulation of excitatory NMDA receptors (NMDAR) suggest that they may be involved in both forms of tolerance (e.g., Khanna et al., 1993) and, on the inhibitory side, mice carrying an alcohol-insensitive mutant of the $\alpha 1$ subunit of the GABA_A receptor showed a decrease in both acute and chronic tolerance (Werner et al., 2009).

There may be broad overlap between some of the molecular and neuronal pathways that mediate acute and more enduring forms of tolerance, but the precise mechanism through which they are engaged may differ. For example, the phosphorylation state of the NMDAR, which dictates the receptor's activity and is altered by acute alcohol exposure, may be the mechanism through which NMDAR participate in acute tolerance (e.g., Hicklin et al., 2011), while NMDAR effects on rapid and chronic tolerance may be mediated by altered subunit expression or composition (e.g., Kalluri et al., 1998). A similar phenomenon can be observed with the large-conductance Ca^{2+} - and voltage-activated K^{+} channel (BK), an important target of alcohol tolerance-related action (Mulholland et al., 2009). A PKA-dependent mechanism may underlie acute tolerance of the BK channel while rapid or chronic tolerance appears to be mediated through a miRNA-dependent mechanism which

alters the splice variant composition and therefore alcohol sensitivity of the channel (Pietrzykowski et al., 2008). Notably, we recently found that K^+ channels were significantly over-represented among genes whose mRNA expression was significantly different between the ILS and ISS, although none of them were in the Ca^{2+} -activated family (Darlington et al., 2013). The specific role of K^+ channels or any other genes on acute or rapid tolerance in the LXS RIs remains to be determined.

Many studies have shown an inverse genetic relationship between acute sensitivity and the capacity to develop rapid or chronic tolerance; i.e., the more sensitive an individual is, the more tolerance they will develop (e.g., Crabbe et al., 1982). This is intuitively satisfying: very insensitive individuals may already be at some kind of sensitivity nadir, unable to become any less sensitive, while more sensitive individuals, prior to alcohol exposure, have not reached that point, but retain the capacity to do so. The negative correlation between ST_{sal} and ST_{RT}, the basis of which can easily be seen by comparing figures 1-a and 1-c, provides support for this inverse relationship, although the highly significant strain-by-pretreatment interaction for ST suggests that it is more complex than the correlation might suggest. Interestingly, 3 of the 8 least sensitive strains actually became significantly sensitized 24 hours after the alcohol pretreatment. This is reminiscent of a similar observation in the ISS from a previous experiment, although that was with the traditional ST test which is procedurally different than the test used in the current study (Radcliffe et al., 2006).

There is clearly a genetic effect on rapid tolerance (Rustay and Crabbe, 2004; current study) and chronic tolerance (Crabbe et al., 1982). This is an important issue because tolerance is one of the DSM-IV criteria for alcohol dependence and, all else being equal, the tolerant individual will drink more thereby increasing the risk for injury to the liver and other organ systems, and possibly may contribute to the perpetuation of the cycle of alcohol dependence. As with the association between ST and drinking behavior, there is conflicting evidence regarding the contribution of rapid or chronic tolerance to drinking, particularly in animal models with which some studies have shown a positive relationship (e.g., Kurtz et al., 1996), while others do not (e.g., Crabbe et al., 2012a). In contrast to AFT, rapid tolerance did not correlate at all to DID in the current study suggesting that genetic influences on rapid tolerance may not be important for variance in drinking behavior, at least in the LXS panel, with an important experimental caveat. While the mice in the DID study had been exposed to alcohol prior to being tested on the third day, the amount they consumed varied by genotype and was almost certainly smaller than the pretreatment dose used for the current rapid tolerance experiment, at least for most of the strains (Saba et al., 2011). It is not known how the LXS strains might have responded in the DID paradigm if they had been pre-exposed to a larger dose similar to that used in the current study. Similarly, the extent (if any) to which the strains would have developed AFT with the doses they were exposed to in the DID experiment also is not known. The current results, however, do not support a genetic relationship between drinking behavior and longer forms of tolerance, in this case, rapid tolerance.

ST is perhaps the most widely used measure of acute alcohol sensitivity in the rodent literature, but its validity as a surrogate for measures of acute sensitivity in the context of

Schuckit's LR hypothesis is unclear and has been questioned (Crabbe et al., 2010a). This is primarily because laboratory-based alcohol challenge studies in human subjects, to our knowledge, have employed exclusively low to moderate doses for which physiological, behavioral, and cognitive measures of sensitivity have included responses such as body sway, subjective feelings of intoxication, endocrine measures, EEG, and others, but not hypnotic sensitivity. Yet it is clear that many people who drink do on occasion, if not frequently, drink to the point of experiencing hypnotic effects, particularly individuals with an AUD (Schuckit et al., 1997). Thus, a question that has not been well addressed is the extent to which high dose sensitivity relates to AUD risk in humans. One clue comes from retrospective studies in which it was found that there was a highly significant negative correlation between subjective feelings of low dose intoxication and the reported number of drinks that it took individuals to "pass out" early in their drinking careers (Schuckit et al., 1997). This observation supports the hypothesis that a low level of response to high dose alcohol is a predictor of AUD risk, similar to the situation with low dose sensitivity.

In conclusion, the current results support that LORR AFT is a predictor of drinking behavior among the LXS RI strains. Moreover, there appears to be an effect of alcohol pre-exposure on AFT highlighting the importance of the tolerance-related plasticity that occurs during early exposure to alcohol as a predictor of genetically-influenced drinking behavior. Indeed, it may be the interaction between AFT and initial exposures to alcohol that is a critical determinant in AUD risk; i.e., it may not be the initial AFT that is important, but rather the way that one's AFT changes in response to prior experience with alcohol that dictates AFT-related AUD risk. The results do not support our previous hypothesis that the pretreatment-mediated change in AFT is responsible for RT, measured as the difference in ST between the saline and alcohol pretreatment groups. Moreover, ST_RT was a poor predictor of drinking behavior in the LXS strains which raises important questions about the genetic relationship between drinking and longer forms of tolerance such as RT. It remains to be seen if these effects are unique to the LXS RIs and, in addition, if the effects are unique to the high-dose LORR response or if AFT following pre-exposure to alcohol for other lower-dose responses, such as the stationary dowel rod, will show a similar kind of relationship.

Acknowledgments

The authors would like to thank Dr. John Crabbe and the reviewers of this article for their helpful comments and suggestions. This research was funded by NIH grant number R01 AA 016957.

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Figure 1.

LXS strain distribution of sleep time (ST) 24 hours after saline (a) or 5 g/kg alcohol (b). ST rapid tolerance is shown in panel c (mean ST_Et – mean ST_sal). Strains are arranged by ST_sal. Asterisks (*) or plus signs (+) indicate a significant decrease or increase in ST_Et from ST_sal, respectively (p<0.05, uncorrected).

Figure 2.

LXS strain distribution of AFT 24 hours after saline (a) or 5 g/kg alcohol (b). Strains are arranged by AFT_{sal}. Asterisks (*) or plus signs (+) in panel b indicate a significant increase or decrease in AFT respectively, in the alcohol pretreatment group ($p < 0.05$; uncorrected).

Figure 3.

Scatterplot of sleep time (ST) vs. BEC2 in the LXS strains 24 hours after saline (a) or 5 g/kg alcohol (b). Least squares linear regression is shown as a solid line. The Pearson product-moment correlation coefficient r is shown ($p < 0.01^{**}$).

Figure 4.

Scatterplot of sleep time (ST) vs. AFT in the LXS strains 24 hours after saline (a) or 5 g/kg alcohol (b). Least squares linear regression is shown as a solid line. The Pearson product-moment correlation coefficient r is shown ($p < 0.05^*$).

Figure 5.

Scatterplot of DID vs. sleep time (ST) in the LXS strains 24 hours after saline (a) or 5 g/kg alcohol (b). Note that the same DID values are shown in panels a and b. Least squares linear regression is shown as a solid line. The Pearson product-moment correlation coefficient r is shown (both values not significant).

Figure 6.

Scatterplot of DID vs. AFT in the LXS strains 24 hours after saline (a) or 5 g/kg alcohol (b). Note that the same DID values are shown in panels a and b. Least squares linear regression is shown as a solid line. The Pearson product-moment correlation coefficient r is shown ($p < 0.01$ **).

Table 1

Results of two-way ANOVA for LORR-related measures in the LXS RIs (strain-by-pretreatment; df = degrees of freedom; n.s. = not significant).

Variable	Strain effect, p (df)	Pretreatment effect, p (df)	Interaction effect, p (df)
Duration of LORR	$<1 \times 10^{-15}$ (46, 904)	0.004 (1, 904)	1×10^{-9} (44, 904)
BEC1	$<1 \times 10^{-15}$ (46, 898)	n.s. (p>0.8)	n.s. (p>0.4)
BEC2	$<1 \times 10^{-15}$ (46, 904)	1×10^{-7} (1, 904)	1×10^{-6} (44, 904)
AFT	$<1 \times 10^{-15}$ (46, 898)	0.009 (1, 898)	0.009 (1, 898)

Table 2

Genetic correlations among the LXS RIs for LORR-related measures 24 hours after saline (sal) or ethanol (Et) pretreatment (Pearson's *r*; *n* = number of strains). Significant correlations shown in bold-faced type (nominal $p < 0.05$ *, $p < 0.01$ **; multiple comparisons corrected by Bonferroni); non-significant correlations shown in parentheses.

	LORR_sal	BEC1_sal	BEC2_sal	AFT_sal	LORR_Et	BEC1_Et	BEC2_Et	AFT_Et
BEC1_sal	(-0.25) n=44							
BEC2_sal	-0.87** n=44	(0.27) n=44						
AFT_sal	-0.45* n=44	-0.67** n=44	0.53** n=44					
LORR_Et	0.83** n=44	(-0.32) n=44	-0.71** n=44	(-0.27) n=44				
BEC1_Et	(-0.33) n=44	0.70** n=44	(0.39) n=44	(-0.32) n=44	(-0.39) n=47			
BEC2_Et	-0.55** n=44	(0.21) n=44	0.63** n=44	(0.30) n=44	-0.58** n=47	(0.37) n=47		
AFT_Et	(-0.05) n=43	-0.56** n=43	(0.05) n=43	0.54** n=43	(-0.03) n=46	-0.75** n=46	(0.35) n=46	
LORR_RT	-0.44* n=44	(-0.08) n=44	(0.40) n=44	(0.38) n=44	(0.14) n=44	(-0.01) n=44	(0.12) n=44	(0.09) n=43

Table 3

Genetic correlations among the LXS RIs between DID and LORR-related measures 24 hours after saline (sal) or ethanol (Et) pretreatment with strain 100 removed (Pearson's r ; n = number of strains). Significant correlations shown in bold-faced type (nominal $p < 0.05$ * , $p < 0.01$ **; multiple comparisons corrected by Bonferroni); non-significant correlations shown in parentheses.

	LORR_sal	BEC1_sal	BEC2_sal	AFT_sal	LORR_Et	BEC1_Et	BEC2_Et	AFT_Et
BEC1_sal	(-0.20) n=43							
BEC2_sal	-0.86** n=43	(0.22) n=43						
AFT_sal	-0.47* n=43	-0.69** n=43	0.56** n=43					
LORR_Et	0.84** n=43	(-0.24) n=43	-0.69** n=43	(-0.31) n=43				
BEC1_Et	(-0.32) n=43	0.69** n=43	(0.37) n=43	(-0.31) n=43	(-0.38) n=46			
BEC2_Et	-0.68** n=43	(0.33) n=43	0.79** n=43	(0.31) n=43	-0.83** n=46	0.44* n=46		
AFT_Et	(-0.14) n=42	-0.52** n=42	(0.17) n=42	0.57** n=42	(-0.20) n=45	-0.76** n=45	(0.26) n=45	
LORR_RT	-0.55** n=43	(0.01) n=43	0.52** n=43	(0.39) n=43	(0.01) n=43	(0.03) n=43	(0.01) n=43	(-0.02) n=42

Genetic correlations among the LXS RIs between DID and LORR-related traits (Pearson's r ; n = number of strains). Significant correlations shown in bold-faced type (nominal $p < 0.05$ *; multiple comparisons corrected by Bonferroni); non-significant correlations shown in parentheses. Strain 100 is not included.

Table 4

	LORR_sal	BEC1_sal	BEC2_sal	AFT_sal	LORR_Et	BEC1_Et	BEC2_Et	AFT_Et	LORR_RT
DID	(-0.30) n=27	(-0.14) n=27	(0.40) n=27	(0.46) n=27	(-0.27) n=29	(-0.39) n=29	(0.28) n=29	0.68** n=28	(0.10) n=27