Published in final edited form as:

Alcohol. 2015 February ; 49(1): 29–36. doi:10.1016/j.alcohol.2014.07.022.

Genotypic and sex differences in anxiety-like behavior and alcohol-induced anxiolysis in high drinking in the dark selected mice

Amanda M. Barkley-Levenson^{a,b,*} and John C. Crabbe^{a,b}

^aDepartment of Behavioral Neuroscience, Oregon Health & Science University, Portland, OR 97239, US

^bPortland Alcohol Research Center, VA Medical Center, Portland, OR 97239, USA

Abstract

Alcohol use disorders (AUDs) and anxiety disorders are highly comorbid in humans. In rodent lines selected for alcohol drinking, differences in anxiety-like behavior have also been seen. The High Drinking in the Dark (HDID) lines of mice have been selectively bred for drinking to intoxication during limited access to alcohol, and these mice represent a genetic model of risk for binge-like drinking. The present studies sought to determine whether these selected lines differ in basal anxiety or in anxiolytic response to alcohol, and if so, whether these differences might contribute to the high drinking phenotype. We also assessed the genetic correlation between alcohol drinking in the dark (DID) and basal anxiety-like behavior using existing inbred strain data. Male and female mice of both HDID replicates (HDID-1 and HDID-2) were tested on an elevated zero maze (EZM) immediately following a DID test. HDID mice of both replicates and sexes showed more time spent in the open arms after drinking alcohol than HS mice, and open-arm time was significantly correlated with blood alcohol concentration. HDID-1 male mice also showed less anxiety-like behavior at baseline (water-drinking controls). In a separate experiment, HDID-1 and HS mice were tested for anxiolytic dose–response to acute alcohol injections. Both genotypes showed increasing time spent in the open arms with increasing alcohol doses, and HDID-1 and female mice had greater open-arm time across all doses. HDID-1 male saline-treated control animals showed lower baseline anxiety-like behavior than the HS control males. Inbred strain data analysis also showed no significant genetic relationship between alcohol DID and anxiety. These findings suggest that selection for drinking to intoxication has not produced systematic changes in anxiety-like behavior or sensitivity to alcohol-induced anxiolysis in the HDID animals, though there is a tendency in the male mice of the first replicate toward reduced basal anxiety-like behavior. Therefore, anxiety state and sensitivity to alcohol's anxiolytic effects do not appear to contribute significantly to the high drinking behavior of the HDID mice under these conditions.

© 2014 Published by Elsevier Inc.

*Corresponding author. Department of Behavioral Neuroscience, Oregon Health & Science University, 3181 SW Sam Jackson Park Rd. L-470, Portland, OR 97239, USA. Tel.: +1 503 220 8262x56675; fax: +1 503 494 6877. barkleya@ohsu.edu (A.M. Barkley-Levenson).

Keywords

Anxiety; Alcohol; Binge drinking; Selected lines; Inbred strains; Behavioral genetics

Introduction

Alcohol use disorders (AUDs) and anxiety disorders have been shown to have a high degree of comorbidity (for review, see Kushner, Abrams, & Borchardt, 2000; Smith & Randall, 2012). Two broad hypotheses that are not mutually exclusive might account for this. Alcohol has significant anxiolytic effects (e.g., Gilman, Ramchandani, Davis, Bjork, & Hommer, 2008), and there is evidence that higher basal anxiety may promote greater alcohol intake which can lead to abuse (Bolton, Cox, Clara, & Sareen, 2006). Another possibility is that anxiety disorders and AUDs may share some of the same underlying genetic risk factors. Family and twin studies have suggested possible common transmission of both anxiety disorders and AUDs, which may represent shared genetic risk (e.g., Merikangas, Risch, & Weissman, 1994; Tambs, Harris, & Magnus, 1997).

There is also evidence from the animal literature to suggest a relationship between anxiety and alcohol consumption. Rats classified as anxious by performance on an elevated plus maze (EPM) voluntarily drink more alcohol in a subsequent test than those classified as non-anxious (Spanagel et al., 1995). Similarly, some rodent lines selected for high vs. low alcohol preference also show innate differences in basal anxiety and sensitivity to alcohol-induced anxiolysis (Colombo et al., 1995; Stewart, Gatto, Lumeng, Li, & Murphy, 1993). These anxiety-related behaviors appear to be correlated responses to selection for high alcohol intake in these lines. However, this relationship is not seen for all rodent lines selected for high vs. low drinking, with some lines showing an opposite relationship or no relationship with drinking (Can, Grahame, & Gould, 2012; Sandbak, Murison, Sarviharju, & Hyytiä, 1998). Another way of examining this relationship is by testing animals selected for anxiety-like behavior for alcohol drinking. Rats selectively bred for high (HAB) or low (LAB) anxiety-related behaviors on an EPM show differences in alcohol preference in a 2-bottle choice test (Henniger, Spanagel, Wigger, Landgraf, & Höltner, 2002). LAB rats have a greater alcohol preference than the HAB rats, but the anxiolytic effect of an injection of alcohol is greater in the HAB rats. Consequently, the possible genetic relationship between alcohol intake and anxiety appears to be complex for both alcohol- and anxiety-related selection phenotypes.

With the exception of the study by Can and colleagues using mice, most of the previous work involves rat lines, and all previous studies have used animals either selected for, or tested on, 2-bottle choice alcohol preference drinking. In the present experiments, we sought to extend these findings to another model animal species and a test of binge-like drinking by determining the relationship between anxiety-like behavior and alcohol drinking in mice selectively bred for drinking to intoxication. The HDID lines of mice were selected for high blood alcohol (ethanol) concentration (BEC) after drinking in the dark (DID), and routinely drink to intoxicating blood levels in a limited-access test (Crabbe et al., 2009, Crabbe et al., 2014). These mice have been extensively behaviorally phenotyped to determine correlated

responses to selection and possible factors promoting their high drinking (for review, see Barkley-Levenson & Crabbe, 2014). Here, we tested whether drinking during the DID test is sufficient to produce alcohol-induced anxiolysis, and whether differences in anxiolytic response to alcohol or basal anxiety may underlie the high drinking phenotype of HDID mice. We also used existing inbred mouse strain data sets to assess the genetic relationship between anxiety-like behavior and alcohol DID.

Materials and methods

Animals and husbandry

Male and female mice of the HDID-1, HDID-2, and HS lines were bred and housed in the Veterinary Medical Unit at the Veterans Affairs Medical Center (Portland, OR, USA). All mice were between 51 and 80 days of age and were experimentally naïve at the start of testing. Mice received *ad libitum* access to food (Purina 5001 chow, LabDiet, St. Louis, MO) and water unless otherwise specified. HDID-1 mice from the 22nd and 27th selection generations were used in Experiment 1 and mice from the 23rd and 28th selection generation were used in Experiment 2. HDID-2 mice from the 19th selection generation were used in Experiment 1. HS/Npt (HS) mice are the starting population from which the HDID lines were selected and are the product of a systematic 8-way inbred strain cross (see Crabbe et al., 2009 for details). These mice are not subjected to selective pressure and represent a genetically heterogeneous population used as a comparator control for the HDID lines. For both Experiments 1 and 2, mice were tested in multiple passes (replicate experiments), with some or all of the sexes and genotypes included in each pass. For Experiment 1, all mice were kept on a 12-h/12-h reverse light/dark cycle with lights off at 9:30 AM. For Experiment 2, one pass of mice was kept on a 12-h/12-h forward light/dark cycle with lights on at 6:00 AM, and a second pass of mice was kept on a reverse light/dark cycle with lights off at 10:30 AM. Both groups were tested at approximately the same time during their circadian light phase, as our laboratory and most others routinely test anxiety-like behavior during the light cycle. All procedures were approved by the local Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Experiment 1: anxiety-like behavior after DID

Seventy-nine male and female mice of the HDID-1, HDID-2, and HS lines were used in this study ($n = 6-9/\text{line/sex/group}$). Mice were tested in 4 passes, with mice of all sexes and genotypes used in each pass except that only female HDID-1 mice were tested in the first pass. At the beginning of the experiment, mice were singly housed and habituated to reverse light/dark for 2 weeks. During this time, mice were given water from polycarbonate bottles with stainless steel sipper tubes attached. After the acclimation period, mice were given a modified version of our standard 2-day DID test. The 2-day DID was chosen because this is the test used in our selection procedure and we were interested in whether alcohol-induced anxiolysis is experienced by these mice under conditions comparable to HDID selection. The DID test is described in detail elsewhere (Crabbe et al., 2009). Briefly, 2–3.5 h after lights off, water bottles were removed and replaced with 10-mL graduated cylinders fitted with stainless steel ball-bearing sipper tubes containing either 20% alcohol or water

depending on group assignment. Start times were staggered by 10-min intervals for every 2 mice to allow for testing on the elevated zero maze (EZM) immediately after drinking on the second day. At the start of the drinking session, fluid levels were recorded and tubes were left in place for 2 h. After 2 h, fluid levels were recorded again and tubes were removed and water bottles were returned to the cages. The next day, the procedure was repeated identically except that tubes were left in place for 4 h. At the end of the 4 h, mice were tested on the EZM in squads of 2 (one mouse per maze) for 5 min. Immediately following the EZM test, a 20 μ L blood sample was taken from the retro-orbital sinus of each alcohol-group mouse to determine BEC.

Experiment 2: dose–response to alcohol-induced anxiolysis

One hundred thirty-three male and female HDID-1 and HS mice ($n = 5\text{--}10/\text{line}/\text{sex}/\text{dose}$) were used in this experiment. Male mice were tested in two passes, and female mice were tested in a single pass. Prior to the start of testing, mice were pseudorandomly assigned to a dose group (saline, 0.5, 1, or 1.5 g/kg alcohol). Behavioral testing in Experiment 2 started at approximately 3 h after lights on for both passes. On the day of testing, mice were moved into the procedure room, weighed, and allowed to habituate for 1 h. Mice were then injected intraperitoneally (i.p.) in squads of 2 with saline or the appropriate dose of alcohol as determined by group assignment. Mice were placed in individual holding cages for 10 min and then given a 5-min test on the EZM.

Experiment 3: genetic correlation of alcohol DID and basal anxiety-like behavior in inbred strains

Our laboratory has previously published 4-day DID consumption data for 23 inbred mouse strains (Crabbe et al., 2012). Intake data for 2-day DID are not presently available for all of these strains, so the 4-day DID data were used for the correlational analysis. In order to correlate DID intake with anxiety-like behavior, inbred strain data were mined from extant data sets in the lab and those available on the Mouse Phenome Database (MPD, The Jackson Laboratory, <http://phenome.jax.org/>). Criteria for selection of anxiety data were: at least 10 strains in common with the DID data set, inclusion of both males and females, and a measure of anxiety-like behavior based on time spent in the anxiogenic region of a test apparatus (e.g., open arms, center region, light compartment). EPM data sets were excluded if the data were reported as time spent in the closed arms, as this could not be converted to a percent of total time in the open arms without knowing center time. Using these criteria, we selected one previously published study from our lab (Milner & Crabbe, 2008) and two data sets from the MPD (Brown1, 2004, and Wahlsten1, 2003). The anxiety measures included were open field (OF) center time (Milner & Crabbe, 2008; Wahlsten1), light–dark box (LDB) light compartment time (Brown1; Milner & Crabbe, 2008), EZM open arm time (Brown1; Milner & Crabbe, 2008), and EPM open arm time (Wahlsten1). All anxiety measures were converted as needed to percent of total time spent in the anxiogenic region. Table 1 summarizes the data sets included in the analysis.

EZM apparatus and testing

Two EZM apparatuses were used, each consisting of a ring-shaped plastic walkway divided into four sections. Two sections of the maze were enclosed on either side by clear plastic walls 11 cm tall (closed arms) and two sections had only a 2-mm tall lip (open arms). Mazes stood 45 cm high and were placed in plastic tubs containing pine chip bedding to prevent injury should an animal fall. Testing was conducted under dim lighting conditions with light levels of approximately 15 lux in the center of each EZM. Mice were tested on both mazes concurrently, with an opaque barrier placed between the mazes so that the mice could not see each other. Mazes were videotaped from above and video was scored by experimenters blinded to treatment and genotype. Variables recorded were the time spent in the open arms as a percent of the total test time and number of line crosses. Eight lines were overlaid onto the video image of the maze such that each arm was divided into three equal segments by the lines. A line crossing was recorded whenever all four feet of an animal crossed the line and this number was used as a measure of locomotor activity. Open arm time was recorded starting when all four feet of a mouse had crossed into an open arm and ending when all four feet of the mouse had crossed back into a closed arm. Percent time spent in the open arms was used as a measure of anxiety-like behavior, with more time in the open arms indicating less anxiety. Mice were placed in the EZM at the start of the test in an open arm facing a closed arm. Between each subject, the floor and walls of the mazes were sprayed with 10% isopropyl alcohol and wiped down to eliminate any odor cues.

Drugs

Alcohol (Decon Laboratories Inc., King of Prussia, PA, USA; 20% v/v) for Experiment 1 was prepared using 100% alcohol diluted with tap water. Alcohol for Experiment 2 was diluted with 0.9% saline (20% v/v) and administered at a 0.5, 1.0, or 1.5 g/kg dose. Saline injections were given at a volume of 10 mL/kg.

Statistical analyses

Statistical analyses were carried out in Systat (version 13; Systat Software, Inc., Chicago, IL, USA). EZM variables were analyzed using multifactorial analysis of variance (ANOVA) with between-group factors of sex, genotype, and fluid group/dose group depending on experiment. Statistically significant interactions were followed up with lower-order ANOVAs, and significant main effects were followed up with Tukey HSD *post hoc* analyses where appropriate. Alcohol drinking data were converted to a g/kg body weight dose and water drinking data were converted to mL/kg. Day 2 intake data were analyzed separately by fluid type by two-way ANOVAs with factors of genotype and sex, as were BEC data. Linear regression was used to correlate BEC with open arm time and line crossings in Experiment 1. In Experiment 3, strain means were computed for each anxiety measure and Day 4 DID alcohol intake. Pearson correlations were used to assess the relationship between alcohol DID and each of the anxiety behaviors, and between anxiety behaviors within and between data sets. The significance level was set at $\alpha = 0.05$ unless otherwise specified.

Results

In Experiment 2, one female HS mouse from the 0.5 g/kg group was given an incorrect dose of alcohol and was excluded from the analysis.

Experiment 1: anxiety-like behavior after DID

Fig. 1 shows the Day 2 alcohol intake and BEC for each line and sex. Alcohol intake showed only a main effect of line [$F(2,34) = 3.976, p = 0.028$], and *post hoc* analyses showed significantly greater intake by the HDID-2 mice than the HS mice ($p = 0.025$). For all other main effects and interactions, $F \leq 1.521$. Analysis of BEC data also revealed only a main effect of line [$F(2,34) = 19.669, p < 0.001$] and *post hoc* analyses showed that both HDID-1 and HDID-2 mice had significantly greater BECs than HS mice ($p < 0.001$ for both). There was no main effect of sex or significant interaction with sex for either intake or BEC. From the EZM, we first analyzed the percent of total time spent in the open arms. There was a main effect of line [$F(2,66) = 4.583, p = 0.014$], and a significant line $\times$ sex $\times$ treatment interaction [$F(2,66) = 3.548, p = 0.034$]. Because of our interest in assessing both basal anxietylike behavior, as well as anxiolytic response to alcohol, we next analyzed the water-drinking group alone by two-way ANOVA. This showed a significant line $\times$ sex interaction [$F(2,31) = 3.709, p = 0.036$]. Results are shown separately for males and females in Fig. 2. One-way ANOVA and subsequent *post hoc* analyses for each sex showed that this interaction was due to a main effect of line only in the male mice [$F(2,14) = 4.907, p = 0.024$]. HDID-1 water-drinking male mice had significantly more time spent in the open arms than HS mice ($p = 0.037$; Fig. 2, left), and trended toward more time in the open arms than HDID-2 mice as well ($p = 0.052$). Water-drinking female mice (Fig. 2, right) did not show any significant differences in open-arm time across genotypes.

Next, to determine the anxiolytic effect of alcohol in each group, we performed one-way ANOVAs with the factor of drinking group for each genotype and sex. A significant anxiolytic effect of drinking was found only in the HDID-1 females [$F(1,16) = 5.734, p = 0.029$] and HDID-2 males [$F(1,9) = 6.09, p = 0.036$], with both groups showing more time spent in the open arms by alcohol-drinking mice than water-drinking mice. All other groups did not have a significant difference in open-arm time between the water and alcohol animals ($F \leq 0.331$ for all; see Fig. 2).

For line crossings, analysis showed main effects of treatment [$F(1,67) = 14.637, p < 0.001$] and sex [$F(1,67) = 4.376, p = 0.04$], and a significant treatment $\times$ line interaction [$F(2,67) = 3.532, p = 0.035$]. In order to facilitate comparison of group differences in alcohol-induced locomotor stimulation with anxiety-like responses, we employed the same strategy as the anxiolysis analysis above and used one-way ANOVA with a factor of drinking group for males and females of each genotype. HDID-2 males (Fig. 3, left) and females (Fig. 3, right) and HDID-1 females (Fig. 3, right) showed significant alcohol-induced locomotor stimulation [$F(1,9-17) = 7.091, p = 0.016$]. Both sexes of HS mice and HDID-1 male mice did not show significant alcohol effects on locomotor activity ($F \leq 1.751$).

Regression analysis of the alcohol-drinking groups combined across all genotypes and sexes showed a moderately strong positive correlation between BEC and both percent time in the

open arms ($r = 0.556$, $n = 40$, $p < 0.001$; see Fig. 4, left) and number of line crosses ($r = 0.623$, $n = 40$, $p < 0.001$; see Fig. 4, right).

Experiment 2: dose–response to alcohol-induced anxiolysis

Main effects were found for dose group [$F(3,11) = 10.835$, $p < 0.001$], line [$F(1,111) = 12.246$, $p = 0.001$], and sex [$F(1,111) = 11.270$, $p = 0.001$], and there were no significant interactions. Due to the lack of significant interactions with sex ($F = 1.284$ for all), Fig. 5 shows the percent time spent in the open arms collapsed on sex. *Post hoc* analyses showed that 1.5 g/kg-treated animals spent significantly more time in the open arms than all other groups ($p = 0.001$ – 0.029), and that 1 g/kg-treated animals spent more time in the open arms than the saline group ($p = 0.04$). HDID-1 mice spent more time in the open arms than HS mice ($p = 0.001$), and female mice spent more time in the open arms than male mice ($p = 0.001$). Due to the results from the water group in Experiment 1, we had an *a priori* interest in whether HDID-1 and HS male mice in the saline group would also show a similar genotypic difference in baseline anxiety-like behavior. Thus, we decided to also analyze open arm time in just the male saline-treated animals. Analysis showed a main effect of line, with HDID-1 male mice spending significantly more time in the open arms than male HS mice [$F(1,17) = 12.557$, $p = 0.002$] (Fig. 5 inset).

For line crossings, there was a main effect of dose group [$F(3,111) = 10.63$, $p < 0.001$] and a trend toward a main effect of line [$F(1,111) = 3.024$, $p = 0.085$]. There were no significant interactions, and Fig. 6 shows the number of line crosses made by each group collapsed on sex. *Post hoc* analyses found that the 1.5 g/kg group made significantly more line crosses than the saline and 0.5 g/kg groups, and that the 1 g/kg group made more line crosses than the saline group ($p = 0.009$ for all).

Experiment 3: genetic correlation of alcohol DID and basal anxietylike behavior in inbred strains

Table 2 shows the correlation matrix for g/kg alcohol intake during DID and all measures of anxiety-like behavior from the selected data sets. No anxiety variable showed a significant correlation with alcohol DID across strains (Table 2, first column). Several anxiety measures showed significant strain correlations with each other both within data sets and between data sets. These correlations are shown in boldface in Table 2.

Discussion

In these experiments, we found that selective breeding for high BECs after limited-access drinking has resulted in replicate- and sex-specific effects on basal anxiety-like behavior and alcohol-induced anxiolysis. In Experiment 1, HDID-1 female mice and HDID-2 male mice showed reductions in anxiety-like behavior after a 4-h alcohol DID session compared to water-drinking controls. These differences appear to be due to the pharmacological effects of alcohol since BEC showed a positive correlation with the anxiety measure used. That is, the higher the BEC achieved following DID, the more open arm time an animal showed. Thus, the line differences in anxiety-like behavior seen following alcohol DID are presumably due to the difference in intake between the HDID and HS lines. We have shown

previously that alcohol intake during DID is sufficient to produce behavioral intoxication as assessed by impairment on the balance beam test (Crabbe et al., 2009), but this is the first study to show DID-induced anxiolysis in these mice. The absence of an apparent anxiolytic effect of alcohol in the HDID-1 male mice is presumably the result of a ceiling effect due to their relatively non-anxious state at baseline (see below). It is less clear, however, why there was no apparent effect of alcohol in the HDID-2 females, as these mice showed normal baseline performance coupled with high alcohol intake. One possibility is that female mice of this genotype have a reduced sensitivity to the anxiolytic effects of alcohol compared to HDID-1 females and HDID-2 males. The HDID-2 mice have not yet been tested for anxiolytic response to acute alcohol injections, and repeating Experiment 2 using the HDID-2 mice might help determine whether females of this line truly show blunted anxiolytic sensitivity.

HDID-1 females and HDID-2 mice of both sexes made more line crosses after alcohol drinking than did the HS mice, indicating that in HDID mice, alcohol intake during DID results in significant locomotor stimulation as well. Because performance on the EZM is activity-dependent, locomotor behavior and anxiety behavior can be closely related in this task (Kliethermes, 2005). Consequently, an increase in locomotor activity could potentially explain an apparent change in anxiety. In this experiment, however, the line differences in anxiety-like behavior do not consistently parallel the activity differences, and therefore are unlikely to be solely a product of changes in locomotion. For example, some groups showing alcohol stimulation (e.g., HDID-2 females) do not also show alcohol-induced anxiolysis. This is supported by the results from Experiment 2, where genotypic differences in anxiety level are seen in the absence of a line difference in activity.

Results from the water-drinking control group in Experiment 1 showed genotypic differences in basal anxiety-like behavior in the males only. Specifically, HDID-1 males showed lower anxiety-like behavior than the HS mice and trended toward a difference from the HDID-2 male mice as well. HDID-2 and HS male mice, and female mice of all three genotypes, did not differ in baseline anxiety levels. In Experiment 2, saline-treated HDID-1 male mice also spent more time in the open arms than saline-treated HS male mice. It appears, therefore, that HDID-1 male mice are unique among these groups in their relatively non-anxious basal state. However, all of the groups spent, on average, at least 30% of the time in the open arms, suggesting that none of the sexes/genotypes have high baseline anxiety as assessed by this task. Previous studies with rats and mice have shown that sex differences in baseline anxiety are influenced by both task and genotype (e.g., Johnston & File, 1991; O'Leary, Gunn, & Brown, 2013; Wilson, Burghardt, Ford, Wilkinson, & Primeaux, 2004), and a different anxiety test could therefore produce different results. Because of the relatively low basal levels of anxiety, testing the animals on a more anxiogenic procedure or on a related behavior such as stress reactivity could better elucidate the replicate- and sex-specific differences among these lines. It should be noted that in these studies we did not control for estrous state in the female mice. Anxiety-like behavior has been shown to fluctuate throughout the estrous cycle in rats (Frye, Petralia, & Rhodes, 2000), and it is possible that if we were to match females for estrous, we would see a different pattern of sex and genotype effects.

In Experiment 2, HDID-1 mice showed generally lower anxiety-like behavior on the EZM than HS mice regardless of alcohol dose, and both genotypes showed increasing anxiolysis with higher doses of alcohol. There is the possibility of a ceiling effect in the HDID-1s, as they started at a relatively low level of anxiety and had little room to show any further anxiolytic effect of alcohol. Due to the nature of the EZM, 50% of time spent in the open arms is the maximum measure for anxiolysis, as this indicates equal preference for (or an inability to distinguish between) the open and closed arms. Consequently, it is difficult to determine whether there were genotypic differences in alcohol-induced anxiolysis between HDID-1 mice and HS from these data. It might be possible to manipulate the parameters of the EZM test (e.g., brighter lighting, shorter open arm lip height) to make the test more anxiogenic in order to produce greater baseline anxiety-like behavior in the HDID-1 mice (Wahlsten, Rustay, Metten, & Crabbe, 2003). With genotypes equated at baseline, it could then be determined more conclusively if there are differences in anxiolytic response to alcohol. From the current study, however, it appears that both HDID-1 and HS mice do show similar dose-dependent decreases in anxiety-like behavior with increasing doses of alcohol.

HDID-1 mice, particularly males, may be less sensitive to the inherent anxiogenic characteristics of the EZM than HS mice. These mice have also been shown to be less sensitive to the aversive effects of alcohol than HS mice in a conditioned taste aversion test that employed only males (Barkley-Levenson, Cunningham, Smitasin, & Crabbe, 2013), and could potentially have a general deficit in sensitivity to aversive stimuli. Rather than a high anxiety/ stress reactive and high drinking phenotype, the HDID-1 mice may instead show more similarity to novelty-seeking or sensation-seeking at-risk human drinkers. Both novelty seeking and sensation seeking have been demonstrated to be associated with risk for AUDs and excessive drinking (Lange, Kampov-Polevoy, & Garbutt, 2010; Manzo et al., 2014; Noël et al., 2011), and it would be interesting to test the HDID-1 mice on a measure of novelty seeking to determine whether this behavior is associated with binge-like drinking in these animals.

Work from other rodent lines selected for alcohol intake measures has shown varied relationships with anxiety-like behavior and alcohol-induced anxiolysis. Some lines, such as the Sardinian and Indiana alcohol-preferring rats, show higher baseline anxiety and greater anxiolytic effect of alcohol than the non-preferring lines (Colombo et al., 1995; Roman et al., 2012; Stewart et al., 1993). In other selected lines, such as the high- and low-alcohol drinking rats and the high- and low-alcohol preferring mice, differences in anxiety-like behavior are not readily apparent or show replicate- and task-dependent differences only (Badia-Elder, Stewart, Powrozek, Murphy, & Li, 2003; Can et al., 2012). Most similar to our findings with the HDID-1 male mice are the Finnish Alko Alcohol rats. These lines show reduced anxiety and enhanced exploratory behavior and risk taking in the high-drinking rats compared to the low-drinking, Alko Non-Alcohol rats (Roman et al., 2012). The HDID-1 male finding is also consistent with the results of the drinking experiment with anxiety-selected lines, where LAB rats drank more alcohol than HAB rats, but showed no significant alcohol anxiolysis (Henniger et al., 2002). Our data appear to continue the trend in the literature that selection for high-drinking behaviors does not reliably produce consistent changes in anxiety.

Analysis of the data from inbred strains in Experiment 3 also supports a lack of genetic association between basal anxiety-like behavior and DID. None of the anxiety variables from the data sets analyzed showed a significant correlation with alcohol DID across strains. Anxiety data used in these analyses included three different tasks and were collected in three different laboratories, and the failure of any of these variables to correlate significantly with DID provides strong support for different genetic factors underlying alcohol DID and basal anxiety-like behavior in mice. However, the anxiety measures were all based on avoidance of an anxiogenic region, and a different result may be found with other anxiety-related behaviors that are not captured by these variables (e.g., fecal boli count, defensive burying, stretch attend positions).

It should be noted that different assays of anxiety-like behavior may not always produce similar results and that a genotype's assessed basal anxiety is likely task-dependent (e.g., Bouwknecht & Paylor, 2002; Griebel, Belzung, Perrault, & Sanger, 2000; Milner & Crabbe, 2008). Consequently, future experiments could look at the HDID lines on a different test of anxiety-like behavior, such as an open field apparatus, to determine whether the reduced basal anxiety-like behavior of HDID-1 male mice generalizes across tests and whether anxiolytic response to alcohol still does not differ from HS. From the present data, however, it seems that selection for drinking to intoxication has not produced systematic changes in alcohol-induced anxiolysis, and that decreases in basal anxiety-like behavior may only be related to selection in a sex- and replicate-specific way. Furthermore, alcohol dependence can itself produce increases in anxiety (e.g., Breese, Overstreet, Knapp, & Navarro, 2005; Schuckit & Hesselbrock, 1994) and it remains to be seen whether post-dependent anxiety-like behavior is elevated differentially between the HDID and HS lines.

Uncited references

Brown et al., Wahlsten and Crabbe.

Acknowledgments

The authors would like to thank Jason Schlumbohm, Wyatt Hack, and Marissa Patterson for their assistance with data collection for Experiment 1, and Andy Cameron for analyzing the blood samples for BEC determination. These studies were supported by NIH-NIAAA grants AA13519, AA10760, AA07702, and AA020245; a grant from the U.S. Department of Veterans Affairs; and the Saturday Academy Apprenticeships in Science and Engineering program. A.M.B.-L. was supported by NIH-NIAAA grants AA007468 and AA02009, the Achievement Rewards for College Scientists Foundation, and an OHSU Graduate Research Scholar fellowship.

References

- Badia-Elder NE, Stewart RB, Powrozek TA, Murphy JM, Li TK. Effects of neuropeptide Y on sucrose and ethanol intake and on anxiety-like behavior in high alcohol drinking (HAD) and low alcohol drinking (LAD) rats. *Alcoholism: Clinical and Experimental Research*. 2003; 27:894–899.
- Barkley-Levenson AM, Crabbe JC. High drinking in the dark mice: a genetic model of drinking to intoxication. *Alcohol*. 2014; 48:217–223. [PubMed: 24360287]
- Barkley-Levenson AM, Cunningham CL, Smitasin PJ, Crabbe JC. Rewarding and aversive effects of ethanol in high drinking in the dark selectively bred mice. *Addiction Biology*. 2013 Epub ahead of print.

- Bolton J, Cox B, Clara I, Sareen J. Use of alcohol and drugs to self-medicate anxiety disorders in a nationally representative sample. *The Journal of Nervous and Mental Disease*. 2006; 194:818–825. [PubMed: 17102705]
- Bouwknicht JA, Paylor R. Behavioral and physiological mouse assays for anxiety: a survey in nine mouse strains. *Behavioural Brain Research*. 2002; 136:489–501. [PubMed: 12429412]
- Breese GR, Overstreet DH, Knapp DJ, Navarro M. Prior multiple ethanol withdrawals enhance stress-induced anxiety-like behavior: inhibition by CRF1- and benzodiazepine-receptor antagonists and a 5-HT1a-receptor agonist. *Neuropsychopharmacology*. 2005; 30:1662–1669. [PubMed: 15726114]
- Brown, RE.; Gunn, RK.; Schellinck, HM.; Wong, AA.; O'Leary, TP. MPD: Brown1 Mouse phenome database web site. The Jackson Laboratory; Bar Harbor, Maine USA: Anxiety, exploratory behavior, and motor activity in 14 inbred strains of mice. <http://phenome.jax.org>
- Can A, Grahame NJ, Gould TD. Affect-related behaviors in mice selectively bred for high and low voluntary alcohol consumption. *Behavior Genetics*. 2012; 42:313–322. [PubMed: 21989731]
- Colombo G, Agabio R, Lobina C, Reali R, Zocchi A, Fadda F, et al. Sardinian alcohol-preferring rats: a genetic animal model of anxiety. *Physiology & Behavior*. 1995; 57:1181–1185. [PubMed: 7652041]
- Crabbe JC, Metten P, Huang LC, Schlumbohm JP, Spence SE, Barkley-Levenson AM, et al. Ethanol withdrawal-associated drinking and drinking in the dark: common and discrete genetic contributions. *Addiction Genetics*. 2012; 1:3–11. [PubMed: 24533180]
- Crabbe JC, Metten P, Rhodes JS, Yu CH, Brown LL, Phillips TJ, et al. A line of mice selected for high blood ethanol concentrations shows drinking in the dark to intoxication. *Biological Psychiatry*. 2009; 65:662–670. [PubMed: 19095222]
- Frye CA, Petralia SM, Rhodes ME. Estrous cycle and sex differences in performance on anxiety tasks coincide with increases in hippocampal progesterone and 3alpha,5alpha-THP. *Pharmacology, Biochemistry, and Behavior*. 2000; 67:587–596.
- Gilman JM, Ramchandani VA, Davis MB, Bjork JM, Hommer DW. Why we like to drink: a functional magnetic resonance imaging study of the rewarding and anxiolytic effects of alcohol. *The Journal of Neuroscience*. 2008; 28:4583–4591. [PubMed: 18448634]
- Griebel G, Belzung C, Perrault G, Sanger DJ. Differences in anxiety-related behaviours and in sensitivity to diazepam in inbred and outbred strains of mice. *Psychopharmacology (Berl)*. 2000; 148:164–170. [PubMed: 10663431]
- Henniger MS, Spanagel R, Wigger A, Landgraf R, Hölter SM. Alcohol self-administration in two rat lines selectively bred for extremes in anxiety-related behavior. *Neuropsychopharmacology*. 2002; 26:729–736. [PubMed: 12007743]
- Johnston AL, File SE. Sex differences in animal tests of anxiety. *Physiology & Behavior*. 1991; 49:245–250. [PubMed: 2062894]
- Kliethermes CL. Anxiety-like behaviors following chronic ethanol exposure. *Neuroscience and Biobehavioral Reviews*. 2005; 28:837–850. [PubMed: 15642625]
- Kushner MG, Abrams K, Borchardt C. The relationship between anxiety disorders and alcohol use disorders: a review of major perspectives and findings. *Clinical Psychology Review*. 2000; 20:149–171. [PubMed: 10721495]
- Lange LA, Kampov-Polevoy AB, Garbutt JC. Sweet liking and high novelty seeking: independent phenotypes associated with alcohol-related problems. *Alcohol and Alcoholism*. 2010; 45:431–436. [PubMed: 20663854]
- Manzo L, Gómez MJ, Callejas-Aguilera JE, Donaire R, Sabariego M, Fernández-Teruel A, et al. Relationship between ethanol preference and sensation/ novelty seeking. *Physiology & Behavior*. 2014; 133:53–60. [PubMed: 24825783]
- Merikangas KR, Risch NJ, Weissman MM. Comorbidity and co-transmission of alcoholism, anxiety and depression. *Psychological Medicine*. 1994; 24:69–80. [PubMed: 8208896]
- Milner LC, Crabbe JC. Three murine anxiety models: results from multiple inbred strain comparisons. *Genes, Brain, and Behavior*. 2008; 7:496–505.
- Noël X, Brevers D, Bechara A, Hanak C, Kornreich C, Verbanck P, et al. Neurocognitive determinants of novelty and sensation-seeking in individuals with alcoholism. *Alcohol and Alcoholism*. 2011; 46:407–415. [PubMed: 21596760]

- O'Leary TP, Gunn RK, Brown RE. What are we measuring when we test strain differences in anxiety in mice? *Behavior Genetics*. 2013; 43:34–50. [PubMed: 23288504]
- Roman E, Stewart RB, Bertholomey ML, Jensen ML, Colombo G, Hyytiä P, et al. Behavioral profiling of multiple pairs of rats selectively bred for high and low alcohol intake using the MCSF test. *Addiction Biology*. 2012; 17:33–46. [PubMed: 21521426]
- Sandbak T, Murison R, Sarviharju M, Hyytiä P. Defensive burying and stress gastric erosions in alcohol-preferring AA and alcohol-avoiding ANA rats. *Alcoholism: Clinical and Experimental Research*. 1998; 22:2050–2054.
- Schuckit MA, Hesselbrock V. Alcohol dependence and anxiety disorders: what is the relationship? *The American Journal of Psychiatry*. 1994; 151:1723–1734. [PubMed: 7977877]
- Smith JP, Randall CL. Anxiety and alcohol use disorders: comorbidity and treatment considerations. *Alcohol Research: Current Reviews*. 2012; 34:414–431. [PubMed: 23584108]
- Spanagel R, Montkowski A, Allingham K, Stöhr T, Shoaib M, Holsboer F, et al. Anxiety: a potential predictor of vulnerability to the initiation of ethanol self-administration in rats. *Psychopharmacology (Berl)*. 1995; 122:369–373. [PubMed: 8657835]
- Stewart RB, Gatto GJ, Lumeng L, Li TK, Murphy JM. Comparison of alcohol-preferring (P) and nonpreferring (NP) rats on tests of anxiety and for the anxiolytic effects of ethanol. *Alcohol*. 1993; 10:1–10. [PubMed: 8095393]
- Tambs K, Harris JR, Magnus P. Genetic and environmental contributions to the correlation between alcohol consumption and symptoms of anxiety and depression. Results from a bivariate analysis of Norwegian twin data. *Behavior Genetics*. 1997; 27:241–250. [PubMed: 9210795]
- Wahlsten, D.; Crabbe, JC. MPD:10852 Mouse Phenome Database web site. The Jackson Laboratory; Bar Harbor, Maine USA: Survey of motor activity, behavior, and forebrain morphometry in 21 inbred strains of mice across two laboratories. <http://phenome.jax.org>
- Wahlsten D, Rustay NR, Metten P, Crabbe JC. In search of a better mouse test. *Trends in Neurosciences*. 2003; 26:132–136. [PubMed: 12591215]
- Wilson MA, Burghardt PR, Ford KA, Wilkinson MB, Primeaux SD. Anxiolytic effects of diazepam and ethanol in two behavioral models: comparison of males and females. *Pharmacology, Biochemistry, and Behavior*. 2004; 78:445–458.

Fig. 1.

Day 2 ethanol intake in g/kg body weight and blood ethanol concentration (BEC) for males (left panels) and females (right panels) of each genotype in the ethanol-drinking group.

Means $\pm$ SEM shown. There was a statistically significant effect of line for both intake and BEC. BEC bar for HS females is absent as no animals in this group had a measurable BEC. $n = 6-9/\text{sex}/\text{line}$.

Fig. 2. Percent time spent in the open arms by males (left) and females (right) of each genotype and drinking group. Means $\pm$ SEM shown. * indicates statistically significant difference from corresponding water group and † indicates statistically significant difference from corresponding HS group ($p < 0.05$). $n = 6-9$ /sex/group/line.

Fig. 3. Number of line crosses made by males (left) and females (right) of each genotype and drinking group. Means $\pm$ SEM shown. * indicates statistically significant difference from corresponding water group ($p < 0.05$). $n = 6-9$ /sex/group/line.

Fig. 4. Percent time spent in the open arms (left) displayed versus blood ethanol concentration (BEC) immediately after testing on the zero maze and line crossings versus BEC (right). All ethanol-drinking animals are shown ($n = 12-17/\text{line}$). Linear regression lines are depicted.

Fig. 5. Percent time spent in the open arms across alcohol doses collapsed on sex. Inset shows males only of the saline-treated control group. Means $\pm$ SEM shown. * indicates statistically significant difference from saline group and † indicates statistically significant difference from 0.5 g/kg alcohol group ($p < 0.05$). $n = 5-10$ /sex/line/dose.

Fig. 6.

Number of line crosses made by each genotype across alcohol dose groups collapsed on sex. Means $\pm$ SEM shown. * indicates statistically significant difference from saline group and † indicates statistically significant difference from 0.5 g/kg alcohol group ($p < 0.05$). $n = 5-10/\text{sex/line/dose}$.

Table 1

Inbred strain panels tested for anxiety-like behavior included in Experiment 3.

Anxiety data set	Anxiety variable	Number of strains in common with DID data	Strains included for correlation analysis
Brown1, EZM	Percent of total time spent in open arms	10	129S1/SvImJ, A/J, AKR/J, BALB/cByJ, BALB/cJ, CAST/EiJ, C57BL/6J, DBA/2J, FVB/NJ, SJL/J
Brown1, LDB	Percent of total time spent in light	11	129S1/SvImJ, A/J, AKR/J, BALB/cByJ, BALB/cJ, C3H/HeJ, CAST/EiJ, C57BL/6J, DBA/2J, FVB/NJ, SJL/J
Milner & Crabbe, EZM	Percent of total time spent in open arms	14	129S1/SvImJ, A/J, AKR/J, BALB/cByJ, C3H/HeJ C57BL/6J, C57 L/J, CBA/J, DBA/2J, FVB/NJ, NZB/B1NJ, PL/J, SJL/J, SWR/J
Milner & Crabbe, LDB	Percent of total time spent in light	14	129S1/SvImJ, A/J, AKR/J, BALB/cByJ, C3H/HeJ C57BL/6J, C57L/J, CBA/J, DBA/2J, FVB/NJ, NZB/B1NJ, PL/J, SJL/J, SWR/J
Milner & Crabbe, OF	Percent of total time spent in center (>7.7 cm from wall)	14	129S1/SvImJ, A/J, AKR/J, BALB/cByJ, C3H/HeJ C57BL/6J, C57L/J, CBA/J, DBA/2J, FVB/NJ, NZB/B1NJ, PL/J, SJL/J, SWR/J
Wahlsten1, EPM	Percent of total time spent in open arms	16	129S1/SvImJ, A/J, AKR/J, BALB/cByJ, BTBR T + tf/J, C3H/HeJ, CAST/EiJ, C57BL/6J, C57L/J, DBA/2J, FVB/NJ, NOD/ShiLtJ, NZB/B1NJ, PL/J, SJL/J, SWR/J
Wahlsten1, OF	Percent of total time spent in center (>10 cm from wall)	16	129S1/SvImJ, A/J, AKR/J, BALB/cByJ, BTBR T + tf/J, C3H/HeJ, CAST/EiJ, C57BL/6J, C57L/J, DBA/2J, FVB/NJ, NOD/ShiLtJ, NZB/B1NJ, PL/J, SJL/J, SWR/J

EZM: elevated zero maze; EPM: elevated plus maze; LDB: light–dark box; OF: open field.

Table 2

Correlation matrix of alcohol DID and anxiety variables for inbred mouse strains.

	DID 4 h g/kg	Brown EZM	Milner EZM	Wahlsten EPM	Brown LDB	Milner LDB	Milner OF
Brown EZM	0.014						
Milner EZM	-0.118	-0.315					
Wahlsten EPM	0.114	0.010	-0.211				
Brown LDB	0.329	-0.233	-0.060	0.664			
Milner LDB	0.065	0.602	0.056	-0.143	0.268		
Milner OF	0.165	0.569	-0.871	0.142	0.123	0.305	
Wahlsten OF	0.357	-0.379	-0.137	0.543	0.315	-0.628	0.058

All anxiety variables represent percent time spent in the anxiogenic region of the apparatus. Boldface indicates statistically significant correlation. Abbreviations: DID: drinking in the dark; EZM: elevated zero maze; EPM: elevated plus maze; LDB: light-dark box; OF: open field.