Published in final edited form as:

Alcohol Clin Exp Res. 2012 July ; 36(7): 1152–1161. doi:10.1111/j.1530-0277.2011.01715.x.

Ethanol Tolerance and Withdrawal Severity in High Drinking in the Dark Selectively Bred Mice

John C. Crabbe, Alexandre M. Colville, Lauren C. Kruse, 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 limited access of ethanol (EtOH) drinking early in the circadian dark phase. High Drinking in the Dark -1 (HDID-1) mice are in selected generation S21, and the replicate HDID-2 line in generation S14. Tolerance and withdrawal symptoms are two of the seven diagnostic criteria for alcohol dependence. Withdrawal severity has been found in mouse studies to be negatively genetically correlated with EtOH preference drinking.

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—Female HDID-1 and HDID-2 mice tended to develop less tolerance than HS to EtOH hypothermia after their 3rd daily injection. A trend toward *greater* tolerance was seen in the HDID males. HDID-1, HDID-2 and control HS lines did not differ in the severity of acute or chronic withdrawal from EtOH as indexed by the handling-induced convulsion (HIC). Both HDID-1 and HDID-2 mice tended to have greater HIC scores than HS regardless of drug treatment.

Conclusions—These results show that tolerance to EtOH's hypothermic effects may share some common genetic control with reaching high BECs after DID, a finding consistent with other data regarding genetic contributions to ethanol responses. Withdrawal severity was not negatively genetically correlated with DID, unlike its correlation with preference drinking, underscoring the genetic differences between preference drinking and DID. HDID lines showed greater basal HIC scores than HS, suggestive of greater central nervous system excitability.

Keywords

Selected mouse lines; High Drinking in the Dark (HDID) mice; hypothermia; tolerance; dependence; genetic correlation; withdrawal

Introduction

The companion paper (Crabbe et al, in press) describes our rationale for developing a new genetic animal model of binge-like EtOH drinking. The assay is called Drinking in the Dark (DID). When offered a 4 hr period of access to a 20% EtOH solution shortly after lights out, some mice will drink enough to become intoxicated (Rhodes *et al.* 2005; Rhodes *et al.* 2007). Studies of the DID phenomenon established that there were significant genetic influences on DID, and that they were partially (but not completely) in common with genetic influences on

two bottle EtOH preference drinking (Rhodes *et al.* 2007). We have been breeding High Drinking in the Dark-1 mice selectively for the BEC attained after a two day DID test. The initial selection response was reported elsewhere (Crabbe *et al.* 2009), and we are also breeding a replicate line, HDID-2, for the same trait (Crabbe *et al.* 2010b). The animals drink to the point of behavioral intoxication (Crabbe *et al.* 2009), but show relatively normal preference for alcohol solutions in a preference test (Crabbe *et al.* 2011b). We review findings from both the selection and the preference drinking studies in the companion paper (Crabbe et al, in press).

We are characterizing the selected lines for other responses to EtOH in order to determine domains of EtOH response that might share common genetic determinants with DID BEC. Studies of the relative sensitivity to EtOH using several behavioral assays across a range of EtOH doses from 1.4 to 4.0 g/kg, reported in a companion manuscript (Crabbe et al, in press), revealed that some, but not all indices of behavioral sensitivity to EtOH were correlates of DID BEC. We restricted those analyses to the behavioral domains of motor stimulation, various movement-based measures of intoxication (balance beam, rotarod, parallel rod floor), and more general sedation (hypothermia, loss of righting reflex). All tests were conducted following single injections of EtOH in naive animals. Our rationale for selecting these traits was grounded in the finding of Marc Schuckit's group that a "low level of response" to alcohol predicted risk for eventual alcohol dependence diagnosis (Schuckit 2009;Schuckit, Smith 2001). We discuss in the companion paper and elsewhere why it is difficult to know which particular measures of acute intoxication in rodents might best "model" human low level of response (Crabbe et al, in press; Crabbe *et al.* 2010a).

When EtOH is administered chronically, tolerance generally develops. This is revealed as either a reduction in response to the same, repeated dose, or the need to raise the dose to maintain the original level of response. Either manifestation is the result of a shift to the right of the dose-effect curve (Kalant *et al.* 1971). Tolerance may arise for pharmacokinetic reasons (e.g., an induction of metabolizing enzyme activity); when this occurs, the proximate cause is a reduction in the EtOH concentration at the target organ site. Because the effector organ for most or all behavioral responses to EtOH is the brain, pharmacokinetic (metabolic) tolerance is not particularly informative as to neural mechanisms. Most studies of EtOH tolerance have, therefore, focused on pharmacodynamic (functional) tolerance, where the same BEC is now less effective due to neuroadaptations to repeated challenges with EtOH (Kalant *et al.* 1971). EtOH tolerance has frequently been studied in rodents [for reviews, see (Kalant 1998;Kalant 1996)].

Several investigators have suggested that a propensity to develop tolerance to EtOH may predispose one to the development of dependence. One way this could occur is that individuals become tolerant to the stimulant, euphorigenic effects of EtOH and so escalate their dose in an attempt to recover the pleasurable "high." Alternatively, or in addition, development of tolerance to the more sedative and unpleasant effects of EtOH may facilitate the escalation of drinking. Dependence is thought to ensue in either case as the total, repeated dose gradually results in neurotoxic effects (Tabakoff, Hoffman 1988;Lê, Mayer 1996;Koob *et al.* 1998).

Dependence is inferred when revealed by signs of withdrawal, which are remarkably similar across mammalian species (Friedman 1980). Manifestations of tolerance and withdrawal are two of the seven possible contributing symptoms leading to a diagnosis of alcohol dependence according to the DSM-IV-R. In mice, the most frequently studied withdrawal sign is the handling-induced convulsion (HIC), a sign of central nervous system excitability that waxes and wanes for several hours after cessation of EtOH administration. This very

sensitive behavioral index is exacerbated for several hours after even a single, anesthetic EtOH dose (Goldstein 1972; Crabbe *et al.* 1991a).

In rodents, sensitivity, tolerance and dependence are all influenced by genetic background. They share some overlap in genetic influences as well, based on studies that estimate genetic correlations from panels of inbred strains or by examining correlated responses in selectively bred lines. However, the areas of commonality do not generally fall along the lines of the behavioral construct. Thus, animals sensitive to one effect of EtOH are not necessarily sensitive to others (Crabbe *et al.* 2005). There are different forms of tolerance to ethanol, usually distinguished by how quickly they develop: they are called acute [within-session, e.g. (Mellanby 1919)], rapid [reduced response to a second administration, e.g. (Crabbe *et al.* 1979)], and chronic [following multiple doses or long-term chronic exposure, e.g. (Chen 1979)]. Genetic contributions to rapid and chronic EtOH tolerance are reasonably consistent, suggesting that they might be early and more fully developed aspects of the same underlying neuroadaptive processes (Kalant 1998; Le, Mayer 1996; Rustay, Crabbe 2004; Hanchar *et al.* 2005). Acute tolerance seems to be a form more genetically distinct from rapid and chronic tolerance (Kalant 1998; Ponomarev 2002). However, there is also evidence that acute and rapid tolerance may share some genetic determinants (Radcliffe *et al.* 2006). Sensitivity and tolerance are sometimes positively genetically correlated in that strains and selected lines more sensitive to an EtOH effect tend to develop more tolerance (Deitrich 1993; Crabbe *et al.* 1982; Phillips, Crabbe 1991) but not all findings are consistent [e.g., (Radcliffe *et al.* 2005; Deitrich *et al.* 2000; Gehle, Erwin 2000; Aufrere *et al.* 1994; Drews *et al.* 2010; Bennett *et al.* 2007)]. The literature on this topic is large, and has not been reviewed systematically for many years (Phillips, Crabbe 1991). Sensitivity and withdrawal severity are not generally correlated (Crabbe *et al.* 1983; Crabbe *et al.* 1994; Drews *et al.* 2010). Chronic tolerance and dependence (i.e., withdrawal severity) may be negatively genetically coupled, but the evidence for this is also mixed [Crabbe 1996; Crabbe *et al.* 1988; Crabbe *et al.* 1983; Drews *et al.* 2010]; for review, see (Crabbe *et al.* in press)]. Overall, it is likely that the genetic relationships among sensitivity, tolerance and dependence vary somewhat not only with the behaviors and the specific forms of tolerance studied but also with the specific genetic animal models employed (Radcliffe *et al.* 2004; Deitrich *et al.* 2000).

In contrast to the mixed results regarding genetic correlations of sensitivity, tolerance and dependence, studies comparing withdrawal severity and preference drinking have consistently reported negative genetic correlations between these phenotypes in both rats and mice [(Chester *et al.* 2003; Chester *et al.* 2002); for reviews, see (Metten *et al.* 1998; Belknap *et al.* 2008)]. Thus, for example, standard inbred mouse strains that prefer to drink EtOH solutions tend to display low withdrawal HICs after being rendered physically dependent. And, lines of mice selectively bred for severe withdrawal HICs tend to drink less than those selected for mild withdrawal (Metten *et al.* 1998). In all these studies, animals are tested for one trait only; thus, the negative genetic relationship does not address post-dependence drinking, of which there are virtually no genetic studies.

We elected to compare HDID-1 mice and HS for their development of EtOH tolerance. Based on the not entirely consistent evidence reviewed above, and the fact that DID and preference drinking are imperfectly genetically correlated, we predicted that HDID-1 mice would develop greater tolerance than HS. We also compared the genotypes for both acute and chronic EtOH withdrawal severity. Again, with the caveat regarding DID and preference, we predicted that HDID-1 would display less severe withdrawal HICs than HS. We elected to employ both HDID-1 and HDID-2 lines, as we were interested in collecting the archival data for HDID-2. The rationale is discussed in the Introduction to the companion manuscript and is not reiterated here (Crabbe *et al.*, in press).

Materials and Methods

Animals and husbandry

All mice were bred and maintained 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) 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. Temperature was maintained at $21 \pm 1^{\circ}\text{C}$.

We tested approximately equal numbers of males and females in each genotype and treatment group in Experiments 1 and 2; only males were available for Experiment 3. 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.* 2010b; Crabbe *et al.* 2009). HDID-1 mice were from the 18th and 19th selected generations, HDID-2 mice from the 11th and 12th. HS mice were from the 70th and 71st filial generations. The HDID lines were selected for the high blood ethanol concentration (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 8 to 15 weeks.

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). Blood ethanol concentrations (BEC) were determined using a standard gas chromatographic procedure (Rustay, Crabbe 2004). Pyrazole HCl (Sigma-Aldrich) was given to all mice in Experiment 3.

Statistical analyses

As discussed in the Introduction to the companion paper (Crabbe et al, in press), we were not interested in assessing potential differences between HDID-1 and HDID-2 lines. Because the HDID-2 line had been selected for many fewer generations, expectations about the relative response of the HDID-2 line vs control HS are less clear than for HDID-1 vs HS. Thus, we conducted two sets of statistical comparisons for each experiment and independently evaluated HDID-1 vs HS and HDID-2 vs HS. We reasoned that the benefit of capturing 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. Differences at $p < 0.05$ were considered significant.

Experiment 1. Tolerance to EtOH hypothermia

Tolerance to EtOH hypothermia was tested using a variant of our standard procedures (Crabbe et al. 1982). These were as described for the acute hypothermia test conducted in Experiment 2 of the companion manuscript (Crabbe et al, in press). Two groups of mice were tested for each genotype. On Days 1 and 2, one group was given a saline injection (Group SSE) and the other group was given EtOH (3.0 g/kg; Group EEE). On Day 3, both groups were given 3.0 g/kg EtOH. Testing for hypothermia before and 30, 60, 90 and 120 minutes after injection was conducted on Days 1 and 3; only injections were given on Day 2 and mice were returned to their home cage. On Day 3, a 20ul blood sample was taken from the tip of the tail to assess BEC after the 120 minute body temperature.

Experiment 2. Acute withdrawal severity

These were the same animals tested simultaneously for loss of righting reflex in Experiment 3 in the companion manuscript (Crabbe et al, in press). Mice were first scored twice for basal HIC severity, 30 minutes apart. These scores were averaged to provide a baseline HIC score. To test for HICs, mice were gently picked up by the tip of the tail. If this failed to elicit a convulsion, mice were gently spun in a 360 degree arc and scored. The HIC scale ranges from 0-7 where 7 is lethal (Crabbe *et al.* 1991a). Scores after acute withdrawal typically range from 0-5. Because most mice had regained righting reflex within 2 hours (see Experiment 3 Results from companion manuscript), mice were tested for EtOH withdrawal HICs starting at hour 2 after injection of 4.0 g/kg, unless they were still on their backs. HICs were scored hourly from hours 2-12, and again the next day at hours 24 and 25. After each HIC test, mice were placed back in their home cages.

Experiment 3. Chronic withdrawal severity

Dependence induction—This study examined withdrawal severity following continuous administration of EtOH vapor by inhalation for 72 hours (Metten, Crabbe 2005). Male mice of each genotype were divided into two groups: one group was exposed only to air in the chambers, while the other group was exposed to volatilized EtOH. Within each genotype, mice within a cage were pseudorandomly assigned to either the air or EtOH condition. Mice in the EtOH groups were initially injected with EtOH (1.75 g/kg, 20% v/v in saline) to establish elevated BECs. Pyrazole HCl (68.1 mg/kg, in saline, ip), an alcohol dehydrogenase inhibitor, was given combined with the EtOH injection to inhibit EtOH metabolism and stabilize BECs. Mice in the Control groups were injected with saline with pyrazole on Day 1 and placed in identical chambers where they inhaled only air. Twenty-four (Day 2) and 48 (Day 3) hours later, all mice were removed from the chambers, injected with pyrazole again, and replaced in the chambers. After 72 hours of inhalation, all mice were removed from the chambers and a 20ul blood sample was drawn from the tip of their nicked tail. Control mice had their tails nicked but no blood was withdrawn.

Each morning during inhalation, a few animals had blood samples taken to assess their BEC before injection of all mice with pyrazole. Adjustments to the EtOH vapor concentration were made in an attempt to maintain average BEC at 2.00 mg/ml.

Withdrawal testing—Immediately after removal from vapor at 72 hours and before blood sampling, all mice were scored for HIC severity and weighed. HIC scoring was repeated each hour for 12 hours and at hours 24 and 25, using the same procedure described above. Because scores at hour 25 had not returned to baseline (see Results), we scored mice again at hours 30 and 31. To index HIC withdrawal severity, the area under the curve (AUC) was computed. A peak value was also computed, defined as the highest running average of three consecutive scores that contained the maximum HIC score for that animal. If there was a tie, the earliest occurrence was considered “peak.” Latency to peak was defined as the withdrawal hour of the first maximum score within the peak.

Results

Experiment 1. Hypothermic tolerance

Day 1 Temperature response time curves for the EEE group receiving their first EtOH injection were very similar to those seen in Experiment 2 in the companion manuscript, while animals given saline showed little temperature change from baseline. Day 3 response time courses were attenuated in the EEE groups, consistent with tolerance development (data and analyses not shown). To assess differences in tolerance between groups, we calculated and analyzed total hypothermic response as the AUC on Day 3, first including sex

as a factor. For both HDID-1 vs HS and HDID-2 vs HS comparisons, EEE groups showed less hypothermia than SSE groups [$F(1,47-48) = 10.3$, $p < 0.01$] and there were significant 3-way interactions of genotype, treatment and sex [$F(1,47-48) = 4.5$, $p < 0.05$]. We therefore analyzed the data from the sexes separately (Figure 1). Main effects of treatment within sex were always significant [all $F(1,21-27) = 5.0$, $p < 0.05$]. For the first replicate, interactions of genotype and treatment tended to reach significance only for the HDID-1 vs HS females [$F(1,27) = 3.1$, $p = 0.09$], where HS ($p = 0.04$) but not HDID-1 female mice showed significant tolerance (Figure 1A: AUC day 3). The apparently similar results in female HDID-2 vs HS mice were not supported by a significant interaction. The apparent difference between HDID-1 and HS males (Figure 1B: AUC day 3), with HDID-1 tending to show *greater* tolerance than HS, also was not supported by a significant interaction ($p = 0.17$), but the second replicate vs HS males tended to show an interaction of genotype $\times$ treatment [$F(1,22) = 3.2$, $p = 0.09$]. HDID-2 ($p = 0.02$) but not HS males showed tolerance.

BECs taken after the 120 minute temperature on Day 3 were analyzed to see whether metabolic tolerance could have developed. Across the 77 animals, average BEC was 2.16 ± 0.05 mg/ml. Mean BECs ranged between 1.77 and 2.61 mg/ml across the 12 genotype $\times$ treatment $\times$ sex conditions (total range = 1.08 - 3.36 mg/ml). ANOVAs including sex showed significant main effects of treatment and sex in both replications [all but one $F(1,43-44) = 3.8$, $p < 0.05$]; however, the main effect of sex only tended toward significance for the HDID-2 vs HS comparison [$F(1,44) = 3.4$, $p = 0.07$]. Neither interaction was significant [both $F(1,43-44) = 2.0$, $p > 0.10$]. Females had higher BECs than males within each genotype $\times$ treatment group comparison. Consistent with the development of metabolic tolerance, EEE groups had lower average BECs than SSE groups within each genotype $\times$ sex comparison.

To estimate the contribution of metabolic tolerance, we regressed Day 3 total hypothermic response on BEC separately for each replicate, including data from both SSE and EEE groups. For HDID-1 vs HS, BEC explained 18% of the variance (R^2) in total hypothermic response ($P=0.002$), and the relationship was even stronger in HDID-2 vs HS ($R^2 = 0.31$, $P<0.0001$). To parallel the analyses of uncorrected temperature responses, we then recalculated the regressions for each sex and replicate combination separately. These relationships showed that BEC explained between 10% (HDID-1 vs HS males) and 41% (HDID-2 vs HS females) of the variance in total hypothermic response.

To estimate the importance of functional tolerance after metabolic tolerance had been accounted for, we therefore expressed each animal's total hypothermic response as a residual from the regression on BEC and analyzed these values on the assumption that comparisons between SSE and EEE groups after the contribution of BEC had been statistically removed provided a reasonable estimate of functional tolerance. For HDID-1 vs HS females, a significant interaction of genotype $\times$ treatment was found [$F(1,24) = 6.0$, $p < 0.05$]; Tukey's *post hoc* tests showed that HS ($p = 0.04$) but not HDID-1 females showed lower total hypothermic responses in their EEE group vs SSE group, i.e., functional tolerance (Figure 1A: residual day 3). For the comparison of male HDID-1 vs HS mice, no main effects or interactions reached significance, but a genotype $\times$ treatment trend [$F(1,19) = 3.2$, $p = 0.09$] suggested that some of the tolerance seen in uncorrected scores in HDID-1 males may have been functional in nature (Figure 1B: residual day 3). Analyses of residual scores for the second replicate yielded results qualitatively similar to those seen in the HDID-1 vs HS comparisons; that is, HDID-2 females appeared to show less, and HDID-2 males greater, tolerance than HS (Figure 1 A,B: residual day 3). The interaction of genotype and treatment approached significance for the HDID-2 males [$F(1,21) = 3.3$, $p = 0.08$]; a *post hoc* test showed that HDID-2 ($p = 0.03$) but not HS showed tolerance.

Across the two analyses, these data suggest that significant tolerance developed to EtOH's hypothermic effects. For the most part, that tolerance appeared to be functional, although a small degree of metabolic tolerance also developed. The genotypic differences in tolerance appeared to be sex-dependent (see Discussion).

Mean body weights at the beginning of tolerance testing were analyzed by genotype and treatment within each sex and replicate comparison to parallel the analyses of hypothermic tolerance. Weights at the beginning were well matched for the main effects of treatment groups (all $F_s = 1.2$, $p_s > 0.10$). Female HDID-1s weighed significantly less than HS [19.8 ± 0.4 g vs 23.0 ± 0.6 g, respectively; $F(1,27)=17.7$, $p < 0.001$], and the interaction of genotype and treatment was not significant ($F = 1.1$, NS). For male HDID-1 vs HS mice, neither main genotypic or treatment group effects, nor their interaction, were statistically significant (all $F_s(1,20) < 1.8$, NS). For HDID-2 vs HS, there were no significant weight differences across treatment groups as either main effects or interactions with genotype for either females or males before injections began (all $F_s(1,22-25) = 1.2$, $p_s > 0.10$). There were trends toward genotypic differences for both sexes (females: $F = 2.7$, $p = 0.12$; males, $F = 3.6$, $p < 0.10$). Females HDID -2 mice tended to weigh less than HS, while males tended to weigh more.

Weight loss during the experiment was approximately -3.3% across all 82 mice. The main effect of treatment with EtOH for three days vs one day was to yield significantly greater weight loss in all genotypes and sexes (all $F_s(1,20-27) = 5.3$, $p_s < 0.05$). Neither genotype nor the interaction with treatment were significant in either replicate (all $F_s < 1$). The exception was HDID-2 vs HS males, where genotype ($F = 5.2$, $p < 0.05$) was significant: male HDID-2 mice did not lose weight overall ($+0.5 \pm 1.7\%$) while HS males did ($-3.3 \pm 0.4\%$). We conclude that weight loss did not contribute to the EtOH tolerance differences seen.

Experiment 2. Acute withdrawal severity

The HIC time course before EtOH injection (Time 0) and during EtOH withdrawal is depicted in Figure 2A. We first analyzed baseline HIC scores. Analyses including sex as a factor revealed a significant main effect of sex (males > females) for HDID-1 vs HS [$F(1,59) = 6.8$, $p < 0.01$], and an interaction of sex and genotype for HDID-2 vs HS [$F(1,60) = 5.0$, $p < 0.05$]. However, all data are reported collapsed on sex because sex-specific analyses did not differ in outcome (data not shown). Figure 2A shows that both HDID-1 and HDID-2 mice had significantly higher baseline HIC scores than HS ($t_s = 3.7$, $df = 61-62$, $p_s < 0.001$).

HIC scores were reduced to near zero when first assessed at 2 hours after EtOH injection, and animals reached their peak withdrawal between 6 and 9 hours after injection. Scores had declined to near baseline by 24 hours later. Figure 2B depicts the area under the 25 hour withdrawal HIC curve, corrected for baseline scores as described in the Methods. All three genotypes showed significant withdrawal (vs an area score of zero, which would represent scores equal to baseline). Neither selected line differed significantly from HS in withdrawal severity (both $t_s < 1$). Neither corrected peak scores nor latency to peak differed between genotypes for any comparison (data not shown).

Experiment 3. Chronic withdrawal severity

The HIC time course during EtOH withdrawal is depicted in Figure 3A. Peak withdrawal was reached between hours 4 and 7 after removal from the chambers, had subsided somewhat by hours 24-25, and even more by hours 30-31. To facilitate comparison with data collected in many other genotypes (Metten, Crabbe 2005; Metten *et al.* 2010; Terdal, Crabbe 1994), we indexed withdrawal severity using the area under the first 25 hours of the

withdrawal curve (Figure 3B). HDID-1 and HS mice displayed equivalent withdrawal severity. Analyses revealed significant main effects of treatment [$F(1,43) = 197.2$, $p < 0.0001$] but not genotype [$F(1,43) = 2.8$, $p = 0.10$]; nor was the interaction significant ($F < 1$). For HDID-2 vs HS, a similar pattern was seen. Main effects of treatment and genotype were significant [$F_s(1,45) = 226.6$ and 8.2 , respectively, $p_s < 0.01$], but the interaction was not ($F < 1$). Given the significantly higher baseline HIC scores seen in both HDID-1 and -2 mice in Experiment 2, we also compared the AUC for the air-treated mice in each replicate. As suggested from Figure 3B, HIC scores for air-treated control mice appeared to be higher in both selected lines than in the HS. The difference in control AUCs was significant for the HDID-2 vs HS comparison [$t(22) = 2.38$, $p = 0.03$] but not for HDID-1 vs HS [$t(22) = 1.28$, $p > 0.10$]. Neither corrected peak scores nor latency to peak differed between genotypes for any comparison (data not shown).

The range of genotypic mean BECs from mice sampled during inhalation was 1.27 - 2.20 mg/ml (Day 1) and 1.69 - 2.23 mg/ml (Day 2). HDID-1 mice tended to reach slightly higher, and HDID-2 mice slightly lower, BECs than HS during the first two days. BECs at the time of withdrawal for the EtOH treated groups were 1.99 ± 0.08 , 1.91 ± 0.08 and 1.84 ± 0.07 mg/ml for HDID-1, HDID-2 and HS, respectively; neither selected line differed significantly from HS in BEC at withdrawal ($t_s < 1.46$, $p_s > 0.10$).

Mean body weights at the beginning of inhalation testing were analyzed by genotype and treatment within each replicate comparison to parallel the analyses of withdrawal severity. Weights at the beginning were well matched across treatment groups ($F(1,43) = 1.3$ but were significantly lower in male HDID-1 than HS mice (29.1 ± 0.7 g vs 31.8 ± 1.0 g, respectively; [$F(1,43) = 4.5$, $p < 0.05$]), and the interaction of genotype and treatment was not significant ($F < 1$). For HDID-2 vs HS, there were no apparent (or significant) weight differences before inhalation [all $F_s(1,45) \leq 2.7$, $p_s > 0.10$]. Mice lost approximately -13% body weight during inhalation exposure. For the HDID-1 vs HS comparison, the main effects of genotype and treatment, and their interactions, were all non-significant [$F_s(1,42) \leq 2.5$, $p_s > 0.10$]. HDID-2 mice lost less weight than HS ($-10.0 \pm 0.9\%$ vs $-14.0 \pm 0.8\%$, respectively; [$F(1,45) = 7.8$, $P < 0.01$]), but percent weight loss did not differ as a function of treatment or the treatment $\times$ genotype interaction ($F_s < 1$).

We conclude that neither any effects of the expected modest weight loss nor differences in delivered EtOH dose could have masked a genotype difference in withdrawal severity. Neither could have the differences in air-treated HIC scores led us to spurious inferences regarding relative sensitivity to EtOH effects.

Discussion

In the hypothermia tolerance study, a complex pattern of results was seen, evidenced by significant 3-way interactions of treatment, genotype and sex for both replicates of the DID selection. Consideration of only uncorrected temperature scores showed clear tolerance development when looking at data within sex for each replicate. Thus, the experiment was successful at engendering EtOH tolerance. Interpretation becomes less clear when the data are considered within each sex separately, because what appear to be substantial genotypic effects interacted with sex. BEC predicted a significant proportion of total hypothermic response in most cases, so we tried to estimate the possible contributions of metabolic vs functional tolerance by comparing uncorrected hypothermia scores with those expressed as a residual from regression on BEC. We acknowledge that statistical rigor for these comparisons was limited by relatively low N for assessing the interactions of genotype and treatment within sex and replicate. We believe that most of the tolerance shown here was functional, and to the extent that metabolic tolerance contributed, it did not affect

interpretation of treatment, sex or genotypic differences. To convince the reader of this interpretation, we suggest visual comparisons of the left with the right half of Figure 1A (for females) and 1B (for males). The pattern of differences for each sex is strikingly similar. When the contribution of BEC was statistically removed (i.e., the residual scores), the genotype $\times$ treatment interactions for females vs HS in both replicates were significant; in contrast, analyses of uncorrected scores resulted only in a statistical trend toward an interaction for HDID-2 vs HS females. The analogous interactions for males yielded only statistical trends; we believe this was likely due to the relatively greater within-group variability of the male vs the female groups. Power to detect interactions in factorial ANOVAs is less than that needed to detect main effects (Wahlsten 2007), and we were not expecting to encounter three-way interactions.

A formal and powerful assessment of metabolic vs functional tolerance would require additional experiments, and could best be addressed by administering multiple injections to one set of groups (like our EEE groups) and then comparing their response on the last day with several groups of "SSE" mice, with each pair of SSE vs EEE groups given different doses of EtOH. This would allow construction of the dose effect curves and allow comparison of their slopes [see (Crabbe *et al.* 1979)]. It should be noted that the overall degree of tolerance seen in the current studies was not large. The three, daily injections we employed resemble both rapid and chronic tolerance, but most studies of chronic tolerance use several more daily (or twice daily) injections or a period of chronic exposure through drinking or vapor inhalation. Thus, extending EtOH injections for more days to elicit maximal chronic rather than rapid tolerance might clarify the picture. Tolerance is seen in nearly all genotypes we have studied when 5-8 daily EtOH injections are used (Crabbe *et al.* 1982;Crabbe 1994;Crabbe *et al.* 2006).

Whether EtOH hypothermic tolerance is a correlated response to selection on DID BEC is a more difficult question to answer. Here, interpretation is first made difficult by the obvious interactions of sex with genotype. Considering only the control HS mice, females clearly developed tolerance, while males did not. HDID-1 and HDID-2 female mice failed to develop significant tolerance, with or without accounting for BEC (Figure 1A). This pattern of results supports the hypothesis that resistance to the development of hypothermic tolerance is a correlated response. Because the degree of hypothermic tolerance development in mice is genetically correlated with initial hypothermic sensitivity [(Browman *et al.* 2000;Crabbe *et al.* 1982;Crabbe *et al.* 2006), but see (Radcliffe *et al.* 2005)], this agrees with our findings in the companion paper (Crabbe et al, in press) that selection for DID BEC resulted in reduced sensitivity to EtOH hypothermia in females. Thus, the lack of hypothermic tolerance in HDID-1 and -2 females may result from their lesser initial response to EtOH than HS. In contrast, both male HDID-1 and HDID-2 mice appeared to develop tolerance while HS did not (Figure 1B), although these differences did not reach statistical significance. It is possible that the males from the selected lines developed tolerance more rapidly than HS males, as they did not differ in initial response in this experiment. We do not know why our earlier study showed less initial hypothermic sensitivity in both female and male HDID mice than in HS (Crabbe et al, in press). We tentatively conclude that hypothermic tolerance is a correlated response, but that it is sex dependent.

We found only four papers reporting tolerance to EtOH hypothermia in rodents of both sexes given multiple injections of EtOH. One paper with rats showed greater tolerance in females (Light *et al.* 1990) while two others favored males (Khanna *et al.* 1985;Webb *et al.* 2002). The Webb study also found greater tolerance in males for the loss of righting reflex, and their data suggest that males developed more acute functional tolerance; this could be taken to support the hypothesis of faster tolerance development in male HDID-1 and -2

mice. A study with mice lacking functional adenosine A2A receptors showed rapid tolerance development to EtOH hypothermia only in female wild types, but not in either knockout females or males of either genotype (Naassila *et al.* 2002).

We found no studies reporting a crossover interaction of sex with genotype for EtOH tolerance. Gehle and Erwin found that female mice of some LSxSS RI strains showed greater acute functional tolerance on the dowel test than males, while for other strains no sex differences were seen (Gehle, Erwin 2000). Gill and Deitrich also examined acute functional tolerance on a fixed speed rotarod task in LS and SS mice of both sexes. They found a genotype $\times$ sex interaction favoring a greater acute functional tolerance difference (SS>LS) between female mice than the difference seen in males (Gill, Deitrich 1998).

The “differentiator hypothesis” (Newlin, Thomson 1990) suggests that human studies of acute alcohol challenge show differences between subjects at low or high risk for developing dependence later in life for two reasons. First, at-risk subjects are more sensitive to effects of EtOH that occur soon after drinking: these include stimulation, elevated mood, and subjective “high.” Some time later during the acute session, at-risk subjects report experiencing less sedation and intoxication, and display other signs of blunted response to EtOH. It is hypothesized that they are less sensitive later due to the development of more acute functional tolerance (Newlin, Thomson 1990). Our companion report (Crabbe et al, in press) found that HDID mice were less sensitive than HS to acute EtOH hypothermia. However, there was sparse evidence for differential sensitivity of HDID vs HS mice across a range of other tasks probing stimulant and sedative responses to acute EtOH challenge. In the current studies, HDID mice tended to develop *less*, not more, tolerance than HS to EtOH hypothermia. However, the form of tolerance probed here resembles rapid or chronic tolerance more than acute functional tolerance. It would be of interest to test HDID and HS mice for acute functional tolerance to EtOH (Ponomarev, Crabbe 2004;Erwin, Deitrich 1996) as this is the closest equivalent to tolerance that could affect apparent sensitivity differences shortly after injections. The extant data from this and the companion report (Crabbe et al, in press) do not align well with the postulate derived from studies of at-risk humans (Schuckit et al. 2009;Schuckit, Smith 2001) that low level of response to EtOH predicted alcoholism risk. However, the animal tests we have employed are not clearly related to the human traits tested, as noted elsewhere (Crabbe *et al.* in press;Crabbe *et al.* 2010a).

There were clear EtOH withdrawal effects in all 3 genotypes, after either a single, acute high-dose injection or following chronic vapor inhalation. However, there were no genetic differences in withdrawal severity. It would not have been a surprise to find that HDID mice displayed less severe acute and chronic EtOH withdrawal HIC scores than HS. A survey of several genetic animal models has revealed a substantial negative genetic correlation between two-bottle preference drinking and withdrawal severity (Metten *et al.* 1998), and subsequent studies in both rats and mice have strengthened the hypothesis of similar genetic control of these traits [e.g., (Chester *et al.* 2003;Chester *et al.* 2002)]. However, DID and two-bottle preference drinking are only partially influenced by common genetic factors (Crabbe *et al.* 2011b;Crabbe *et al.* 2009). The current data suggest that the genetic influence shared between withdrawal and preference drinking is largely different from that shared between the two drinking phenotypes.

It is interesting that both selected lines seem to have developed greater basal handling induced convulsion scores than HS, a sign of a correlated response. The HIC was first reported under that name by Goldstein (Goldstein 1972) as a sensitive sign of EtOH withdrawal severity. The HIC resembles signs from earlier reports by Chance (Chance, Yaxley 1950;Chance 1953a;Chance 1953b), who elicited a convulsion by holding a mouse

by the tail and jerking his hand down rapidly. That the HIC reflects central nervous system excitability may be inferred from several reports that it is exacerbated by a wide range of doses of convulsant drugs that are too low to elicit the full tonic and/or clonic convulsions characteristic of the particular drug (Crabbe *et al.* 1991b; Crabbe *et al.* 1981). HICs are exacerbated during withdrawal from many agents that depress central nervous system activity, including barbiturates, benzodiazepines, alcohols, and other sedative-hypnotics (Belknap *et al.* 1987; Goldstein 1972; Crabbe *et al.* 1991a; Reilly *et al.* 2000; Crabbe 1992), and withdrawal-related HICs are suppressed by those same agents and other drugs (Littleton *et al.* 1990; Olive, Becker 2008; Beadles-Bohling, Wiren 2006).

Despite the wide use of the HIC to index drug withdrawal, little is known about its physiology, and its pharmacology is obviously promiscuous. Elevated baseline HICs emerged as a correlated response to selection for chronic EtOH withdrawal HIC severity in both Withdrawal Seizure-Prone selected lines, and virtually disappeared from the corresponding Withdrawal Seizure-Resistant lines (Crabbe *et al.* 1985). This likely occurred because selection was not based on a difference score between EtOH-withdrawing mice and saline-treated mice. WSP mice are generally about 10% more sensitive than WSR mice to the effects of convulsants, but this is not true for all drugs and the pharmacological differences suggested from seizure susceptibility are complex [for review, see (Metten, Crabbe 1996; Crabbe 1996)]. It would be of interest to compare the HDID selected lines and HS for susceptibility to a range of convulsants and other seizure-inducing treatments to explore the basis for the elevated basal HIC further.

We found that differences in EtOH metabolism could not explain the observed differences between HDID lines and HS in acute sensitivity to EtOH (Crabbe et al, in press). For the chronic withdrawal data in Experiment 3, there was no serious possibility that differences in metabolism could affect results, as such differences were explicitly disallowed by matching BECs across genotypes during each day of inhalation. Neither could BEC at the time of the test explain differences in hypothermic tolerance in Experiment 1. Much prior data suggests that genetic differences in behavioral response to EtOH are unrelated to genetic differences in BEC. The modest reductions in body weight seen during the chronic hypothermia and withdrawal tests also could not explain the patterns of genotypic differences.

As we explained in the companion manuscript (Crabbe et al, in press), it is somewhat difficult to interpret the results from the studies with HDID-2 at this relatively early point in selection. Thus, it is possible that some of the responses we tested here could come to differ between HDID-2 and HS in future generations. In conclusion, selection for high BEC after DID has not yielded genotypes that differ in withdrawal severity from unselected HS mice. However, female mice from these selected lines appear to develop less EtOH hypothermic tolerance than HS. Whether male HDID mice develop more tolerance, or develop it faster than HS will require further experiments. Finally, many other behavioral domains remain unexplored in HDID mice. Anxiety- or depression-like behaviors, impulsive behaviors, sensitivity to reward or punishment, learning ability, and the sensitivity to effects of EtOH in any of these domains could be related to the elevated DID-BEC phenotype, and will be the subject of future experiments.

Acknowledgments

These studies were supported by grants AA010760, AA013519, and T32 AA07468 from the NIAAA, and by a grant from the US Department of Veterans Affairs. AMC and LCK were supported by grant AA07468 from the NIAAA. We thank Mark Rutledge-Gorman for assistance with the manuscript.

References

- Aufreere G, Menez JF, Beauge F, Duffy O, Le Bourhis B. Tolerance and dependence in high alcohol sensitivity (HAS) and low alcohol sensitivity (LAS) rats after chronic alcohol intoxication by inhalation. *Alcohol Alcohol*. 1994; 29(4):397–401. [PubMed: 7986276]
- Beadles-Bohling AS, Wiren KM. Anticonvulsive effects of kappa-opioid receptor modulation in an animal model of ethanol withdrawal. *Genes Brain Behav*. 2006; 5:483–496. [PubMed: 16923153]
- Belknap JK, Laursen SE, Crabbe JC. Ethanol and nitrous oxide produce withdrawal-induced convulsions by similar mechanisms in mice. *Life Sci*. 1987; 41:2033–2040. [PubMed: 3669909]
- Belknap JK, Metten P, Beckley EH, Crabbe JC. Multivariate analyses reveal common and drug-specific genetic influences on responses to four drugs of abuse. *Trends Pharmacol Sci*. 2008; 29:537–543. [PubMed: 18774184]
- Bennett B, Downing C, Carosone-Link P, Ponicsan H, Ruf C, Johnson TE. Quantitative trait locus mapping for acute functional tolerance to ethanol in the L $\times$ S recombinant inbred panel. *Alcohol Clin Exp Res*. 2007; 31:200–208. [PubMed: 17250610]
- Browman KE, Rustay NR, Nikolaidis N, Crawshaw L, Crabbe JC. Sensitivity and tolerance to ethanol in mouse lines selected for ethanol-induced hypothermia. *Pharmacol Biochem Behav*. 2000; 67:821–829. [PubMed: 11166073]
- Chance MR, Yaxley DC. Central nervous function and changes in brain metabolite concentration. I. Glycogen and lactate in convulsing mice. *J Exp Biol*. 1950; 27:311–323. [PubMed: 14803615]
- Chance MRA. Central nervous function and changes in brain metabolite concentration. III. Glycogen in the normal behaviour of mice. *J Exp Biol*. 1953a; 30:468–474.
- Chance MRA. The posture of a falling mouse. *Br J Anim Behav*. 1953b; 1:118–119.
- Chen CS. Acquisition of behavioral tolerance to ethanol as a function of reinforced practice in rats. *Psychopharmacology*. 1979; 63:285–288. [PubMed: 113815]
- Chester JA, Blose AM, Froehlich JC. Further evidence of an inverse genetic relationship between innate differences in alcohol preference and alcohol withdrawal magnitude in multiple selectively bred rat lines. *Alcohol Clin Exp Res*. 2003; 27:377–387. [PubMed: 12658103]
- Chester JA, Price CS, Froehlich JC. Inverse genetic association between alcohol preference and severity of alcohol withdrawal in two sets of rat lines selected for the same phenotype. *Alcohol Clin Exp Res*. 2002; 26:19–27. [PubMed: 11821650]
- Crabbe JC. Antagonism of ethanol withdrawal convulsions in Withdrawal Seizure-Prone mice by diazepam and abecarnil. *Eur J Pharmacol*. 1992; 221:85–90. [PubMed: 1360903]
- Crabbe JC. Tolerance to ethanol hypothermia in HOT and COLD mice. *Alcohol Clin Exp Res*. 1994; 18:42–46. [PubMed: 8198225]
- Crabbe JC. A genetic animal model of alcohol withdrawal. *Alcohol Clin Exp Res*. 1996; 20(8):96A–100A.
- Crabbe JC, Belknap JK, Buck KJ. Genetic animal models of alcohol and drug abuse. *Science*. 1994; 264:1715–1723. [PubMed: 8209252]
- 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, Janowsky JS, Young ER, Kosobud A, Stack J, Rieger H. Tolerance to ethanol hypothermia in inbred mice: genotypic correlations with behavioral responses. *Alc Clin Exp Res*. 1982; 6:446–458.
- Crabbe, JC.; Kendler, KS.; Hitzemann, RJ. Modeling the diagnostic criteria for alcohol dependence with genetic animal models. In: Spanagel, R.; Sommer, WH., editors. *Curr Topics Behav Neurosci*. Springer-Verlag; Berlin: in press
- Crabbe, JC.; Kosobud, A.; Feller, DJ.; Phillips, TJ. Use of selectively bred mouse lines to study genetically correlated traits related to alcohol. In: Kuriyama, K.; Takada, A.; Ishii, H., editors. *Biomedical and Social Aspects of Alcohol and Alcoholism*. Elsevier Science Publishers BV; Amsterdam: 1988. p. 427–430.
- Crabbe JC, Kosobud A, Young ER, Tam BR, McSwigan JD. Bidirectional selection for susceptibility to ethanol withdrawal seizures in *Mus musculus*. *Behav Genet*. 1985; 15:521–536. [PubMed: 4096679]

- Crabbe JC, Merrill CD, Belknap JK. Acute dependence on depressant drugs is determined by common genes in mice. *J Pharmacol Exp Ther.* 1991a; 257(2):663–667. [PubMed: 2033513]
- Crabbe JC, Merrill CD, Belknap JK. Effects of convulsants on handling-induced convulsions in mice selected for ethanol withdrawal severity. *Brain Res.* 1991b; 550:1–6. [PubMed: 1888987]
- 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 CH, 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, Phillips TJ, Belknap JK. The complexity of alcohol drinking: studies in rodent genetic models. *Behav Genet.* 2010b; 40:737–750. [PubMed: 20552264]
- Crabbe JC, Rigter H, Uijlen J, Srijbos C. Rapid development of tolerance to the hypothermic effect of ethanol in mice. *J Pharmacol Exp Ther.* 1979; 208(1):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.* 2011b; 45:427–440. [PubMed: 21194877]
- Crabbe JC, Young ER, Janowsky J, Rigter H. Pyrazole exacerbates handling-induced convulsions in mice. *Neuropharmacology.* 1981; 20:605–609. [PubMed: 7242876]
- Crabbe JC, Young ER, Kosobud A. Genetic correlations with ethanol withdrawal severity. *Pharmacol Biochem Behav.* 1983; 18(1):541–547. [PubMed: 6685310]
- Deitrich RA. Selective breeding for initial sensitivity to ethanol. *Behav Genet.* 1993; 23:153–162. [PubMed: 8512530]
- Deitrich RA, Bludeau P, Erwin VG. Phenotypic and genotypic relationships between ethanol tolerance and sensitivity in mice selectively bred for initial sensitivity to ethanol (SS and LS) or development of acute tolerance (HAFT and LAFT). *Alcohol Clin Exp Res.* 2000; 24:595–604. [PubMed: 10832900]
- Drews E, Racz I, Lacava AD, Barth A, Bilkei-Gorzo A, Wienker TF, Zimmer A. Quantitative trait loci contributing to physiological and behavioural ethanol responses after acute and chronic treatment. *Int J Neuropsychopharmacol.* 2010; 13:155–169. [PubMed: 19691874]
- Erwin VG, Deitrich RA. Genetic selection and characterization of mouse lines for acute functional tolerance to ethanol. *J Pharmacol Exp Ther.* 1996; 279:1310–1317. [PubMed: 8968355]
- Friedman, HJ. Assessment of physical dependence on and withdrawal from ethanol in animals. In: Rigter, H.; Crabbe, JC., editors. *Alcohol Tolerance and Dependence.* Elsevier/North-Holland Biomedical Press; Amsterdam: 1980. p. 93-121.
- Gehle VM, Erwin VG. The genetics of acute functional tolerance and initial sensitivity to ethanol for an ataxia test in the LSxSS RI strains. *Alc Clin Exp Res.* 2000; 24:579–587.
- Gill K, Deitrich RA. Acute tolerance to the ataxic effects of ethanol in short-sleep (SS) and long-sleep (LS) mice. *Psychopharmacology.* 1998; 136:91–98. [PubMed: 9537687]
- Goldstein DB. Relationship of alcohol dose to intensity of withdrawal signs in mice. *J Pharmacol Exp Ther.* 1972; 180:203–215. [PubMed: 5062297]
- Hanchar HJ, Dodson PD, Olsen RW, Otis TS, Wallner M. Alcohol-induced motor impairment caused by increased extrasynaptic GABA(A) receptor activity. *Nat Neurosci.* 2005; 8:339–345. [PubMed: 15696164]
- Kalant H. Current state of knowledge about the mechanisms of alcohol tolerance. *Addict Biol.* 1996; 1:133–141. [PubMed: 12893473]
- Kalant H. Research on tolerance: What can we learn from history? *Alcohol Clin Exp Res.* 1998; 22:67–76. [PubMed: 9514287]
- Kalant H, LeBlanc AE, Gibbins RJ. Tolerance to, and dependence on, some non-opiate psychotropic drugs. *Pharmacol Rev.* 1971; 23:135–191. [PubMed: 4398655]

- Khanna JM, Le AD, LeBlanc AE, Shah G. Initial sensitivity versus acquired tolerance to ethanol in rats selectively bred for ethanol sensitivity. *Psychopharmacology (Berl)*. 1985; 86:302–306. [PubMed: 3929300]
- Koob GF, Sanna PP, Bloom FE. Neuroscience of addiction. *Neuron*. 1998; 21:467–476. [PubMed: 9768834]
- Lê, AD.; Mayer, JM. Aspects of alcohol tolerance in humans and experimental animals. In: Deitrich, RA.; Erwin, VG., editors. *Pharmacological Effects of Ethanol on the Nervous System*. CRC Press; Boca Raton, FL: 1996. p. 251-268.
- Light KE, Goad LD, Fletcher SK, Serbus DC. Tolerance to ethanol-induced hypothermia in adult rats following postnatal ethanol exposure. *Alcohol*. 1990; 7:517–522. [PubMed: 2261090]
- Littleton JM, Little HJ, Whittington MA. Effects of dihydropyridine calcium channel antagonists in ethanol withdrawal; doses required, stereospecificity and actions of Bay K 8644. *Psychopharmacology*. 1990; 100:387–392. [PubMed: 1690434]
- Mellanby, E. Alcohol: its absorption into and disappearance from the blood under different conditions. London: Medical Research Committee; 1919.
- Metten, P.; Crabbe, JC. Dependence and withdrawal. In: Deitrich, RA.; Erwin, VG., editors. *Pharmacological Effects of Ethanol on the Nervous System*. CRC Press; Boca Raton FL: 1996. p. 269-290.
- Metten P, Crabbe JC. Alcohol withdrawal severity in inbred mouse (*Mus musculus*) strains. *Behav Neurosci*. 2005; 119:911–925. [PubMed: 16187819]
- Metten P, Phillips TJ, Crabbe JC, Tarantino LM, McClearn GE, Plomin R, Erwin VG, Belknap JK. High genetic susceptibility to ethanol withdrawal predicts low ethanol consumption. *Mamm Genome*. 1998; 9:983–990. [PubMed: 9880664]
- Metten P, Sorensen ML, Cameron AJ, Yu CH, Crabbe JC. Withdrawal severity after chronic intermittent ethanol in inbred mouse strains. *Alc Clin Exp Res*. 2010; 34:1552–1564.
- Naassila M, Ledent C, Daoust M. Low ethanol sensitivity and increased ethanol consumption in mice lacking adenosine A2A receptors. *J Neurosci*. 2002; 22:10487–10493. [PubMed: 12451148]
- Newlin DB, Thomson JB. Alcohol challenge with sons of alcoholics: a critical review and analysis. *Psychol Bull*. 1990; 108(3):383–402. [PubMed: 2270234]
- Olive MF, Becker HC. Effects of the mGluR2/3 agonist LY379268 and the mGluR5 antagonist MPEP on handling-induced convulsions during ethanol withdrawal in mice. *Alcohol*. 2008; 42:191–197. [PubMed: 18420113]
- Phillips, TJ.; Crabbe, JC. Behavioral studies of genetic differences in alcohol action. In: Crabbe, JC.; Harris, RA., editors. *The Genetic Basis of Alcohol and Drug Actions*. Plenum Press; New York: 1991. p. 25-104.
- Ponomarev I, Crabbe JC. Characterization of acute functional tolerance to the hypnotic effects of ethanol in mice. *Alc Clin Exp Res*. 2004; 28:991–997.
- Ponomarev, I. Ph D. Thesis. Portland, OR: Oregon Health & Science University; 2002. Mechanisms of rapid adaptation to hypnotic effects of ethanol in mice: a pharmacogenetic approach; p. 1-115.
- Radcliffe RA, Floyd KL, Drahnak JA, Deitrich RA. Genetic dissociation between ethanol sensitivity and rapid tolerance in mouse and rat strains selectively bred for differential ethanol sensitivity. *Alcohol Clin Exp Res*. 2005; 29:1580–1589. [PubMed: 16205358]
- Radcliffe RA, Floyd KL, Lee MJ. Rapid ethanol tolerance mediated by adaptations in acute tolerance in inbred mouse strains. *Pharmacol Biochem Behav*. 2006; 84:524–534. [PubMed: 16899285]
- Radcliffe RA, Hoffmann SE, Deng XS, Asperi W, Fay T, Bludeau P, Erwin VG, Deitrich RA. Behavioral characterization of alcohol-tolerant and alcohol-nontolerant rat lines and an F(2) generation. *Behav Genet*. 2004; 34:453–463. [PubMed: 15082942]
- Reilly MT, Crabbe JC, Rustay NR, Finn DA. Acute neuroactive steroid withdrawal in Withdrawal Seizure-Prone and Withdrawal Seizure-Resistant mice. *Pharmacol Biochem Behav*. 2000; 67:709–717. [PubMed: 11166061]
- 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 CH, 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]
- 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]
- Schuckit MA. An overview of genetic influences in alcoholism. *J Subst Abuse Treat.* 2009; 36:S5–14. [PubMed: 19062348]
- 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]
- Tabakoff, B.; Hoffman, PL. A neurobiological theory of alcoholism. In: Chaudron, CD.; Wilkinson, DA., editors. *Theories on Alcoholism.* Addiction Research Foundation; Toronto, CA: 1988. p. 29-72.
- Terdal ES, Crabbe JC. Indexing withdrawal in mice: matching genotypes for exposure in studies using ethanol vapor inhalation. *Alcohol Clin Exp Res.* 1994; 18:542–547. [PubMed: 7943652]
- Wahlsten, D. Sample size requirements for experiments on laboratory animals. In: Jones, BC.; Mormede, P., editors. *Neurobehavioral Genetics Methods and Applications.* Second. CRC Press; Boca Raton, FL: 2007. p. 149-168.
- Webb B, Burnett PW, Walker DW. Sex differences in ethanol-induced hypnosis and hypothermia in young Long-Evans rats. *Alcohol Clin Exp Res.* 2002; 26:695–704. [PubMed: 12045479]

Figure 1.

Mean $\pm$ total hypothermic response on Day 3 in SSE groups receiving their first EtOH injection (dark bars) and EEE groups receiving their third (light bars). Panel A shows data for females, Panel B for males. Left half of each panel shows uncorrected scores [Total hypothermic response (AUC day 3)], and right half shows residuals from regression of uncorrected scores on BEC [Total hypothermic response (residual day 3)]. $N = 5-8$ per sex/genotype/treatment group. Note that the bars depicting HS scores for residuals [Total hypothermic response (residual day 3)] are a composite, based on averaging the two values derived from the separate regressions of each replicate HDID line and HS. These two values did not differ by more than 11% for any average shown.

Figure 2.

Panel A: Mean $\pm$ SE handling induced convulsion scores before (Time 0) and after injection (arrow) with 4.0 g/kg EtOH. N = 32, 31, and 32 for HS, HDID-1 and HDID-2, respectively. Mice were scored for LORR between hours 0 and 2 (see companion manuscript, Crabbe et al, in press). Panel B: Corrected area under the HIC curve for data depicted in Panel A.

Figure 3.

Panel A: Mean $\pm$ SE handling induced convulsion scores from the time of removal from the inhalation chambers (Time 0) and 31 hr later. N = 11, 12 and 14 for HS, HDID-1 and HDID-2 EtOH groups, respectively. N = 11, 10 and 11 for the three respective air control groups. Panel B: Area under the HIC curve for hours 0-25 for the data depicted in Panel A. EtOH treated mice are shown in dark bars, and air treated controls in light bars.