Published in final edited form as:

Alcohol Clin Exp Res. 2012 July ; 36(7): 1162–1170. doi:10.1111/j.1530-0277.2012.01735.x.

Ethanol Sensitivity in High Drinking in the Dark Selectively Bred Mice

John C. Crabbe, Lauren C. Kruse, Alexandre M. Colville, Andy J. Cameron, Stephanie E. Spence, Jason P. Schlumbohm, Lawrence C. Huang, and Pamela Metten

Portland Alcohol Research Center, Department of Behavioral Neuroscience, Oregon Health & Science University, and VA Medical Center, Portland, Oregon 97239 USA

Abstract

Background—Mouse lines are being selectively bred in replicate for high blood ethanol concentrations (BECs) achieved after a short period of ethanol drinking early in the circadian dark phase. High Drinking in the Dark -1 (HDID-1) mice were in selected generation S18, and the replicate HDID-2 line in generation S11.

Methods—To determine other traits genetically correlated with high DID, we compared naive animals from both lines with the unselected, segregating progenitor stock, HS/Npt. Differences between HDID-1 and HS would imply commonality of genetic influences on DID and these traits.

Results—HDID-1 showed less basal activity, greater ethanol stimulated activity and greater sensitivity to ethanol-induced foot slips than HS. They showed lesser sensitivity to acute ethanol hypothermia and longer duration loss of righting reflex (LORR) than HS. HDID-1 and control HS lines did not differ in sensitivity on two measures of intoxication, the balance beam and the accelerating rotarod. None of the acute response results could be explained by differences in ethanol metabolism. HDID-2 mice differed from HS on some, but not all of the above responses.

Conclusions—These results show that some ethanol responses share common genetic control with reaching high BECs after DID, a finding consistent with other data regarding genetic contributions to ethanol responses.

Keywords

Selected mouse lines; High Drinking in the Dark (HDID) mice; activity; hypothermia; loss of righting reflex; ataxia; genetic correlation

INTRODUCTION

Risk for alcohol dependence (alcoholism) shows substantial genetic contributions in human studies, and genes appear to explain approximately 60% of individual differences in risk in human twin studies (Goldman and Ducci, 2007). Alcohol dependence is a complex trait, and no genetic animal model addresses all the diagnostic features of the disease (Crabbe, 2008). Rather, animal experiments target specific symptoms that are deemed important, and most study the tendency of rats or mice to elect to drink alcohol solutions. Most employ preference drinking tests, where an animal chooses between a 10–15% ethanol (EtOH) solution or water. While animal studies have taught us a great deal about the neurobiology of EtOH and genetic contributions to individual differences in EtOH-related behaviors, the drinking studies have generally not overcome one issue. Even animals genetically

predisposed to drink EtOH rarely drink enough to achieve intoxicating blood ethanol concentrations (BECs) [for review, see (Crabbe et al., 2010b)].

In an attempt to provide an additional genetic animal model that addresses this limitation, we have been breeding mice selectively for the BEC attained after a 4 hr period of access to a 20% EtOH solution offered early during their circadian dark phase. This test has come to be called Drinking in the Dark (DID), and genotypes that genetically prefer EtOH solutions will drink enough to become intoxicated (Rhodes et al., 2005, 2007). High Drinking in the Dark-1 mice were selected from a segregating stock, HS/Npt (HS), and after 9 generations of selection (S9) were found to reach BECs after drinking that led to behavioral intoxication on a balance beam (Crabbe et al., 2009). A replicate line, HDID-2, has also been bred and the course of response to selection in both lines is nearly identical (Crabbe et al., 2010b). One way that HDID-1 mice achieve higher BECs at the end of a drinking period is by drinking more EtOH solution, because g/kg intake has approximately doubled while BEC has tripled. Because selection has only been based on BEC, this indicates pleiotropic gene effects on drinking, and the substantial genetic correlation between the two traits is consistent with other evidence (Rhodes et al., 2007).

Because genes influencing DID BEC are expected to have other pleiotropic effects, we are beginning to characterize the selected lines for other responses to EtOH. Strain mean DID BEC and two bottle preference were significantly correlated in earlier studies (Rhodes et al., 2007), but when HDID-1 mice were compared with HS for two-bottle EtOH preference, they did not differ significantly (Crabbe et al., 2011). However, after weeks of access to 15% EtOH vs water during limited duration sessions, HDID-1 mice drank modestly more than HS. HAP-1 mice, selected for high two bottle preference drinking, drank more than the LAP-1 low preference line in a DID test (Grahame et al 1999). This suggests that some genes affecting DID also play a limited role in other forms of EtOH drinking. HDID-1 and HS mice were also tested for preference drinking of two concentrations each of sucrose, saccharin, and quinine, and the genotypes showed dose-dependent (but equivalent) preference for the former two and avoidance of the latter tastant. HS mice drank significantly more total fluid per kg body weight than HDID-1 across most alcohol concentrations tested, but the genotypes did not differ in body weight at the beginning or the end of the test for either sex (Crabbe et al, 2011).

“Low level of response” to EtOH during an alcohol challenge has been suggested to be a genetic predictor of alcohol dependence diagnosis risk in human studies (Schuckit et al., 2009; Schuckit and Smith, 2001). However, the particular methods employed to assess low level of response can affect this relationship [for reviews, see (Crabbe et al. 2010a; Newlin and Thomson, 1990). Most human subjects studies have concentrated on measures of subjective intoxication or mood state (e.g., subjective “high”), and on a measure of body sway. Schuckit’s group measures body sway by attaching the subject to a harness with pulleys. Body sway is detected by magnetic sensors and converted to an index of low or high level of response by summing anterior/posterior and lateral sway (Schuckit, 1985).

Animal studies of EtOH have explored many features of the initial sensitivity of naive organisms to a first alcohol challenge and have found significant genetic contributions to individual differences in level of response. Responses to EtOH in rodents are dose-dependent (as they are in humans), and differ in certain respects between rats and mice. Mice typically respond to low-dose EtOH with locomotor stimulation, and this response is sometimes taken as a surrogate for EtOH’s euphorogenic effects in humans (Phillips, 1993). However, the genetic contributions to stimulation in mice do not robustly predict two-bottle preference drinking (Crabbe et al., 2010a). Most rat genotypes respond to low-dose EtOH with only modest locomotor stimulation, and in that species, genetic studies generally

support a positive genetic correlation between EtOH stimulation and preference drinking (Crabbe et al., 2010a; Rodd et al., 2004). Thus, if we take preference drinking as a model of alcohol abuse, rodent locomotor stimulation does not parallel drinking according to the relationship proposed by Schuckit and others.

After moderate to high doses of EtOH, rats and mice have very similar response profiles. The responses most frequently studied in rodents include hypothermia, and many different behaviors that are most often characterized as “ataxia” or “sedation.” A thorough discussion of all the various behavioral assays that have been used (e.g., balance beam, observer-rated wobbling gait, rotarod test, tilting plane) is beyond the scope of this paper, but it should be apparent that it is difficult to envision an obviously close surrogate for the human body sway measure in a mouse or rat. After high (i.e., anesthetic) EtOH doses, both rats and mice lose the ability to right themselves when placed on their back. This loss of righting reflex (LORR) has been a frequently studied phenotype in genetic studies in both species.

The pleiotropic effects of genes affecting any one of these sensitivity traits in rodents are complex. That is, genotypes sensitive to one behavioral response are not necessarily sensitive to others. We systematically evaluated the extent of such genetic correlations by comparing the behavioral responses of eight inbred mouse strains across 18 variables, using 11 behavioral assays, after giving EtOH as an intoxicant. While the genetic influences on behavior and sensitivity to EtOH were pronounced, as expected, the strain sensitivities were generally only modestly correlated (Crabbe et al., 2005).

Thus, when we set out to perform a broad initial screen to establish whether high DID appeared to be genetically correlated with other acute responses to EtOH, we necessarily had to choose from a very large range of available behavioral assays. We used tasks from our laboratory's repertoire that were sensitive to EtOH doses ranging from 1.4 to 4.0 g/kg. For reasons discussed in detail elsewhere (Crabbe et al., 2005, 2010a), because we really could not predict in advance which responses might be correlated with DID, we selected tasks in widespread use. With the human low level of response data in mind, we also explicitly elected to restrict our survey to behavioral assays thought to represent stimulant and depressant responses to EtOH. Thus, we did not attempt to include mouse models of anxiety-like behavior, depression-like behavior, learning and memory, impulsivity, sensitivity to EtOH reinforcement, or any of the other behavioral domains of interest. In a companion paper, however, we extended the range of this survey to address EtOH tolerance and physical dependence/withdrawal (Crabbe et al, in press).

We elected to employ both HDID-1 and HDID-2 lines. The predicted outcome for HDID-1 vs HS controls was straightforward. Although the direction of a predicted difference between them is not obvious, a significant difference in an unselected trait is *prima facie* evidence for genetic correlation. As we have argued elsewhere, finding a difference in both pairs of replicate selected lines in an experiment examining them simultaneously to offer the best possible control over environmental contributions offers the strongest evidence for genetic correlation (Crabbe et al., 1990; Crabbe, 1999). However, our HDID-2 line had only been subjected to selection pressure for 11–12 generations at the time of these tests. Thus, a finding that HDID-1 differed significantly from controls but HDID-2 did not is harder to interpret, as the HDID-2 line may simply not yet have diverged enough from HS for some correlated responses to emerge (see Discussion). However, we wanted to collect data from HDID-2 at a relatively early point during selection. Cases where continued selection yields parallel changes in the magnitude of response differences between genotypes in both the selected trait and in a putative correlated response to selection provide very strong evidence for pleiotropy [see (Belknap et al., 1989) for a clear example].

MATERIALS AND METHODS

Animals and husbandry

All mice were bred and maintained in our colonies at the Portland VA Veterinary Medical Unit, an AAALAC approved facility. Lights were on from 0700 – 1900 hours, and all behavioral testing was conducted between 0900 and 1400 hours except as noted. Mice were housed 2–5 to a polycarbonate or polysulfone cage (used interchangeably) with Bedocob bedding in ventilated (Thoren) racks with *ad libitum* access to tap water and food (Purina 5001). Temperature was maintained at $21 \pm 1^\circ\text{C}$.

Males and females were used in all studies, with approximately equal division across sexes in each genotype and treatment group. Mice were High Drinking in the Dark -1 (HDID-1) and -2 (HDID-2) selected lines and the heterogeneous stock (HS/Npt; HS) from which the selections were initiated (Crabbe et al., 2009, 2010b). HDID-1 mice were from the 18th selected generation and HDID-2 mice from the 11th. Mice from this generation of HDID-1 mice averaged 1.04 ± 0.06 mg/ml BECs at the end of their drinking in the dark test; the equivalent value for S11 HDID-2 mice was 0.86 ± 0.05 mg/ml (unpublished data). HS/Npt mice were from the (unselected) G70 generation. HS mice from both generations G51 and G69 tested for drinking in the dark averaged BECs of approximately 0.30. The HDID animals were selected for the high BEC they displayed at the end of a 4 hr session of drinking 20% (v/v) EtOH starting 3 hours after the beginning of their circadian dark period. All mice were naive at the beginning of the behavioral test (or pair of tests) and ranged in age from 7 to 12 weeks. All three genotypes were compared contemporaneously for each response. Mice were moved to the testing room approximately one hour before testing and returned to their colony after all testing for the day was complete.

Drugs and injections

EtOH was mixed 20% (v/v) in physiological saline on the morning of the test. Injections were given intraperitoneally at a volume adjusted for dose of EtOH (g EtOH/kg body weight). Mice were weighed at the time of injection. BECs were determined using a standard gas chromatographic procedure (Rustay and Crabbe, 2004).

Statistical analyses

As discussed in the Introduction, there is not as firm an expectation for the relative response of the HDID-2 line vs control HS as there is for HDID-1 vs HS. Furthermore, we were not interested in assessing potential differences between HDID-1 and HDID-2 lines. Thus, we conducted two sets of statistical comparisons for each experiment, independently evaluating HDID-1 vs HS and HDID-2 vs HS. We reasoned that the benefit of capturing and analyzing archival data on the HDID-2 mice was worth enduring the potential statistical confound of using the control HS data twice. Analyses of variance (ANOVAs) or t-tests were used to evaluate all dependent variables. Significant interactions were pursued with Tukey's HSD tests. Initial ANOVAs included sex as a factor. In most cases, the lack of systematic interactive effects with sex led us to report only subsequent analyses collapsed across sex. Differences at $p < 0.05$ were considered significant. For body weights, there were few significant interactions involving sex and none involving treatment condition, so we report only analyses of genotypic means.

Experiment 1. Balance beam and parallel rod floor

Mice were first tested for foot slip errors on a balance beam (Crabbe et al., 2003). Six days later, they were tested for activity and foot slip errors in the parallel rod floor apparatus (Kamens et al., 2005).

For the balance beam, on Day 1, all mice were weighed, given an ip injection of saline, and placed in individual holding cages 10 minutes prior to placement on the balance beam. Next, mice were placed on one end of the 19 mm balance beam and were required to traverse the beam once. A gentle nudge to the hind haunches was applied if mice did not cross willingly. Twenty-four hours later, mice were pseudorandomly divided into groups given saline or 1.4 g/kg EtOH and the same procedure was followed. During the test, number of hind foot slips was recorded as the mice performed one transit of the balance beam. Immediately following testing, a periorbital sinus blood sample (20 μ l) was taken from the left eye of the EtOH group mice and all were returned to their home cages.

The parallel rod floor test was conducted 6 days later. Mice were pseudorandomly reassigned to receive either saline or 2.0 g/kg EtOH. On Days 1 and 2, all mice were given an ip saline injection immediately before placement into the apparatus, a 15 $\times$ 15 cm plastic box with a floor made of parallel steel rods (1.6 mm diameter) spaced 6 mm apart. Foot slips (contacts with a plate 1 cm below the grid floor) and locomotor activity (beam interruptions from a set of 4 infrared beams crisscrossing the apparatus) were automatically recorded for 15 minutes. After each test, mice were placed back in their home cages. These days served as habituation and baseline activity level days, respectively. The same procedure was followed for Day 3, except that half the mice were given EtOH instead of saline. A periorbital sinus blood sample was taken from the right eye of the EtOH group immediately after the test.

Experiment 2. Accelerating rotarod and acute hypothermia

Mice were first tested for acquisition and then EtOH intoxication on the accelerating rotarod (Rustay et al., 2003). Six days later, they were tested for acute hypothermia (Crabbe et al., 2006).

For the accelerating rotarod (6.3 cm), on Day 1, 4 mice at a time were placed on individual lanes of the apparatus and given 10 successive training trials. The rod started at 0 RPM and accelerated at 20 RPM/minute until all mice fell. Latency to fall was recorded for each mouse, and 30–120 seconds' rest was given to all. The same procedure was followed for the 9 succeeding trials. Mice were placed back in their home cages following the 10th trial. Mice were then pseudorandomly assigned to saline and 2.0 g/kg EtOH groups. On Day 2, 24 hours after the training trials, mice were placed back on the rotarod and allowed 3 successive practice trials. After all mice had received practice, mice were injected with either saline or EtOH and were placed in individual holding cages. Starting 30 minutes after injection, mice were given 8 successive trials on the accelerating rotarod. After all mice had been tested, mice were placed back in their home cages.

For hypothermia testing, mice were separated into individually ventilated hypothermia chambers to acclimate for 60 minutes. Mice were then removed from the chambers to record baseline temperatures with a glycerol-lubricated probe (1.2 mm ball $\times$ 2cm length; Senterex Thermalert TH-8) that was inserted into the rectum for 5 seconds. Immediately following baseline recording, each mouse was given a 3.0 g/kg ip injection of EtOH and placed back in its chamber. Rectal temperatures were taken again at 30, 60, 90, and 120 minutes following the EtOH injection.

Experiment 3. Loss of righting reflex

Mice were simultaneously tested for LORR (Crabbe et al., 2006) and acute withdrawal severity; the acute withdrawal data are reported in the companion manuscript (Crabbe et al, in press). Mice were tested in two passes due to limited availability; results were combined across passes before analysis. All genotype $\times$ sex combinations were represented by

multiple mice in each pass. Each mouse was given a 4.0 g/kg EtOH injection. Starting about 1–2 minutes later, the mouse was manually restrained until it appeared to be sufficiently intoxicated, and was placed on its back in a V-shaped trough. Once the mouse was unable to right itself within 30 seconds, the time of LORR was recorded, with the proviso that LORR occurred within 3 minutes after injection time. Mice were then observed for the ensuing 2–3 hours. When a mouse righted itself, the time was recorded, and it was again placed on its back and given 30 seconds to right itself. If the mouse succeeded, the time of regaining righting reflex was recorded and the mouse was placed back in its home cage. If the mouse failed to regain the righting reflex on the second test, the same procedure was repeated until each mouse had regained righting reflex twice within 30 seconds.

RESULTS

Results across all experiments are summarized in Table 1.

Experiment 1

Balance beam—Figure 1 shows the results of the balance beam test. EtOH significantly intoxicated mice of all 3 genotypes. For both analyses, initial ANOVAs showed no significant main or interactive effects of sex [all $F_s < 3.7$, $p_s > 0.05$]. Subsequent genotype X drug ANOVAs showed significant main effects of EtOH treatment for both HDID-1 vs HS [$F(1,60) = 54.5$, $p < 0.0001$] and HDID-2 vs HS [$F(1,61) = 88.3$, $p < 0.0001$]. The main effects of genotype and the interactions were all non-significant [all $F_s(1,60–61) < 1.7$, $p_s > 0.10$]. BECs at the end of the test were 1.64 ± 0.11 , 1.58 ± 0.11 , and 1.76 ± 0.09 mg/ml for HS, HDID-1, and HDID-2, respectively. BECs did not differ significantly (both $t_s < 1$). Body weights tended to be significantly lower in HDID-1 (23.8 ± 0.6 g) than HS mice (25.9 ± 0.9 g); $t(61) = 1.9$, $p = 0.06$. HDID-2 (25.1 ± 0.7 g) did not differ significantly from HS ($t < 1$).

Parallel rod floor—To follow our established method, data were collected in three 5-minute bins. Data from two mice were eliminated due to injection errors. Figure 2, Panels A–C show the number of foot slips by each group. Because there was an apparent genotype-specific effect of EtOH on activity (Panels D–F), the raw foot slip data are not easily interpretable (mice that are more active will necessarily make more foot slips than those moving less) and are shown only for reference. Panels G–I show the foot slip data corrected for activity.

We first analyzed both the activity data and the corrected foot slip data using ANOVAs examining effects of genotype, drug, and sex, with epoch as a repeated measure. We found significant interactions of drug X epoch for the corrected foot slip data [HDID-1 vs HS, $F(2,104) = 4.4$, $p < 0.05$] and HDID-2 vs HS [$F(2,100) = 12.4$, $p < 0.0001$]. The only significant effect involving sex was the drug X sex X epoch interaction for HDID-1 vs HS [$F(2,104) = 3.6$, $p < 0.05$]. We therefore report analyses of (and show) the data separately for each epoch collapsed on sex.

For the HDID-1 vs HS comparison, mice were significantly stimulated by EtOH in all three epochs [$F_s(1,59) = 24.1, 14.1$, and 7.8 , respectively, $p_s < 0.01$]. A significant genotype X drug interaction was found for all 3 epochs [$F_s(1,59) = 6.7, 8.3$ and 7.1 , respectively, $p_s < 0.01$]. *Post hoc* tests confirmed that for all three epochs, only the HDID-1 mice showed significantly greater activity after treatment with EtOH. Both genotypes showed equivalent activity after saline in epoch 1, but HDID-1 showed significantly lower activity than HS in epochs 2 and 3 ($t_s = 3.1$ and 3.7 , $dfs = 30$, $p_s < 0.01$, respectively). For corrected foot slips, there was a significant effect of EtOH to increase errors in all 3 epochs [$F_s(1,59) = 13.4, 45.8$ and 29.9 , respectively, all $p_s < 0.001$]. A significant genotype X drug interaction was

seen only in epoch 2 [$F(1,59) = 5.4, p < 0.05$]. This was due to significant impairment by EtOH in the HDID-1 mice, but not the HS. Both genotypes showed equivalent corrected foot slips after saline.

The HDID-2 vs HS comparisons differed substantially from the HDID-1 vs HS (see Table 1). There were no significant effects for activity in the first two epochs. Overall, the significant main effect of genotype showed that HDID-2 mice were less active than HS in epoch 3 [$F(1,58) = 5.6, p < 0.05$]. No stimulation or sedation by EtOH was seen in any epoch. For corrected foot slips, EtOH significantly increased errors only in epochs 2 and 3 [$F(1,58) = 6.0$ and 28.1 , respectively, $ps < 0.05$] and there were no significant genotype X drug interactions.

BECs taken after the parallel rod floor test were 2.42 ± 0.18 , 2.70 ± 0.22 , and 2.24 ± 0.18 mg/ml for HS, HDID-1, and HDID-2, respectively. BECs did not differ significantly (both $ts < 1$). Body weights did not differ for either comparison (HS, 25.5 ± 0.8 g; HDID-1, 23.8 ± 0.6 g; HDID-2, 25.5 ± 0.7 g; both $t = 1.6, p > 0.10$).

Experiment 2

Accelerating rotarod—Figure 3A shows the acquisition of accelerating rotarod performance across trials in the three genotypes on Day 1, in the absence of drug treatment. To summarize performance, we computed average latency to fall across the 10 test trials (data not shown). There was a significant main effect of sex (females performed worse than males; data not shown) only for the HDID-2 vs HS comparison [$F(1,63) = 4.8, p < 0.05$]. Sex did not interact significantly with genotype for either replicate comparison (both $Fs < 3.0, ps > 0.05$). We therefore analyzed average acquisition performance collapsed across sex. Neither selected line differed significantly from HS [$F(1,64-65) < 0.1$; data not shown]. When tested after EtOH or saline the next day, we saw no significant main or interacting effects of sex. Both selected lines showed impairment by EtOH equivalent to that in HS (Figure 3B), as main effects of genotype and genotype X treatment effects were not significant [$F(1,62-63) < 0.3$]. EtOH treated mice performed worse than their saline groups [both $F(1,62-63) = 4.2, ps < 0.05$]. Body weights did not differ between HS (24.9 ± 0.5 g) and HDID-1 mice (24.7 ± 0.7 g, $t < 1$). HDID-2 mice tended to weigh less (23.5 ± 0.6 g) than HS [$t(65) = 1.9, p = 0.07$].

Hypothermia—Figure 4 shows the results for the acute hypothermic response to EtOH, injected at Time 0. Temperature was maximally reduced at 30 minutes after injection and recovered gradually over the next 90 minutes (Figure 4A). To summarize each animal's total hypothermic response, we summed the difference scores from baseline temperature for each post-baseline time (Figure 4B). Initial analyses revealed no significant main or interactive effects of sex [$F(1,60) = 2.2, ps > 0.10$] so data were analyzed and are reported collapsed across sex. Both selected lines showed significantly less hypothermic response than HS (for HDID-1 vs HS: $t = 2.85, df = 62$; for HDID-2 vs HS: $t = 4.51, df = 62$. Both $ps < 0.01$). Genotypes did not differ significantly in body weight (both $ts < 1$). HS mice weighed 24.6 ± 0.5 g, HDID-1 weighed 24.6 ± 0.7 g, and HDID-2 mice weighed 24.5 ± 0.5 g.

Experiment 3

Loss of righting reflex—All mice were injected with EtOH for this study. Thirteen mice (5 HS, 4 HDID-1 and 4 HDID-2) failed to lose righting reflex and their data were eliminated. Initial analyses for each selected line vs HS revealed no significant main or interactive effects of sex [$F(1,51) = 2.8, ps > 0.10$], so data were analyzed collapsed across sex. LORR durations were: 93.8 ± 4.5 minutes for HS ($N = 28$); 120.2 ± 5.5 for HDID-1 ($N = 27$); and 77.1 ± 4.9 for HDID-2 ($N = 27$). The HDID-1 mice had significantly longer

duration LORR than HS ($t = 3.7$, $df = 53$, $p < 0.05$) while the HDID-2 mice had significantly shorter duration LORR than HS ($t = 2.5$, $df = 53$, $p < 0.05$). Body weight of HDID-1 mice was significantly lower (23.4 ± 0.6 g) than that of HS mice (27.9 ± 1.0 g), $t(61) = 3.8$, $p < 0.001$. For HDID-2 (26.0 ± 0.6 g), the difference from HS did not reach significance [$t(62) = 1.6$, $p > 0.10$].

DISCUSSION

A global summary of pleiotropic gene effects for those genes predisposing to high drinking in the dark is given in Table 1. The best test of common gene action comes from comparisons between HDID-1 and HS mice. These genotypes differed for some acute behavioral responses to EtOH, but not for others. HDID-1 mice showed less basal activity, greater sensitivity to EtOH's locomotor stimulant effects, and were more likely to commit foot slip errors in the parallel rod floor apparatus after EtOH than HS. In the parallel rod floor, HDID-2 mice also showed less basal activity, but no stimulant response to EtOH and equivalent intoxication compared to HS. HDID-1 had longer duration LORR than HS after a high EtOH dose, while HDID-2 had shorter responses. HDID-1 and HDID-2 were less sensitive than HS to EtOH-induced hypothermia (but see companion paper, Crabbe et al, in press). The 3 genotypes did not differ in performance on the accelerating rotarod, either before or after EtOH, and were equally intoxicated on the balance beam.

Differences in EtOH pharmacokinetics could underlie the observed patterns of acute behavioral differences (Kalant et al, 1971). For tests assessed at a single time point, if genotypes experienced different effective doses of EtOH (e.g., due to differential absorption and/or distribution into brain) different BECs would have been seen. However, specific measurements of BEC after 1.4 or 2.0 g/kg injections in Experiment 1 did not reveal any differences in BEC across genotypes. An assessment in earlier generations of HDID-1 and HS mice of BECs and rate of metabolism after an acute injection of 2.0 g/kg revealed slightly higher BECs in HDID-1, but no differences in EtOH elimination rate (Crabbe et al., 2009). In the current generations, the line differences in BEC were no longer evident, but we did not assess rate of metabolism. For EtOH hypothermia, different rates of EtOH clearance during the test could have led to differences in response. However, the body temperature differences were already evident at 30 minutes after injection (Figure 4A), before substantial elimination would have been seen. Furthermore, there was no evidence of genetic differences in rate of recovery to baseline temperatures. We conclude that differences in the dose of EtOH experienced are unlikely to explain the differences in acute hypothermic sensitivity, which agrees with much previous data (Crabbe et al., 2005, 2006). We decided not to take blood EtOH samples at recovery of righting reflex as we felt this might influence the scoring of acute withdrawal severity reported in the companion manuscript (Crabbe et al, in press).

Another correlated response seen in HDID-1 mice was in the parallel rod floor test, where they engaged in less locomotion than HS during the latter two epochs of the test when given saline. HDID-2 mice also showed less activity than HS during the 3rd epoch. For the comparison of HDID-2 vs HS, the interaction of treatment and genotype did not reach significance. Basal activity is not thought to be a strong genetic predictor of EtOH preference drinking. However, we previously reported that g/kg EtOH intake on the first day of the DID test was strongly correlated with a measure of activity across ten inbred strain mean values for both traits (Rhodes et al., 2007). The activity measure, however, was quite unusual, i.e., cumulated beam interruptions over 3 days of constant exposure to an initially novel apparatus. And, the reported correlations were opposite in sign to those suggested here, where lower activity was seen in lines selected for high DID. Because the activity differences became more pronounced later during the 15 minute parallel rod floor test, it

would be interesting to explore other measures of activity in these genotypes and explore tests of longer duration.

Initial locomotor stimulant response to EtOH differed markedly between HDID-1 and HS, and may also be a correlated response. In a relatively novel activity chamber (i.e., in a situation more similar to the parallel rod floor test), a study that tested many recombinant inbred strains derived from the intercross of C57BL/6 and DBA/2 inbreds reported that low initial stimulant response to EtOH predicted preference for 3% (but not 10%) EtOH in a continuous-access 2-bottle test (Phillips et al., 1995). However, this relationship is opposite to what we saw in HDID-1 vs HS mice. The previous study also did not report a predictive relationship for basal activity (Shen et al., 1996). Nor did we see a difference in HDID-2 vs HS. Finally, although LORR duration differed significantly from HS in both selected lines, the opposite direction of those differences makes it unlikely that LORR is a correlated response to selection on DID BEC.

Overall, the suggestion that a low level of initial response to EtOH on assays reflecting ataxia or balance or postural control might predict predisposition to drink excessively (or, in our case, reach high BECs after DID) was not supported by most of our data. HDID-1 were more sensitive to impairment in the parallel rod floor apparatus than HS mice, but they did not differ in EtOH-induced foot slips on the balance beam or in performance impairment on the rotarod. Previous human studies are also equivocal on the relationship between EtOH sensitivity and risk for subsequent alcohol dependence. A persuasive hypothesis (the “differentiator hypothesis”) suggests that genetically at-risk humans are more sensitive to effects of EtOH that occur shortly after ingestion, but less sensitive to effects later in a drinking session due to the development of more acute functional tolerance (Newlin and Thomson, 1990). However, for HDID-1 vs HS mice, results of the two tests performed shortly after injection (balance beam, parallel rod floor) yielded different results. Furthermore, HDID-1 mice tended to develop less, not more tolerance than HS to EtOH hypothermia when it was assessed in separate experiments (Crabbe et al, in press).

Carryover effects could have influenced the results of the parallel rod floor and hypothermia tests, as these mice had been given a single dose of EtOH 6 days earlier. For example, the initial EtOH treatment could have led to tolerance development, expressed as a reduced response in either the parallel rod floor or hypothermia tests (Kalant et al., 1971). The pattern of results seen does not correspond well with this hypothesis as a general explanation for genotypic differences in these two tests (see Table 1). Tolerance to a single injection of EtOH persists for only 48 hr in an acute hypothermia test (Crabbe et al., 1979). In the companion manuscript (Crabbe et al, in press), we studied hypothermic tolerance explicitly and found *less* tolerance development in HDID-1 mice of both sexes than in HS, a pattern contrary to a tolerance explanation for the reduced sensitivity to EtOH hypothermia we saw in Experiment 2. We selected the 6 day interval between acute tests in both Experiments 1 and 2 to minimize the possibility of carryover effects. Nonetheless, some effect of prior handling other than tolerance may have influenced the results with the parallel rod floor and in the hypothermia test.

We have reported a modest positive genetic correlation between drinking in the dark-BEC and ethanol preference drinking (Rhodes et al., 2007; Crabbe et al., 2011). It is reasonable to ask whether the pattern of genetically correlated responses with drinking in the dark-BEC we report here resembles the relationships among those and similar variables from the literature on ethanol preference. Systematically exploring the genetic correlational architecture of all the traits we report here would be an immense undertaking (for a worked example seeking patterns of correlations among different ataxia measures after ethanol challenge in mice see Crabbe et al, 2005) and well beyond the scope of this paper. However,

we explored the limited question of whether acute hypothermic response to ethanol (here found to be negatively genetically correlated with drinking in the dark BEC) was also negatively genetically correlated with ethanol preference drinking, given the modest positive genetic correlation between the drinking in the dark BEC and preference. This required reviewing 18 papers and book chapters published between 1979 and 2008. We looked at available data from rat lines selectively bred for high or low preference drinking – most such data supported the negative genetic relationship. The mouse data, however, were largely derived from correlations among inbred strain means. These data generally showed small, non-significant *positive* genetic correlations between hypothermia and preference, and also between hypothermia and drinking in the dark-BEC. There are no rat data on drinking in the dark-BEC. In other words, it is difficult to glean a consistent pattern of relationships from the published data against which the hypothesized similarity of correlations in the current data could be tested. We believe this to be due to the combination of comparing across species; across differences in procedure, dose, age, and sex; and the low heritabilities of the drinking in the dark-BEC phenotype plus the modest heritabilities of preference drinking and hypothermic sensitivity.

Data from the HDID-2 line were provided largely to benchmark the HDID-2 and HS genotypes for future assessments of correlated responses after the HDID-2 mice have responded to further selection. It is difficult to predict when truly correlated responses should begin to differentiate these genotypes, as this would depend on the heritability of both traits (i.e., DID and the putatively correlated response), the intensity of selection, and the underlying genetic complexity of both traits. DID has been under rather intense selection in the HDID-2 line, and the heritability of DID in this line appears very similar to the estimate derived from the response in HDID-1. In the HDID-1 line, h^2 was estimated to be only 0.09, which is quite low (Crabbe et al., 2009). Thus, we believe that it is still possible that some of the responses we tested here will come to differ between HDID-2 and HS in future generations.

In conclusion, it is not possible to characterize HDID-1 mice as generally “more” or “less” sensitive to EtOH than HS. Some clear indications of pleiotropic gene effects emerged, but they did not systematically align with constructs such as “sensitivity,” “tolerance” or “dependence.” This is consistent with analyses of multiple inbred strains’ sensitivity to EtOH on multiple behaviors, where we found no strains with generally enhanced or reduced sensitivity across tasks (Crabbe et al., 2005; Munn et al., 2011). As response to selection continues to develop in HDID-1 and HDID-2 mice, it should become easier to isolate genetic correlates that offer possible explanations of the genetic basis for high DID.

Acknowledgments

These studies were supported by grants AA10760 and AA13519 from the NIAAA, and by a grant from the US Department of Veterans Affairs. LCK and AMC were supported by grant AA07468. We thank Mark Rutledge-Gorman for assistance with the manuscript.

References

- Belknap JK, Crabbe JC, Laursen SE. Ethanol and diazepam withdrawal convulsions are extensively codetermined in WSP and WSR mice. *Life Sci.* 1989; 44:2075–2080. [PubMed: 2501607]
- Crabbe, JC. Animal models in neurobehavioral genetics: Methods for estimating genetic correlation. In: Mormède, P.; Jones, BC., editors. *Neurobehavioral Genetics: Methods and Applications*. CRC Press; Boca Raton FL: 1999. p. 121-138.
- Crabbe JC. Neurogenetic studies of alcohol addiction. *Philos Trans R Soc Lond B Biol Sci.* 2008; 363:3201–3211. [PubMed: 18640917]

- Crabbe JC, Bell RL, Ehlers CL. Human and laboratory rodent low response to alcohol: is better consilience possible? *Addict Biol.* 2010a; 15:125–144. [PubMed: 20148776]
- Crabbe, JC.; Colville, AM.; Kruse, LC.; Cameron, AJ.; Spence, SE.; Schlumbohm, JP.; Huang, LC.; Metten, P. Alcoholism Clinical and Experimental Research. Ethanol tolerance and withdrawal severity in High Drinking in the Dark selectively bred mice. in press
- Crabbe JC, Metten P, Cameron AJ, Wahlsten D. An analysis of the genetics of alcohol intoxication in inbred mice. *Neurosci Biobehav Rev.* 2005; 28:785–802. [PubMed: 15642621]
- Crabbe JC, Metten P, Ponomarev I, Prescott CA, Wahlsten D. Effects of genetic and procedural variation on measurement of alcohol sensitivity in mouse inbred strains. *Behav Genet.* 2006; 36:536–552. [PubMed: 16619134]
- Crabbe JC, Metten P, Rhodes JS, Yu C-H, Brown LL, Phillips TJ, Finn DA. A line of mice selected for high blood ethanol concentrations shows drinking in the dark to intoxication. *Biol Psychiat.* 2009; 65:662–670. [PubMed: 19095222]
- Crabbe JC, Metten P, Yu C-H, Schlumbohm JP, Cameron AJ, Wahlsten D. Genotypic differences in ethanol sensitivity in two tests of motor incoordination. *J Appl Physiol.* 2003; 95:1338–1351. [PubMed: 12704090]
- Crabbe JC, Phillips TJ, Belknap JK. The complexity of alcohol drinking: studies in rodent genetic models. *Behav Genet.* 2010b; 40:737–750. [PubMed: 20552264]
- Crabbe JC, Phillips TJ, Kosobud A, Belknap JK. Estimation of genetic correlation: interpretation of experiments using selectively bred and inbred animals. *Alcohol Clin Exp Res.* 1990; 14:141–151. [PubMed: 2190477]
- Crabbe JC, Rigter H, Uijlen J, Strijbos C. Rapid development of tolerance to the hypothermic effect of ethanol in mice. *J Pharmacol Exp Ther.* 1979; 208:128–133. [PubMed: 759607]
- Crabbe JC, Spence SE, Brown LL, Metten P. Alcohol preference drinking in a mouse line selectively bred for high drinking in the dark. *Alcohol.* 2011; 45:427–440. [PubMed: 21194877]
- Goldman D, Ducci F. Deconstruction of vulnerability to complex diseases: enhanced effect sizes and power of intermediate phenotypes. *Sci World J.* 2007; 7:124–130.
- Grahame NJ, Li T-K, Lumeng L. Limited access alcohol drinking in high- and low-alcohol preferring selected lines of mice. *Alcoholism Clinical and Experimental Research.* 1999; 23:1015–1022.
- Kalant H, LeBlanc AE, Gibbins RJ. Tolerance to, and dependence on, some non-opiate psychotropic drugs. *Pharmacol Rev.* 1971; 23:135–191. [PubMed: 4398655]
- Kamens HM, Phillips TJ, Holstein SE, Crabbe JC. Characterization of the parallel rod floor apparatus to test motor incoordination in mice. *Genes Brain Behav.* 2005; 4:253–266. [PubMed: 15924557]
- Munn E, Bunning M, Prada S, Bohlen M, Crabbe JC, Wahlsten D. Reversed light-dark cycle and cage enrichment effects on ethanol-induced deficits in motor coordination assessed in inbred mouse strains with a compact battery of refined tests. *Behav Brain Res.* 2011; 224:259–271. [PubMed: 21664382]
- Newlin DB, Thomson JB. Alcohol challenge with sons of alcoholics: a critical review and analysis. *Psychol Bull.* 1990; 108(3):383–402. [PubMed: 2270234]
- Phillips TJ. Use of genetically distinct mouse populations to explore ethanol reinforcement. *Alcohol Alcohol Suppl.* 1993; 2:451–455. [PubMed: 7748338]
- Phillips TJ, Huson M, Gwiazdon C, Burkhardt-Kasch S, Shen EH. Effects of acute and repeated ethanol exposures on the locomotor activity of BXD recombinant inbred mice. *Alcohol Clin Exp Res.* 1995; 19:269–278. [PubMed: 7625557]
- Rhodes JS, Best K, Belknap JK, Finn DA, Crabbe JC. Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. *Physiol Behav.* 2005; 84:53–63. [PubMed: 15642607]
- Rhodes JS, Ford MM, Yu C-H, Brown LL, Finn DA, Garland T Jr, Crabbe JC. Mouse inbred strain differences in ethanol drinking to intoxication. *Genes Brain Behav.* 2007; 6:1–18. [PubMed: 17233637]
- Rodd ZA, Bell RL, McKinzie DL, Webster AA, Murphy JM, Lumeng L, Li TK, McBride WJ. Low-dose stimulatory effects of ethanol during adolescence in rat lines selectively bred for high alcohol intake. *Alcohol Clin Exp Res.* 2004; 28:535–543. [PubMed: 15100603]
- Rustay NR, Crabbe JC. Genetic analysis of rapid tolerance to ethanol's incoordinating effects in mice: Inbred strains and artificial selection. *Behav Genet.* 2004; 34:441–451. [PubMed: 15082941]

- Rustay NR, Wahlsten D, Crabbe JC. Assessment of genetic susceptibility to ethanol intoxication in mice. *Proc Natl Acad Sci USA*. 2003; 100:2917–2922. [PubMed: 12584362]
- Schuckit MA. Ethanol-induced changes in body sway in men at high alcoholism risk. *Arch Gen Psychiat*. 1985; 42:375–379. [PubMed: 3977555]
- Schuckit MA, Smith TL. The clinical course of alcohol dependence associated with a low level of response to alcohol. *Addiction*. 2001; 96:903–910. [PubMed: 11399221]
- Schuckit MA, Smith TL, Trim R, Fukukura T, Allen R. The overlap in predicting alcohol outcome for two measures of the level of response to alcohol. *Alcohol Clin Exp Res*. 2009; 33:563–569. [PubMed: 19120060]
- Shen EH, Dorow JD, Huson M, Phillips TJ. Correlated responses to selection in FAST and SLOW mice: effects of ethanol on ataxia, temperature, sedation, and withdrawal. *Alcohol Clin Exp Res*. 1996; 20:688–696. [PubMed: 8800386]

Figure 1. Mean $\pm$ SE hind foot slips on the balance beam. Saline groups: N = 17, 15, and 16 for HS, HDID-1 and HDID-2, respectively. EtOH groups: N = 16 for each genotype.

Figure 2.

Mean $\pm$ SE for scores in the parallel rod floor apparatus for three successive 5 minutes intervals after injection. Saline groups: N = 16 for all 3 genotypes. EtOH groups: N = 16, 15, and 14 for HS, HDID-1 and HDID-2, respectively. Panels A–C: Foot slips. Panels D–F: Locomotor activity (beam interruptions). Panels G–I: Foot slips corrected for activity (data from Panels A–C divided by data from panels D–F).

Figure 3.

Mean $\pm$ SE latency to fall from the accelerating rotarod. Saline groups: N = 17, 16, and 17 for HS, HDID-1 and HDID-2, respectively. EtOH groups: N = 16, 17, and 17 for HS, HDID-1 and HDID-2, respectively. Panel A: Performance across the 10 acquisition trials on Day 1 for each genotype. Panel B: Average latency to fall across the 8 trials on Day 2 starting 30 minutes after saline or 2.0 g/kg EtOH.

Figure 4.

Mean $\pm$ SE body temperature ($^{\circ}$ C) before (Time 0) and after 3.0 g/kg EtOH. All mice (N = 32/genotype) were given EtOH. Panel A: Body temperature before and at 30 minute intervals after injection. Panel B: Total hypothermic response; approximate area under the hypothermia curves from Panel A.

Table 1

Behavioral performance and sensitivity to EtOH in HDID-1, HDID-2 and HS mice.

Task	Measure	HDID-1 vs HS*	HDID-2 vs HS*	Notes
Balance beam	EtOH -induced foot slips	=	=	Significant impairment
	BEC	=	=	
Parallel rod floor	Basal activity	<		Differences emerged later during test
	EtOH stimulated activity	>	=	No stimulation in HS and non-significant sedation in HDID-2
	Increase in corrected foot slips after EtOH	>	=	Only in HDID-1 in epoch 2 All genotypes = after saline
	BEC	=	=	
Accelerating rotarod	Acquisition	=	=	Females performed worse than males for HDID-2 vs HS
	Performance impairment after EtOH	=	=	Significant impairment
Hypothermia	Reduced temperature after EtOH	<	<	See companion paper, Crabbe, Colville et al., submitted
Loss of righting reflex	Duration after EtOH	>	<	

* Comparisons reflect either relative performance after saline, or relative response to EtOH.