A Line of Mice Selected for High Blood Ethanol Concentrations Shows Drinking in the Dark to Intoxication

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Background: Many animal models of alcoholism have targeted aspects of excessive alcohol intake (abuse) and dependence. In the rodent, models aimed at increasing alcohol self-administration have used genetic or environmental manipulations, or their combination. Strictly genetic manipulations (e.g., comparison of inbred strains or targeted mutants, selective breeding) have not yielded rat or mouse genotypes that will regularly and voluntarily drink alcohol to the point of intoxication. Although some behavioral manipulations (e.g., scheduling or limiting access to alcohol, adding a sweetener) will induce mice or rats to drink enough alcohol to become intoxicated, these typically require significant food or water restriction or a long time to develop. We report progress toward the development of a new genetic animal model for high levels of alcohol drinking.

Methods: High Drinking in the Dark (HDID-1) mice have been selectively bred for high blood ethanol concentrations (BEC, ideally exceeding 100 mg%) resulting from the ingestion of a 20% alcohol solution.

Results: After 11 generations of selection, more than 56% of the population now exceeds this BEC after a 4-hour drinking session in which a single bottle containing 20% ethanol is available. The dose of ethanol consumed also produced quantifiable signs of intoxication.

Conclusions: These mice will be useful for mechanistic studies of the biological and genetic contributions to excessive drinking.

Key Words: DID, ethanol consumption, genetic animal models, HDID, intoxication, mouse, pharmacogenetics, selective breeding

Alcoholism is a complex psychiatric disorder with strong genetic as well as environmental risk factors, the interactions of which affect individual risk (1). Most laboratory neurobiological research targeting alcoholism has employed rodents, and the usual measure of self-administration is the two-bottle preference test in which animals are offered a bottle of tap water versus a bottle containing an alcohol solution. That genotype strongly influences preference drinking has been known for many years (2,3), and differences in preference for drinking alcohol among inbred mouse strains are stable across laboratories and over decades (4). However, inbred strains differ for many phenotypes, and not all alleles leading to high drinking are overrepresented in the genotypes of even the highest drinking strains. Thus, alcohol research has benefited from frequent use of the technique of selective breeding. By mating highly preferring individuals repeatedly over generations, several lines of rats and mice have been produced that preferentially drink nearly all their daily fluid from a bottle of unflavored 10% ethanol despite the availability of unadulterated water. Because all of these lines have been selected for nearly identical drinking phenotypes, the comparison of results across such lines as Preferring (P) versus Non-Preferring (NP) rats, High and Low Alcohol Drinking (HAD/LAD) rats, Alko Alcohol (AA) and Non-Alcohol (ANA) rats, and High and Low Alcohol Preferring (HAP/LAP) mice has been very informative (see several recent reviews of these lines in *Addiction Biology* 2006, Vol 11). We have learned, for example, that low levels of brain serotonin are associated with high-preferring genotypes (5).

One of the limits of alcohol preference drinking studies has been that rodents, unlike humans, rarely self-administer enough alcohol to become intoxicated. Rodents drink in bouts rather than continuously, and most intake occurs during the circadian dark. It appears that as their rate of intake approaches the maximal rate at which they can metabolize ethanol and eliminate it, they slow their drinking (6,7). Many humans would like to reach this level of self-regulation! Even those lines genetically selected for preference generally stop drinking when their blood ethanol concentration (BEC) reaches about 50 to 70 mg% (7). These levels correspond roughly to the legal driving limit in most states.

However, some of the genetically predisposed rodent lines will self-administer significant amounts of alcohol under certain conditions (8,9). Thus, selection can produce animals that will voluntarily exceed BECs of 100 mg% and that become physically dependent. However, the existing protocols for achieving this behavior involve selectively examining those animals at the extremely high end of the drinking distribution, as well as either many weeks of testing or technically challenging procedures such as gastric cannulation. Many other procedures for achieving high BECs in rats or mice are also available, but they either involve food or water restriction, relatively complicated operant schedules, or long periods of study. For discussion of these requirements, see Supplement 1.

Therefore, there remains the need for rodent models of one of the key behavioral aspects of abusive human drinking, the tendency to drink excessively. In a recent study, C57BL/6J mice were exposed to gradually increasing concentrations of ethanol in water solutions for a 30-min period restricted to their circadian

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dark phase, when they are normally engaged in their highest levels of eating, drinking, and activity. After about a week, these mice were drinking enough alcohol to show signs of intoxication (10,11). We adapted these procedures substantially, and developed a procedure in which mice would drink ethanol to intoxication by the second day of exposure (12,13). Inbred strains differed in their propensity for drinking in the dark (DID), and the behavior appeared to be heritable (13). Here, we report a new genetic animal model, High Drinking in the Dark (HDID-1) mice, developed by selectively breeding for high BEC after a short period of access to an alcohol solution during the circadian dark.

Methods and Materials

Animals and General Husbandry

See Supplement 1. All procedures were approved by our Institutional Animal Care and Use Committee.

Drinking in the Dark

The mouse colony was illuminated during the "dark" phase with a red bulb (2 lumen/square foot, about 21.5 lux). Mice were individually housed in the same type of caging for 5–9 days before testing. During this period, mice were trained to drink from a water spout using a polycarbonate bottle with a stainless steel drinking spout (Ancare Corp., Bellmore, New York). For DID testing, the same type of drinking spout was inserted into 9 mL, calibrated polystyrene tubes for measurement of intake (for details, see Supplement 1). Testing was conducted starting between 50 and 119 days of age with the exception of one family of selected generation 9 (S9) tested at 47 days of age. Details regarding the drinking in the dark phenotype and how it is ascertained have been published (12,13). In addition, a detailed procedural protocol is available at http://www.scripps.edu/cnad/inia/modelmousedrinkingindark.pdf or from the authors on request. Previous studies showed that individual intake values for C57BL/6J mice for DID on the first day of exposure were not highly correlated with DID intakes on Days 2–4 but that intakes on the second day were well correlated with Days 3 and 4 (12) and for up to 12 further days of drinking (data not shown). Therefore, we elected to use a 2-day DID test.

Each mouse was weighed on Day 1 before the lights went out. On the same day, starting at 3 hours after lights off, the single water bottle was removed from the cage of each mouse and replaced with a single tube containing 20% ethanol in tap water (v/v). Tubes were read again at 2 hours, the volume change recorded, and each tube was replaced by the standard water bottle. On Day 2, the procedure was repeated exactly, but tubes were left in place at the 2 hours reading for an additional 2 hours. At 4 hours, a 20-μL blood sample was drawn from the periorbital sinus with a capillary tube. Care was taken to be as quiet as possible and to disturb the mice minimally.

The Selection Phenotype

We wanted to select mice drinking to intoxication without behaviorally testing them for intoxication. Because intake was imperfectly predictive of BEC and because the many lines selected for high intake rather than for intoxication do not readily drink to intoxicating BECs, we elected to breed selectively on BEC attained rather than intake in g/kg.

Selective Breeding

We initiated this selection with a founding population (selection generation zero, S0), of 82 female and 76 male HS/Npt mice, all the offspring of 25 HS/Npt pregnant dams (see Supplement 1). All mice were tested for DID for 2 days and the BEC determined. Generations S0 through S2 were maintained and tested within the Oregon Health & Science University (OHSU) facility. When S3 offspring were about 12–17 days old, the colony was moved to the Portland VA Medical Center Veterinary Medical Unit. VA and OHSU husbandry conditions were very similar. For the first five generations, we employed within-family selection, using a method we have employed for prior selections for alcohol phenotypes (14,15). Because response to selection was slower than anticipated, when selecting the S5 breeders to produce S6, we shifted to the use of individual selection, which we have since employed. Details for numbers of mice tested each generation are given in Table 1. For methods, see the Supplement 1.

Mice are deemed "HDID-1" because we are in the process of breeding a replicate line, using generally the same procedures. Data from HDID-2 mice, which are being selected from the outset using individual selection, will be reported after the selection has proceeded for more than the current five generations.

Ethanol Metabolism

Naive HDID-1 mice from the S11 generation were compared with HS/Npt mice after an injection of 2 g/kg ethanol (20% v/v in saline, intraperitoneally). Mice were 132–143 days old and comprised half male and half female per genotype (n = 6–7/line). Blood samples (20-μL) were obtained from the periorbital sinus from alternating eyes at four time points after injection: 15, 30, 60, and 120 min.

Two-Bottle DID

Naive male mice from second litters of the S9 generation, aged 55–68 days, were given a two-bottle preference version of the DID test (13). Thirty-eight mice were tested for 3 days with 2 hours access starting 3 hours after lights off. On the fourth day, access was extended for 4 hours. Half the mice were offered a single tube of 20% ethanol (v/v) each day (standard DID group). The other half of the mice were offered two identical tubes, one containing water and one containing ethanol, each day (two-bottle choice DID group). Mice within the latter groups were assigned to either left or right position of the ethanol tube, which remained the same each day, to mimic the procedure previously followed (13). A blood sample was taken from all mice at the end of the test on Day 4.

Intoxication Experiments

Naive mice from second litters of the S9 generation were pretested on the balance beam, tested for DID as described earlier, and then tested for performance on the balance beam and accelerating rotarod immediately following the second day of DID testing. For testing procedures, see Supplement 1.

Results

Response to Selection for DID

Selection on BEC at the end of the 4 hours drinking session on Day 2 of the DID test resulted in a 3.6-fold increase in the average BEC across 11 generations. Figure 1A shows the average BEC for each generation. BEC in the foundation population of HS/Npt mice (S0) averaged .30 mg/mL. Inverted open triangles indicate the average BEC of individuals selected as parents for each subsequent generation. For example, by S4, BEC in HDID-1 mice averaged .43 mg/mL. From this population, BEC in the 13 mating pairs that were chosen to produce the S5 generation averaged .83

Table 1. Generational Data on Selection for BEC after Drinking in the Dark

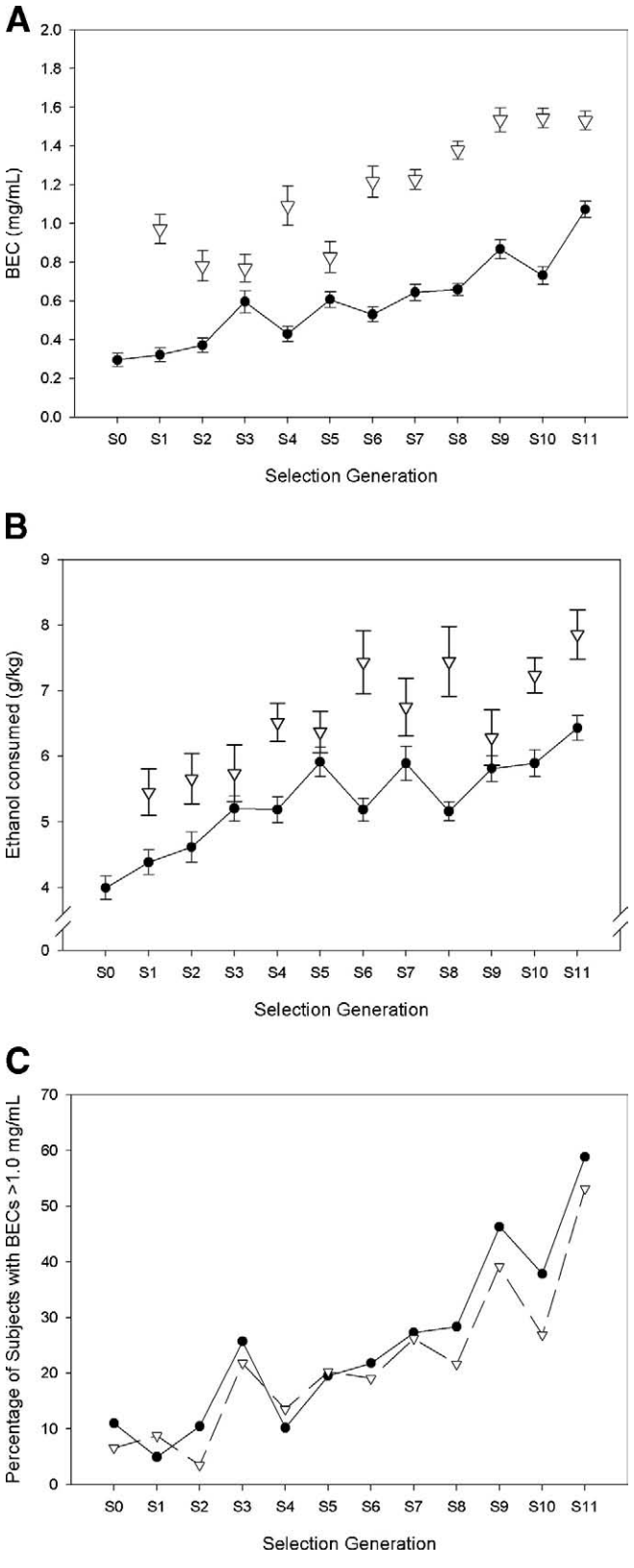
Generations of Selection	*N*	BEC (mg/mL)	4-Hour Consumption (g/kg)	Age (days)	Body Weight (g)	No. Families	N_e[a]	Cum ΔF[b]
S_0 Female	82	.35 ± .05	4.7 ± .26	87.8 ± .91	20.9 ± .26	15	—	—
S_0 Male	76	.24 ± .04	3.2 ± .20	87.0 ± .88	25.8 ± .32			
S_0 Parents	30	.97 ± .07 (S^c = .676)	5.0 ± .36	—	—			
S_1 Female	81	.31 ± .04	4.6 ± .23	66.3 ± .47	20.1 ± .23	15	62	.008
S_1 Male	57	.34 ± .06	4.0 ± .31	67.2 ± .45	25.7 ± .46			
S_1 Parents	30	.78 ± .08 (S = .460)	6.5 ± .60	—	—			
S_2 Female	67	.47 ± .05	5.1 ± .27	65.7 ± .53	21.2 ± .19	15	62	.016
S_2 Male	58	.25 ± .04	4.0 ± .37	66.3 ± .52	26.7 ± .34			
S_2 Parents	30	.77 ± .07 (S = .398)	5.7 ± .43	—	—			
S_3 Female	70	.61 ± .08	5.6 ± .27	56.1 ± .14	19.3 ± .19	15	62	.024
S_3 Male	55	.58 ± .08	4.7 ± .26	56.1 ± .13	24.4 ± .33			
S_3 Parents	30	1.09 ± .10 (S = .496)	6.5 ± .30	—	—			
S_4 Female	49	.40 ± .06	5.5 ± .29	82.4 ± .37	22.0 ± .27	13	54	.033
S_4 Male	59	.45 ± .05	4.9 ± .27	82.9 ± .24	26.5 ± .27			
S_4 Parents	26	.83 ± .08 (S = .397)	6.5 ± .39	—	—			
S_5 Female	82	.62 ± .05	6.5 ± .31	65.5 ± .48	20.3 ± .21	13	54	.042
S_5 Male	69	.60 ± .06	5.2 ± .30	62.6 ± .71	24.6 ± .32			
S_5 Parents	26	1.22 ± .08 (S = .608)	7.4 ± .48	—	—			
S_6 Female	78	.56 ± .05	5.9 ± .24	70.7 ± .26	21.0 ± .26	13	28	.060
S_6 Male	84	.50 ± .05	4.5 ± .23	70.7 ± .21	25.0 ± .28			
S_6 Parents	26	1.23 ± .05 (S = .697)	6.8 ± .44	—	—			
S_7 Female	66	.66 ± .06	6.3 ± .37	70.3 ± .48	19.0 ± .24	15	32	.076
S_7 Male	84	.63 ± .06	5.6 ± .36	70.7 ± .40	24.3 ± .32			
S_7 Parents	30	1.38 ± .05 (S = .733)	7.4 ± .53	—	—			
S_8[d] Female	127	.70 ± .05	6.0 ± .21	103.3 ± .90	22.2 ± .16	15	32	.092
S_8 Male	139	.63 ± .04	4.4 ± .17	101.9 ± .96	27.6 ± .25			
S_8 Parents	30	1.54 ± .06 (S = .877)	6.3 ± .43	—	—			
S_9[d] Female	67	.90 ± .07	6.1 ± .26	60.7 ± .87	18.9 ± .20	20	42	.104
S_9 Male	69	.84 ± .08	5.6 ± .29	60.3 ± .86	23.5 ± .33			
S_9 Parents	40	1.54 ± .05 (S = .677)	7.2 ± .27	—	—			
S_{10} Female	74	.83 ± .07	6.6 ± .28	73.4 ± .63	20.3 ± .21	20	42	.116
S_{10} Male	93	.66 ± .06	5.4 ± .28	73.6 ± .53	25.3 ± .33			
S_{10} Parents	40	1.53 ± .05 (S = .800)	7.9 ± .38	—	—			
S_{11} Female	85	1.12 ± .05	7.1 ± .26	71.2 ± .60	20.2 ± .27	20	42	.128
S_{11} Male	81	1.02 ± .07	5.7 ± .26	70.4 ± .65	25.0 ± .32			
S_{11} Parents	40	1.78 ± .04 (S = .703)	7.5 ± .33	—	—			

BEC, blood ethanol concentration.
[a] N_e, the "effective" breeding population size, changes as a function of number of breeders and breeding scheme (see text).
[b] ΔF is the inbreeding coefficient; Cum ΔF is the cumulative inbreeding over generations.
[c] S is selection differential (see text).
[d] In this generation, first and second litters were tested.

mg/mL. Their S5 offspring had average BECs of .61 mg/mL. By S11, the average BEC was 1.07 mg/mL for the offspring population, an increase over the foundation population of 262%.

Table 1 gives the number of offspring that were tested each generation and their average BEC, 4 hours consumption in g/kg, age, and body weight (at the time of DID testing). The mean BEC and consumption values for the selected parents of each generation also are provided. The selection differentials (S) were calculated from the mean BECs of the mice selected to breed the next generation minus the mean of the population from which they were selected. The number of breeder pairs (families) varied slightly across generations because some pairs were infertile or offspring were too few or died before testing. Figure 2 shows the realized response to selection R, the change in BEC from the S0 foundation mean value (e.g., S2–S1), plotted against the cumulative S. The regression of R on cumulative S gave an estimate of

heritability from the slope of the regression line (h^2 = .09). Response to selection has not slowed, suggesting that additive genetic variability remains in the population (see Supplement 1).



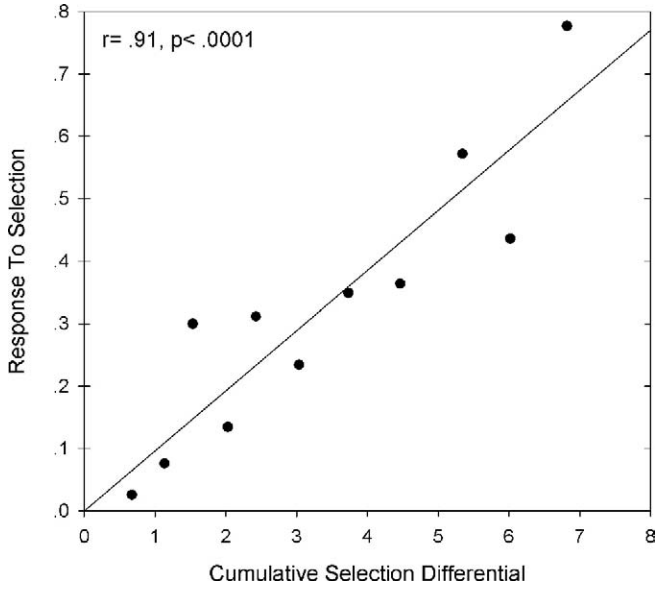


Figure 2. Realized response to selection in High Drinking in the Dark (HDID-1) mice. Total realized response to selection in each generation (R) is plotted versus the cumulative selection differential (S) at that generation. R_N is the difference between population mean BEC at the Nth generation and mean BEC in generation S0. S is the difference between BEC of individuals selected as parents and the population from which they were selected (see Figure 1A, Table 1). Thus, for example, as described in the text, the mean BEC of generation S4 was .43, whereas that of the foundation population was .30 (see Table 1). The fourth dot from the left depicts R_4, the total realized response to selection for S0-S4, as .13 mg/mL. The values for S can be estimated from Figure 1A as the difference between the S_N parents (inverted open triangle) minus the S_N population mean (black dot), or [(.97–.30) = .67] for S0. This value is added to [(.78–.32) = .46], [(.77–.37) = .40], [(1.09–.60) = .49] to obtain cumulated S_4 = 2.02. This value appears on the x axis for generation S4. The linear regression of R on cumulative S values is shown. From the slope of this line, heritability is estimated to be h^2 = .096. Data from males and females were combined for this estimate and are given in Table 1. Units for both axes are in mg EtOH/ml blood, but axes of R on S plots are usually not labeled as such by convention. As explained in Supplement 1, the goodness of fit to a linear regression (r = .91, $p < .0001$) is an indication that additive genetic variability has not yet been exhausted by selective pressure and that the line will continue to show increased response. Once additive variability begins to diminish significantly, the R/S plot will begin to flatten as it reaches an asymptote, and this method of estimating heritability will no longer be valid.

Correlated Response to Selection for DID

Selection strictly on BEC at the end of the 4 hours drinking session on Day 2 of the DID test also produced concomitant increases in g/kg consumption across generations (Figure 1B), which are considered a correlated response to selection. Whereas BEC had more than tripled over generations, intake increased from 4.00 g/kg in S0 to 6.43 g/kg in S11, an increase of 60.8%. Figure 1C shows the percentage of animals in each

Figure 1. Response to selection for high blood ethanol concentration (BEC) across 11 selected generations in High Drinking in the Dark (HDID-1) mice. **(A)** Mean ± SEM BEC is shown. Solid circles represent the total population tested each generation. Open inverted triangles give values of the animals chosen as parents from the preceding generation: their offspring are represented in solid circles directly below. For numbers of mice, see Table 1. **(B)** Corresponding ethanol intake (g/kg) for the mice depicted in panel A is shown. **(C)** Increase in the frequency of HDID-1 subjects with BEC > 1.0 mg/mL across generations is shown. Solid circles depict females, inverted triangles depict males.

generation for which BEC exceeded 100 mg%. This value is consistent with behavioral intoxication in mice. By S11, 53% of males and 58% of females exceeded this threshold. There has been no apparent change in average body weight across generations, nor has the general health of the colonies appeared to change (data not shown).

Sex Differences and Estimate of Inbreeding Coefficient

From Table 1, it appeared that females achieved slightly higher BECs than males and drank more. Although both measures increased over generations, the small sex differential remained stable (see Supplement 1). By S11, the estimate of inbreeding (see Supplement 1) was 12.8% through S11. These values are also given in Table 1. The rate of inbreeding during S6–S11 (approximately 1.4% per generation) is what would be expected for at least the next several generations.

Intake Across the 4-hour Session

Mice achieving higher BECs displayed modestly greater intakes, so we asked whether ethanol intake during the first versus the second 2-hour portions of the DID test had changed over generations. Figure 3 shows the (2 hours) intake on the first day of DID testing, and the intake on Day 2 during the first and last 2 hours of testing, after which the BEC was taken for selection. Three S11 mice were excluded from this analysis because their total consumption values on Day 2 were excessive as discussed in the Supplement 1. Generation 11 mice drank more on the first day of testing than S0 mice [$F(1,319) = 41.5$, $p < .0001$]. When we analyzed the two 2-hour periods of drinking on Day 2 across generations, there was a significant overall increase in S11 versus S0 mice when data were collapsed on time [$F(1,319) = 96.8$, $p < .0001$]. Mice of both generations drank more during the second 2-hour period on Day 2 [$F(1,319) = 114.9$, $p < .0001$], and the interaction was significant [$F(1,319) = 7.2$, $p < .01$]. Mice of S11 increased their drinking during the second period relative to the first by about .5 g/kg as compared to S0 mice (increases of 1.37 ± .16 and .82 ± .13 g/kg, respectively).

Figure 4 shows the modest predictive value of intake in determining BEC in S11 mice. The left panel shows that intake from the final 2 hours was modestly associated with BEC, predicting 17% of the variance. Total intake (right panel) predicted 20% of the variance in BEC. These correlations are consistent with those seen in C57BL/6J mice with the DID procedure (12).

Ethanol Metabolism

Figure 5 shows the results of this study. BECs were somewhat higher (about 10%) in the HDID-1 than in HS/Npt mice, but lines did not differ in rate of metabolism (see Supplement 1).

Two-Bottle DID

In this study, mice from S9 had 4 days of ethanol access (2 hours on Days 1–3, 4 hours on Day 4), with separate groups of animals having a single ethanol bottle (standard DID) versus two bottles (ethanol vs. water). An initial analysis compared placement of ethanol tube (left vs. right) on intake on Day 4 for mice whom the ethanol tube was placed in the position of the water bottle versus mice for whom ethanol was placed on the other side of the cage top. Placement of the ethanol tube did not significantly alter ethanol intake in either the standard DID group [$F(1,16) = .03$, $p = .86$] or the two-bottle choice group [$F(1,18) = .45$, $p = .51$]. Thus, data for the counterbalanced groups were combined for analyses of intake (see Figure 6). Mean BEC in the single-bottle group was .47 mg/mL and average intake on the last day of testing was 4.91 g/kg ethanol. Mean BEC in the two-bottle group was .12 mg/mL, and intake averaged 2.98 g/kg. BEC and ethanol intake differed significantly in the single-bottle versus two-bottle groups [$F(1,36) = 5.6$ and 9.9, respectively, $p < .05$]. Only 3 of the 20 mice in the two-bottle choice group had detectable BEC (>.05 mg/mL), whereas 15 of the 18 mice in the single-bottle group had detectable BEC values. Total fluid consumption also differed significantly between groups. The two-bottle group drank 1.15 ± .11 mL fluid, whereas the single-bottle group drank .71 ± .06 mL [$F(1,36) = 11.6$, $p < .01$].

Balance Beam

Results are given in Figure 7. A preliminary analysis showed that mice tested first on the balance beam and subsequently on the rotarod had BECs at the end of testing equivalent to mice tested in the opposite order. Mean BECs were .64 ± .15 and .84 ± .13 mg/mL, respectively [$F(1,40) = 1.03$, $p > .10$]. Analysis of foot slips showed that mice from S9 of the HDID-1 selection were clearly intoxicated, averaging more than three foot slips on

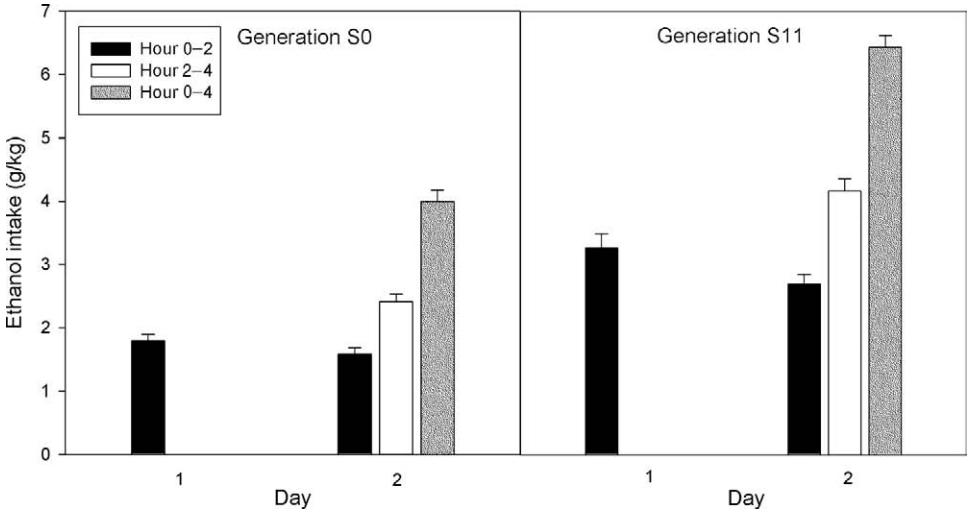


Figure 3. Increase in intake from S0 to S11 in High Drinking in the Dark mice. Each bar represents the mean ± SEM ethanol intake (g/kg) during the 2-hour drinking in the dark (DID; Day 1) and the first and last 2 hours of the 4-hour DID test on Day 2. Inset gives key. Data for all generations for total intake on Day 2 are provided in Table 1. For statistical analyses, see Results.

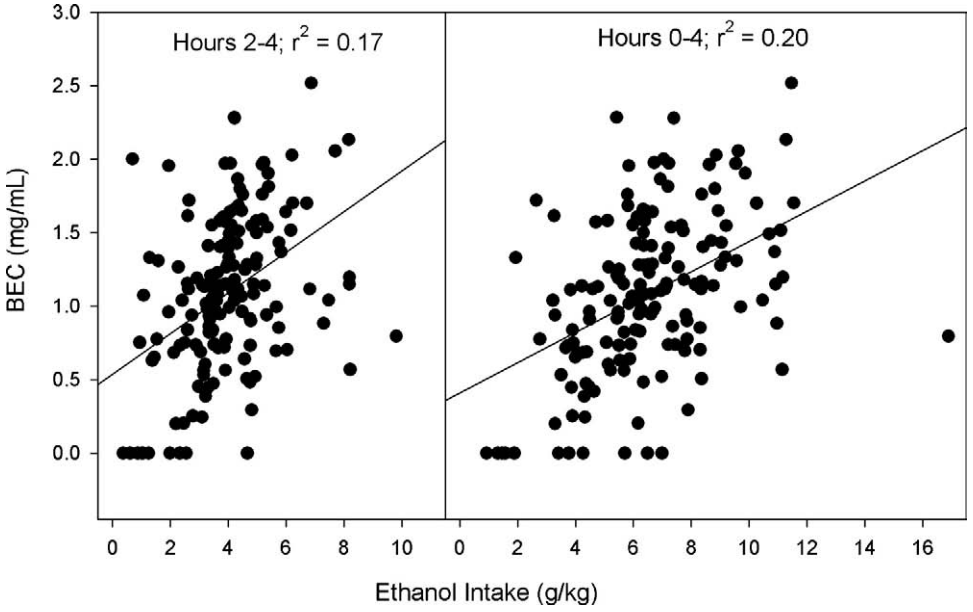


Figure 4. Blood ethanol concentration (BEC) at the end of drinking in the dark testing displayed versus ethanol intake in 163 High Drinking in the Dark mice from generation S11. Individual BECs are plotted versus intake during hours 2–4 (left panel) or hours 0–4 (right panel). Data from males and females are combined, and the linear regression lines are depicted.

the balance beam. Comparable mice given access only to water averaged fewer than one foot slip, and these group differences were significant [$F(1,55) = 12.3$, $p < .001$]. Neither the effect of test order on foot slips ($F = 1.19$) nor the group × test order interaction ($F < 1$) were significant.

Rotarod

For the rotarod, even though BECs were equivalent for those mice tested first and second, the results were more complex. A preliminary analysis of mean trial latencies showed a trend toward significant effects of test order [$F(1,53) = 3.6$, $p = .07$]. Those mice tested first on the rotarod performed more poorly than those tested after balance beam testing, and there also was a trend toward a significant interaction of group and test order [$F(1,53) = 2.9$, $p = .10$]. We therefore performed separate analyses of the mice tested first and those tested second on the rotarod. In all analyses, performance improved significantly over trials, assessed by calculating the difference between Trial 1 and Trial 3 latencies. This improvement index was slightly skewed to the left, so we performed a reflection of the data (largest score +1) and then took the square root of the reflected difference score to obtain a normal distribution (16). Analyses of these transformed data showed that ethanol drinking in the dark led to nearly significant impairment for those mice tested second on the

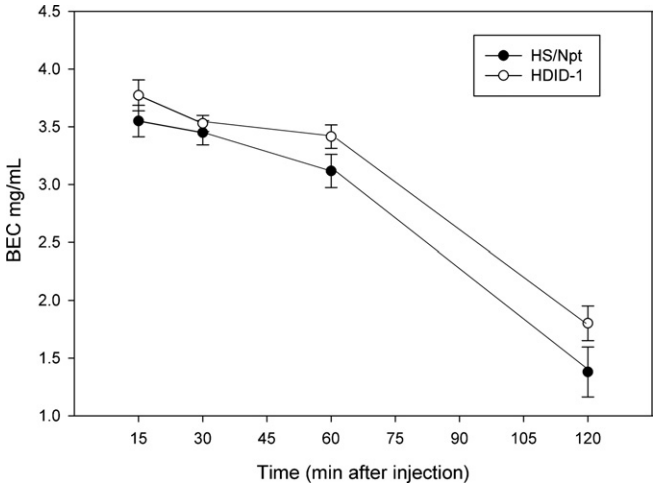


Figure 5. Blood ethanol concentration (BEC) at four time points after 2g/kg ethanol (intraperitoneal) in High Drinking in the Dark (HDID-1) mice from S11 and HS/Npt mice. Closed circles depict HS/Npt mice. Open circles depict HDID-1 mice. Symbols and y-error bars represent the mean ± SEM BEC for each group at each time point. For statistical comparisons, see Results.

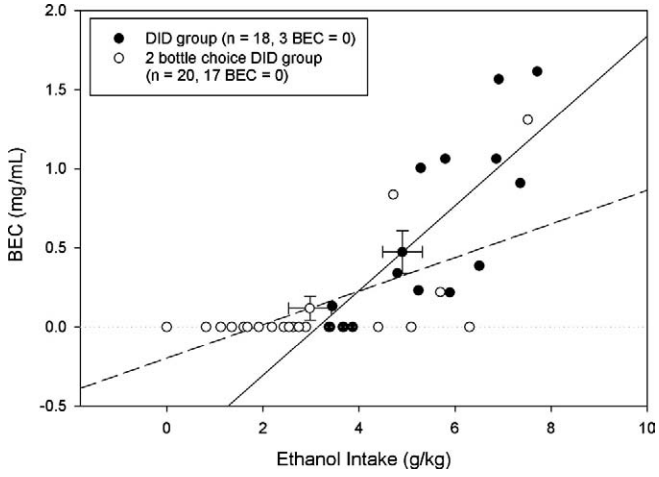


Figure 6. Comparison of intake and blood ethanol concentration (BEC) in High Drinking in the Dark mice from S9 with a single-bottle or two-bottle tests (see inset key). Closed circles depict mice tested with a single ethanol tube (standard drinking in the dark [DID] test) and the linear regression of their BEC on their intake (solid line). Open circles depict mice also offered a tube containing water, with a dashed line reflecting the linear regression of their BEC and intake. Symbols with x- and y-error bars represent the mean ± SEM intake and BEC for each group. For statistical comparisons, see Results.

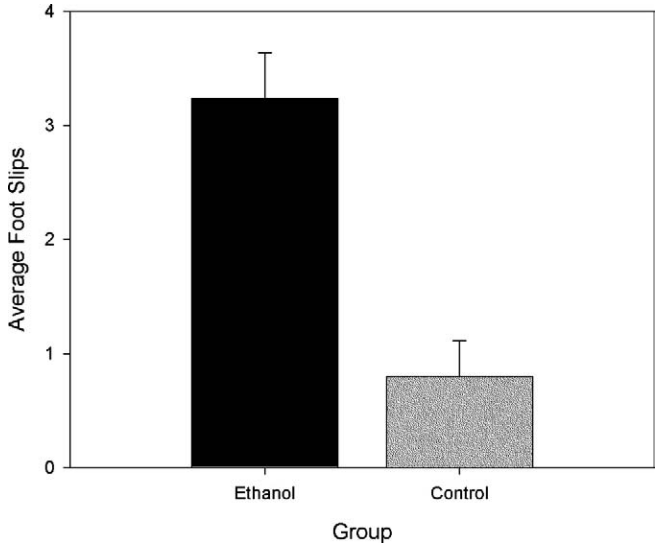


Figure 7. Intoxication on the balance beam in High Drinking in the Dark mice following drinking in the dark testing. Mean ± SEM foot slip errors are shown for mice offered ethanol versus those offered water (Control). For statistical comparisons, see Results.

rotarod [$F(1,25) = 3.96$, $p = .058$] but not in those tested first [$F(1,28) = .5$, $p = .47$; see Figure 8].

Discussion

HDID-1 mice drink substantial amounts of a relatively high concentration of ethanol (20%) in limited access tests during the circadian dark. The realized heritability of the BEC developed from this behavior across 11 generations of selection is low (approximately 9%), which has undoubtedly contributed to the slow increase in BEC and ethanol intake over generations. The approximate realized heritability of $h^2 = .096$ was lower than that estimated from inbred strains, in which it ranged from .46 to .74 (13). Differences between such estimates assessed in inbred strains and selected lines are not unexpected. They can arise from many features that distinguish the experimental populations (e.g., no heterozygotes in inbreds, many in selected lines; for discussion see ref. 17).

This selection represents the first to our knowledge in which the blood level of a drug served as the selection index. Selection has commonly been employed by the drug abuse (particularly alcohol) research community, but the target of selection has always been either a behavioral response to or the amount of a drug consumed. We elected to target BEC rather than amount consumed because we were interested in developing an animal model of self-intoxication. We reasoned that there were many ways an animal might pattern its intake over a 4-hour test period, and not all of those would be consistent with behavioral intoxication at the end. Indeed, it might have been expected that by targeting high BEC, we would have been choosing those animals that drank more overall, and especially later during the session. However, Figure 3 shows that mice in the foundation population also tended to drink more in the second half of the 4-hour DID test and that the allocation of consumption changed only mildly over selected generations. HDID-1 mice are clearly drinking more overall. A more fine-grained temporal analysis of intake such as the lickometer-derived data we reported for C57BL/6J mice (13) will be required to determine the role of pattern of intake on BEC. HDID-1 mice reached BEC levels greater than those seen in C57BL/6J mice tested under similar conditions (see Supplement 1).

HDID-1 mice were clearly intoxicated when tested on the balance beam. The sensitivity of this task to detect intoxication is high (18), and the effective dose range across multiple inbred strains was between 1.0 and 1.4 g/kg ethanol (19). The lower end of this dose range would be expected to yield BECs in the range

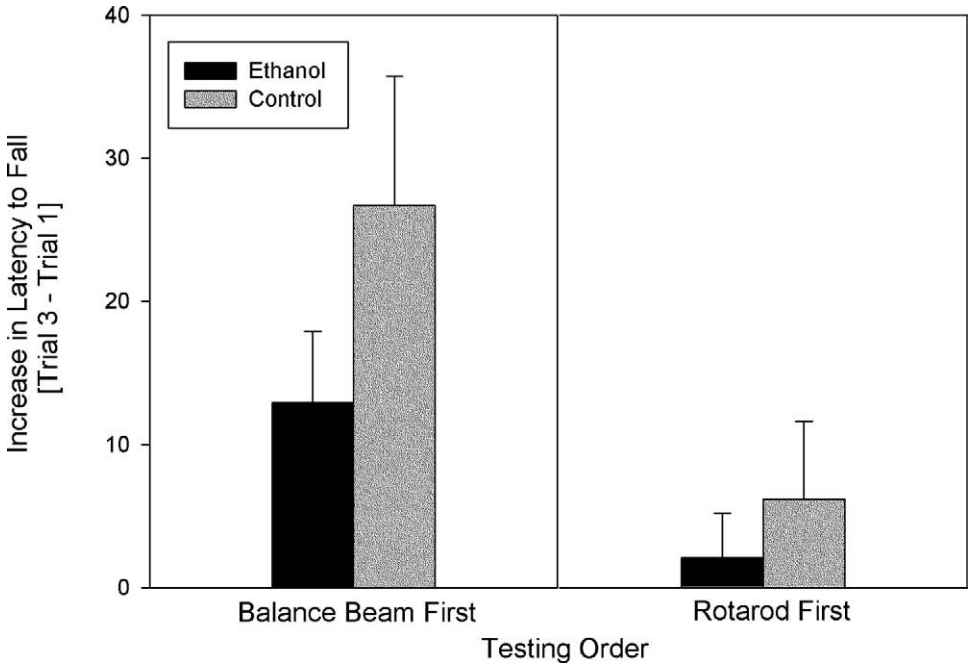


Figure 8. Intoxication on the accelerating rotarod in High Drinking in the Dark mice following drinking in the dark testing in groups offered ethanol versus water. The improvement in rotarod performance is given as the increase in latency to fall (sec) between the third and first trial. Mice tested first on the balance beam are shown in the left panel, and those tested first on the rotarod on the right. For statistical comparisons, see Results.

of many of the tested HDID-1 mice. Consistent with this notion, 39%–46% of mice in the S9 generation exhibited BECs that exceeded 100 mg% (Figure 1C), which is a value that has been shown to produce behavioral intoxication in mice (18,20). However, results with the accelerating rotarod (ARR) were more equivocal, because only mice tested second on the ARR showed signs of intoxication. We speculate that the very limited testing (three trials) may have contributed and that those mice for which the ARR experience was their first behavioral test while intoxicated may have been adapting nonspecifically to handling and novelty. One reason we suspect this may be true is that the control group tested first on the rotarod also showed substantially lower performance than the water-drinking control mice tested second (Figure 8). Also, we have observed that BECs in the range we obtained here cause *improvements* in performance on the ARR in some genotypes (21), so it is possible that we were assessing a mixture of performance decrements and improvements in the HDID-1 mice. In a study that employed a different ethanol drinking schedule and C57BL/6J mice, mice were impaired in a different variant of the rotarod task (fixed speed) after extensive pretraining on the accelerating rotarod. In that study, average BECs were 1.3–1.4 g/kg (22). We also note that levels of intake and BECs were lower in the second litters of HDID-1 mice that were tested for intoxication than those seen across the first litters of S9 HDID-1 mice tested for selection. We usually do not see such a difference between first and second litters; one potential explanation for the lower intakes (and BECs) is that concurrent testing for intoxication and intake in the same room may have disrupted ethanol consumption.

The test of two-bottle DID intake was consistent with previous studies with inbred mice. Multiple inbred strains of mice were given three DID tests, each lasting 4 days. The first two, a week apart, were with a single ethanol bottle. The final test, 2 weeks later, was with two bottles (ethanol vs. water). In that study, mean strain intake of ethanol was quite stable across all three tests. However, mice also drank some water in the two-bottle test, and mean BECs were considerably lower than in the single-bottle DID test (13). Given that only about 43% of animals in the HDID-1 line were drinking to intoxication by S9, it is perhaps understandable that the phenotype did not generalize to a two-bottle choice situation in S9. However, we plan to test future generations on a regular basis as their DID response becomes more extreme.

Most selection programs perform bidirectional selection, with two lines selected from the same starting population for the opposite responses. Occasionally, a nonselected (quasi-randomly mated) control line is maintained. The rationale, advantages, and disadvantages of the various mating schemes have been discussed in detail elsewhere (23). Because most genetically heterogeneous mice drink very little (and therefore reach very low BECs; in S0, only 8.9% had BECs of 1 mg/mL or more) in the DID procedure, we did not deem it useful to try to breed a line selectively for low DID. Rather, we have elected to use the foundation population of HS/Npt mice as a control group for comparisons with HDID-1 mice. This colony is maintained with 48 mating pairs and, in generation 44, was genotyped using a panel of 1532 single nucleotide polymorphism markers. Allele loss was estimated at 5.1% (R. Hitzemann, personal communication). This index suggests that inbreeding by G50 is likely to be low. HS/Npt mice are currently maintained at the original animal facility (Department of Comparative Medicine, OHSU, Portland, Oregon). Both facilities are Association for Assessment and Accreditation of Laboratory Animal Care–approved. Unfortunately, the HS/Npt colony experienced an outbreak of mouse parvo virus (MPV) during 2007. The pathogen has been eliminated, but we were only able to move HS/Npt mice into the VA facility in April, 2008. Our plan is to maintain this subset of HS/Npt breeders by quasi-random mating (excluding common grandparents) so that matched sets of HDID-1 and control mice can be made available for experiments.

Our data suggest that the HDID phenotype is polygenic, and that the limits of selection have not been reached. Greater expansion of the phenotype, as well as the existence of a replicate selected line (HDID-2 mice) will be useful for detecting other correlated responses and understanding the biological basis of the excessive drinking (see Supplement 1).

We have discussed elsewhere many other procedures that have been effectively used to increase drinking in rodents (12) (see also Supplement 1). These have their uses, and some can lead to very high BECs, but nearly all require a significantly greater degree of training over a longer period. Alternatively, they may require either food or water deprivation (or both). The animals in the DID procedure are never food or fluid deprived. A nonpreferring mouse must withhold drinking for the 4-hour period of the test, but this is easily tolerated without adverse physiological consequences (24). We do not know why some mice elect to drink a great deal during the DID procedure and others do not. Taste is a complex phenotype, and genetic influences are an important contributor to taste preferences for various tastants (e.g., salt, sweet) (25). An extensive literature supports a role for taste in two-bottle ethanol preference drinking (for reviews, see refs. 26,27). Thus, it will be important to explore taste sensitivity and preferences in the HDID-1 mice. We would predict that a genotype that voluntarily drinks 20% ethanol solutions also will ingest sucrose solutions avidly, on the basis of the substantial common genetic influences on alcohol and sucrose preference drinking (28–30).

We reiterate that the HDID-1 mouse is not intended to serve as a genetic model for alcoholism. Like McClearn (31), we do not believe that a plausible rodent model that fully resembles clinical alcoholism is a feasible goal (32). This is primarily because many of the diagnostic criteria for alcohol dependence are behavioral and are defined in ways that undermine the face validity of rodent models (e.g., difficulty with relationships or work). Rather, we are attempting to model one salient feature, a single binge episode. The DID model is rapid and simple, and this is its greatest strength. It is clearly different from human alcoholic drinking in several obvious ways. Whatever its pattern, alcoholic drinking is developed after years, and we would not expect the neurobiological changes seen after DID in mice to reflect the same changes achieved by a chronic alcoholic. In the current generation of HDID-1 mice, DID intakes are reduced when there is water available. However, if intakes continue to increase with further selection, we may well see significant intoxication in HDID-1 mice even when water is available. Maximal intakes are seen during the circadian dark, when feeding and drinking are normally highest. We do not know whether prandial drinking differs in significant ways from drinking at other times during the day, although one might suspect that some prandial drinking is motivated by feeding-associated thirst. Despite these limitations, the genes predisposing to high DID may well influence other alcohol-related traits; this remains to be demonstrated in future studies.

One of the daunting features of undertaking a selective breeding project is the need to convince the relevant research community that the resultant selected lines will be useful. These studies were conducted as a part of the Integrated Neuroscience

Initiative on Alcoholism (INIA-West), a consortium effort supported by the National Institute on Alcohol Abuse and Alcoholism. Because one goal is to provide tools to the research community (http://www.scripps.edu/cnad/inia/), and because the idea of creating these lines emerged consensually, there is broad interest in studying HDID-1 mice among other laboratories. For example, the phenotype (DID) has been used in INIA-West and other laboratories to analyze the pharmacology of high DID (33–36). We anticipate the use of HDID-1 mice in studies exploring the neurocircuitry, neurophysiology, and neurochemistry underlying the drinking response, as well as in further behavioral analyses (e.g., will future generations of HDID-1 mice drink sufficient ethanol to display withdrawal signs on cessation? Are there other responses genetically correlated with their propensity to drink to intoxication?). Interested investigators are invited to contact us with ideas for the use of these mice and/or requests for their provision.

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Supplementary material cited in this article is available online.

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