

Mouse inbred strain differences in ethanol drinking to intoxication

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Recently, we described a simple procedure, Drinking in the Dark (DID), in which C57BL/6J mice self-administer ethanol to a blood ethanol concentration (BEC) above 1 mg/ml. The test consists of replacing the water with 20% ethanol in the home cage for 4 h early during the dark phase of the light/dark cycle. Three experiments were conducted to explore this high ethanol drinking model further. In experiment 1, a microanalysis of C57BL/6J behavior showed that the pattern of ethanol drinking was different from routine water intake. In experiment 2, drinking impaired performance of C57BL/6J on the accelerating rotarod and balance beam. In experiment 3, 12 inbred strains were screened to estimate genetic influences on DID and correlations with other traits. Large, reliable differences in intake and BEC were detected among the strains, with C57BL/6J showing the highest values. Strain means were positively correlated with intake and BEC in the standard (24 h) and a limited (4 h) two-bottle ethanol vs. water test, but BECs reached higher levels for DID. Strain mean correlations with other traits in the Mouse Phenome Project database supported previously reported genetic relationships of high ethanol drinking with low chronic ethanol withdrawal severity and low ethanol-conditioned taste aversion. We extend these findings by showing that the correlation estimates remain relatively unchanged even after correcting for phylogenetic relatedness among the strains, thus relaxing the assumption that the strain means are statistically independent. We discuss applications of the model for finding genes that predispose pharmacologically significant drinking in mice.

Keywords: C57BL/6J, drinking pattern, drinking, ethanol, inbred mouse strains, intoxication, Mouse Phenome

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Alcoholism is a complex disorder influenced by many genes and their interactions with each other and the environment (Heath *et al.* 2002; Kendler *et al.* 1995). Partial mouse models have been developed to study features of alcoholism such as excessive ethanol drinking behavior (Crabbe *et al.* 1994; Finn *et al.* 2005). Many procedures have been developed to study ethanol drinking in mice, but it has been a challenge to find a procedure in which mice drink to the point of reaching intoxication (Dole & Gentry 1984). Recently, a simple procedure was described, which we call Drinking in the Dark (DID), in which a majority of individuals within the genetically predisposed strain, C57BL/6J, drink to a blood ethanol concentration (BEC) above 1 mg/ml blood (Rhodes *et al.* 2005). In brief, the water bottle is replaced with a bottle containing 20% ethanol for 2 or 4 h in the home cage starting 3 h after lights shut off. A majority (>50%) of C57BL/6J mice achieve a BEC above 1 mg/ml after 4 h of ethanol exposure using this procedure (Rhodes *et al.* 2005). We propose that this constitutes a model with which to study genetic predisposition to initiate high ethanol drinking, possibly analogous to 'binge' drinking. This paper explores this high ethanol-drinking model further.

Although C57BL/6J mice self-administer relatively high levels of ethanol in the DID procedure, the pattern of drinking the ethanol solution that leads to the pharmacologically significant BEC at the end of the 4-h period has not yet been elucidated. Rodents tend to drink water in discrete bouts usually after eating (i.e. postprandial), and it has been suggested that C57BL/6J mice drink ethanol as rapidly as they drink water (Dole *et al.* 1988). The goal of the first experiment was to characterize the pattern of drinking 20% ethanol as compared with water using the DID procedure.

The goal of the second experiment was to test the hypothesis that DID causes behavioral intoxication (Crabbe *et al.* 2003b; Crabbe *et al.* 2005; Cronise *et al.* 2005; Rustay *et al.* 2003a; Sharpe *et al.* 2005). Although it is known that BECs above approximately 1 mg/ml cause motor impairment (Crabbe *et al.* 2003b; Rustay *et al.* 2003a), and DID produces BECs above this level (Rhodes *et al.* 2005), it has never been directly demonstrated that DID produces motor impairment.

Therefore, we tested whether DID impairs performance on the rotarod and balance beam, two tests that reflect motor impairment from a variety of causes (Crabbe *et al.* 2003a; Crabbe *et al.* 2005; Cronise *et al.* 2005; Rustay *et al.* 2003a).

One of our long-term goals is to use the DID procedure to study the genetic basis of pharmacologically significant ethanol drinking in mice (Rhodes *et al.* 2005). For the DID procedure, we have published only data for C57BL/6J and DBA/2J (Rhodes *et al.* 2005), and a large difference in intake and BEC was observed between these two strains. However, to find genes that underlie ethanol drinking using such strategies as quantitative trait locus (QTL) analysis or *in silico* mapping, it is useful to have multiple genotypes showing different values for the traits (Belknap & Atkins 2001; Wang *et al.* 2005). Moreover, at least three and preferably more than 10 strains are needed to estimate strain mean correlations between traits (Crabbe *et al.* 1990; Crabbe *et al.* 1994). This strategy is useful to determine whether a common group of genes influences both the DID trait and other known physiological or behavioral traits that may underlie or contribute to DID. Because same sex individuals within an inbred strain are genetically identical, strain means can be correlated, even though different animals are measured for each trait. This provides a basis for the building of cumulative data sets for comparisons across studies (Crabbe *et al.* 1990; Crabbe *et al.* 1994). Hundreds of basic behavioral and physiological traits for a variety of inbred strains are now available in The Mouse Phenome Project (MPP) public database (<http://aretha.jax.org/pub/cgi/phenome/mpdcgi?rtn=docs/home>) provided by The Jackson Laboratory (Bar Harbor, ME) (Grubb *et al.* 2004).

Previous reports of strain mean correlations among ethanol responses and other traits have used conventional statistical methods (Crabbe *et al.* 2003b; Metten & Crabbe 1994, 2005; Metten *et al.* 1998), thus implicitly assuming that each strain mean represents a statistically independent (and identically distributed) data point. However, the historical record shows that certain strains are more recently derived than others, and this information can be used to trace the pattern of divergence (hence genetic relatedness) among the strain lineages (Beck *et al.* 2000). The strain relationships can also be estimated using such polymorphic genetic markers as single nucleotide polymorphisms (SNPs) and microsatellites (Atchley & Fitch 1991; Petkov *et al.* 2004). Phylogenies constructed using each of these methods have been published and generally agree (Atchley & Fitch 1991; Beck *et al.* 2000; Petkov *et al.* 2004).

The goals of experiment 3 were (a) to screen 12 inbred strains to determine the reliability and effect sizes for DID strain differences in ethanol intake and BEC and (b) to examine genetic correlations between the DID traits and 609 other traits using the public MPP database and our own unpublished and published datasets. The genetic correlations were estimated in the conventional way, assuming that each strain mean represents an independent data point, and also using a

method that takes into account the hypothesized pattern of genetic relatedness among the strains.

Materials and methods

Subjects and husbandry

Male and female mice were purchased from The Jackson Laboratory except in experiment 1 and the second batch of experiment 2, for which C57BL/6J from our own colony were used. Every three generations, we re-establish our C57BL/6J colony with newly purchased mice from The Jackson Laboratory to avoid substrain divergence. Animals that were directly purchased arrived at our facility at 5–6 weeks of age and were acclimated for 3 weeks prior to testing. Mice were housed three to four per cage in standard, polycarbonate or polysulfone shoebox cages with Bed-o-Cob™ bedding until 1 week prior to the start of an experiment when they were transferred to individual housing. Rooms were controlled for temperature (21 ± 1 °C) and photo-period (12:12 L : D). In the colony, lights turned on at 0600 h and off at 1800 h, Pacific Standard Time. Approximately 2 weeks prior to the start of an experiment, the mice were switched to a reverse light/dark schedule such that lights turned on at 2030 h and off at 0830 h. Food (Purina 5001™) was always provided *ad libitum*. Water was provided *ad libitum* except when ethanol was substituted for water for 2 or 4 h per day as described below. The animals were approximately 60 days old at the time of testing. All mice were housed and tested in the Department of Comparative Medicine at the Oregon Health & Science University or in the Veterinary Medical Unit at the Portland VA Medical Center, both AAALAC-approved facilities. All procedures were approved by the appropriate Institutional Animal Care and Use Committee and adhered to NIH Guidelines.

DID procedure

Four-day version

Following Rhodes *et al.* (2005), starting 3 h after lights off in the animal rooms, the water bottles were replaced with 10 ml graduated cylinders fitted with sipper tubes containing 20% (v/v) ethanol in tap water. This was done in the home cages where animals were singly housed (see above). The ethanol cylinders remained in place for 2 h. After the 2-h period, intakes were recorded, and the ethanol cylinders were replaced with water bottles. This procedure was repeated on days 2 and 3. On day 4, the procedure was again repeated except that the ethanol cylinders were left in place for 4 h, and intakes were recorded after 2 and 4 h. Immediately after recording intakes following the 4-h access, a 20- μ l sample of blood was taken from the peri-orbital sinus. Following the blood sample, mice were returned to their cages, and the ethanol cylinders were replaced with water

bottles. Blood samples were processed and analyzed by gas chromatography according to previously published methods to determine BEC (Ponomarev & Crabbe 2002).

Two-day version

The same procedure described above was followed, except that on day 2 the ethanol cylinders were left in place for 4 h instead of 2 h. A blood sample was taken following the 4-h session on day 2.

Experiment 1: Microanalysis of Drinking in the Dark in C57BL/6J mice

The 4-day DID procedure was conducted as described above with some modifications. When the animals ($n = 10$ males and 13 females) were transferred to individual housing (1 week prior to the start of the experiment), they were transferred into lickometer chambers (described below) instead of standard shoebox cages. The animals were housed in these chambers 24 h per day for 3 weeks (1 week of acclimation and 2 weeks of experiments). Thus, the lickometer chambers became the home cages of these animals. Food was placed on the floor of the cages *ad libitum* instead of in a food hopper, and water was available via a sipper tube throughout the experiment except when ethanol was substituted for water. After 1 week of acclimation, the animals were tested using the 4-day version of the DID procedure except that in the first 4 days of testing, the water bottles were replaced with 10-ml graduated cylinders containing water instead of ethanol to collect baseline data on drinking patterns for water. Blood samples were not taken after the water tests. Three days later (the following week), the DID procedure was repeated in the same individuals (within subjects design) using 20% ethanol. A blood sample was taken on day 4 after the 4-h test. An IBM-compatible computer running MED Associates software (St. Albans, VT) was used to record automatically each time a mouse contacted a sipper tube. These data were used to calculate several summary statistics to characterize an individual's drinking pattern. Only data on day 4 during the 4-h water and 4-h ethanol tests were analyzed. The following traits were calculated for each individual: number of drinking bouts, average duration of a bout (min), average number of sipper tube contacts within the bouts, average duration of the interbout intervals (min), average rate of sipper tube contacts within the bouts (contacts/min) and latency to the first bout (min). A drinking bout was defined as a series of at least 20 contacts on the sipper tube with less than 1-min separating contacts (Ford *et al.* 2002; Ford *et al.* 2005). The summary statistics were also recalculated using an alternate definition of a bout, a series of at least 40 contacts with less than 1-min separation. Results were qualitatively the same for either definition; hence, only results for the first definition of a bout (at least 20 contacts with less than 1 min between contacts) are shown.

Lickometer apparatus

Chambers consisted of four walls of Plexiglas (creating a space 7" long \times 4" wide \times 7" high for the animals to explore) with a stainless steel wire floor (VWR, Tualatin, OR) and a perforated Plexiglas-hinged lid. The entire chamber was placed into a standard shoebox cage. Bed-o-Cob™ bedding was placed below the wire floor. Sipper tubes were inserted through a hole in the Plexiglas. The steel wire floor and metal sipper formed an open electrical circuit. When an animal contacted the metal sipper tube while standing on the metal floor, the circuit would become closed. A lickometer device (MED Associates, St. Albans, VT) recorded each time this happened.

Experiment 2: Drinking in the Dark – demonstration of intoxication in C57BL/6J mice

Batch 1 (n = 32 females)

After pretraining on the balance beam (see below), half the mice (ethanol group; $n = 16$) received the 2-day version of the DID procedure (see above) except that, on day 2, immediately after the 4-h drinking period, they were tested using either five trials on an accelerating rotarod or a single trial on a balance beam (see below). Mice did not receive any pretraining on the rotarod, because preliminary data suggested that pretraining abolished differences between ethanol- and water-exposed mice. Immediately following the motor performance test (which lasted approximately 2 min for the balance beam and 5 min for the rotarod), a blood sample was taken. The other half (control group; $n = 16$) were treated similarly except that each water bottle was replaced with a 10-ml cylinder containing water instead of 20% ethanol to serve as the between-subjects control, and no blood samples were taken. One week later, the procedure was repeated. Animals that underwent the rotarod test for the first time were tested on the balance beam the second time and vice versa.

Batch 2 (n = 31 females)

Mice in the ethanol group ($n = 21$) received the 2-day version of the DID procedure and were tested (immediately after 4 h of access) on the balance beam (see below). Following the test, a blood sample was taken. Control mice ($n = 10$) were treated similarly except they were given water instead of 20% ethanol to serve as the between-subjects control.

Accelerating rotarod

The apparatus is described in detail elsewhere (Rustay *et al.* 2003b). The procedure was modified from Rustay *et al.* (2003b). Mice were placed on a modified AccuRotor Rota Rod (Accuscan Instruments, Columbus, OH) with a 63-cm fall height and 6.5-cm-diameter rotating dowel starting at 0 r.p.m. A timer was started, and the dowel was then accelerated at a constant rate of 30 r.p.m./min. A photobeam at

the base of the apparatus was used to stop the timer automatically when a mouse fell from the dowel. The mouse was then placed back on the apparatus at 0 r.p.m., and this was repeated for a total of five trials.

Balance beam

The apparatus and procedure are described in detail elsewhere (Crabbe *et al.* 2003a). The day before their first ethanol (or water) exposure, the mice were encouraged, if necessary, by a light touch of the tail, to walk across the 104.1-cm length of the 19.6-mm-wide beam (elevated 54.5 cm above the table below) in both directions twice. We have found that, after this training, the mice traverse the beam during a subsequent test without any encouragement. On the test days, mice were placed at one end of the beam, and the number of hind foot slips was recorded as they traversed to the other end. These tests were conducted by an investigator who was blind to the assignment of treatment groups.

Experiment 3: Drinking in the Dark: strain differences and relationship to two-bottle choice

A panel of 12 standard inbred strains ($n = 6$ males and 6 females per strain) was studied: C57BL/6J, 129S1/SvImJ, A/J, BALB/cByJ, BTBR T+tf/J, C3H/HeJ, DBA/2J, FVB/NJ, AKR/J, BALB/cJ, CBA/J and LP/J. All mice were screened for the 4-day version of DID (see above). This was accomplished in two batches. The first batch comprised the first eight strains listed above, and the second batch comprised the last four strains. In the second batch, we also tested C57BL/6J mice from our own colony ($n = 9$ females and 10 males), along with the four other strains, to serve as a reference for comparison between batches. We have shown previously that C57BL/6J mice from two different colonies of The Jackson Laboratory (East and West) and those from our own colony drink a similar amount using this procedure (Rhodes *et al.* 2005). These strains were chosen because they have been used in many of our own previous studies (e.g. Crabbe *et al.* 2003b; Metten & Crabbe 2005) and because behavioral, physiological and morphological data are available for these strains in the MPP database (<http://aretha.jax.org/pub/cgi/phenome/mpdcgi?rtn=docs/home>). In addition, genotyping projects are underway for these strains (Wang *et al.* 2005).

Retest

The entire procedure described above was repeated with the same animals following a 10-day recovery period during which time the animals had *ad libitum* access to water and were not disturbed except for a routine cage change.

Limited access two-bottle choice test (only conducted for the first eight strains on the list above)

After the retest, the animals were again left undisturbed, this time for 8 days (except for a routine cage change), and then tested a third time. In the third test, ethanol was offered in a similar schedule (2 h the first 3 days and 4 h the 4th day

starting 3 h after lights off each day), except that this time the animals were given a choice between 20% ethanol and tap water. Thus, each time ethanol was offered, instead of replacing the water bottle with one cylinder containing 20% ethanol, the mice received two cylinders: one with 20% ethanol and one with tap water. To alleviate the need to consider a side bias in the analysis (i.e. some animals might prefer to drink out of a cylinder on one side of the cage regardless of its contents), we placed the ethanol cylinders on alternate sides in a counterbalanced fashion for each strain-sex combination (i.e. half the females and half the males in each strain received the ethanol cylinder on the left side, whereas the other half received it on the right side). The side harboring the ethanol cylinder remained the same for each individual mouse on the first 2 days and then was switched for the last 2 days.

DID strain mean correlations with traits in the Mouse Phenome Project database (males only)

The MPP database was combined with our own unpublished and published datasets, which together consisted of 609 behavioral, morphological and physiological traits measured across panels of standard inbred strains. The strain means in the MPP database are separated by sex, and many of the traits were only measured in males; therefore, we focused on male strain means only. We used a private portal into the MPP site to correlate strain means for each trait in MPP with the male strain means for DID intake (g/kg) and BEC (mg/ml, first test, day 4 after 4 h ethanol exposure). Results were automatically sorted based on raw Pearson's correlation coefficient, r . Because of the multiple comparisons (609 tests were conducted), these data were statistically analyzed using the false discovery rate method (see *Statistical analysis*).

Statistical analysis

SAS (Release 8.01) procedures MIXED and GLM, as well as R (Release 2.0.1), were used for most analyses. A P value less than 0.05 was used to evaluate significance except where indicated. Drinking pattern variables (experiment 1) were analyzed using a two-way repeated measures ANOVA with fluid type (water vs. 20% ethanol) as the within-subjects factor (because the treatments were given sequentially to the same animals) and sex (male vs. female) as the between-subjects factor. Latency to fall from the rotarod (experiment 2) was analyzed using a two-way repeated measures ANOVA with fluid type (water vs. 20% ethanol) entered as the between-subjects factor and trial as the within-subjects factor. Foot slips on the balance beam (experiment 2) were analyzed using an unpaired t -test (water vs. ethanol).

Effect sizes (R^2 values) for strain differences (experiment 3) were estimated using 1-way ANOVA with strain entered as the factor. Polynomial regression was used to test whether the relationship between ethanol intake and BEC across strains was linear.

The consistency of ethanol intake across days was assessed for each strain separately using repeated measures one-way ANOVA with day entered as the repeated measures factor. Effect sizes (R^2 values) for the reliability of strain means were estimated using simple linear regression with retest strain means regressed on test strain means. Paired t -tests were used separately for each strain to determine whether individuals within a strain tended to increase or decrease their consumption of ethanol in the retest relative to the first test. For sex differences (experiment 3), data were analyzed using a two-way ANOVA with strain, sex and strain-by-sex interaction entered as factors.

To determine whether intake and BEC differed for the single bottle DID tests vs. the limited two-bottle test across strains (experiment 3), we used a mixed effects repeated measures analysis with strain entered as a random effect and test (3 levels: first single bottle DID test, DID retest and 2-bottle test) entered as the repeated measures factor. Significant ANOVA main effects were followed with Tukey *post hoc* tests to determine which pair-wise comparisons were significant.

Correlations among strain means

To evaluate the significance of correlations (Pearson's r) among strain means for DID traits (collapsed across sex) and between strain means for DID intake (males only) and four other ethanol traits (intake in standard two-bottle choice, taste aversion to saline solutions conditioned by 4 g/kg ethanol, chronic ethanol withdrawal severity and ethanol-induced hypothermia 30 min after 3g/kg ethanol; males only), a P value less than 0.05 was used, because these were planned comparisons with *a priori* hypotheses generated from the literature (Bachtell *et al.* 2002; Bachtell *et al.* 2003; Cunningham *et al.* 1991; Metten *et al.* 1998; Phillips *et al.* 2005). Pearson's correlations and sample sizes (number of strains in common between the pairs of traits) for

comparisons between the strain means for DID intake (males only) and 609 other morphological, physiological and behavioral traits were extracted from the MPP database. These 609 traits were selected because they contained at least eight strains in common with the 12 strains measured for DID intake. The 'Best Pearson's correlations' were extracted, which represent the most statistically significant estimates based on comparisons of untransformed or log-transformed strain means (<http://aretha.jax.org/pub/cgi/phenome/mpdcgi?rtn=docs/corrinfo>). These correlation estimates were squared to produce R^2 values, and then these R^2 values and sample sizes were imported into R to generate P values using the 'pbeta' function with shape parameters 1/2 and $(n-2)/2$. To control for the 609 multiple comparisons, we determined the P value cutoff that would yield a global, experiment-wise, false discovery rate of 5%. This was done using open source software called QVALUE (see URL: <http://faculty.washington.edu/~jstorey/qvalue/>) (Storey 2002). We show only those traits for which the correlations were significant at the experiment-wise $P < 0.05$ level. These traits showed significant correlations for raw, untransformed means; hence, only raw values are shown and were used for phylogenetic analyses (see below).

Phylogenetically independent contrasts

To account for the fact that the mouse strains we studied are genetically related in a hierarchical fashion (Atchley & Fitch 1991; Beck *et al.* 2000; Petkov *et al.* 2004), we also calculated correlations of strain means using phylogenetically independent contrasts (Felsenstein 1985; Garland *et al.* 1992). This and related methods, such as phylogenetic generalized least-squares models, are reviewed in Garland *et al.* (2005). In brief, Felsenstein's (1985) algorithm uses the specified phylogenetic information (represented by the cladogram; Fig. 1) to transform the original N -trait values (i.e. in

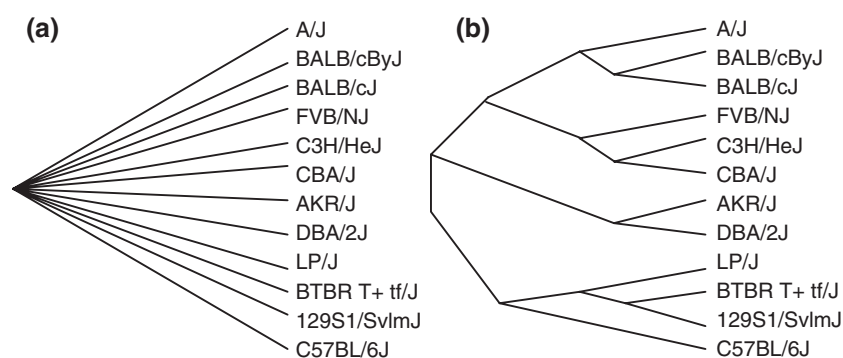


Figure 1: Assumptions for carrying out correlations of strain means using conventional vs. phylogenetically based methods.

(A) Conventional: a star phylogeny of the 12 inbred strains studied herein depicts each strain as independent (no hierarchical relationships). (B) Phylogenetically based methods: a phylogeny, constructed using parsimony analysis of 144 single locus genotypes, was redrawn and slightly modified from Fig. 1 in Atchley and Fitch (1991). As Atchley and Fitch (1991) did not include FVB/NJ and BTBR T+tf/J and did not distinguish BALB/cJ from BALB/cByJ, these strains were incorporated into the tree using Fig. 5 from Petkov *et al.* (2004). Branch lengths shown here are arbitrary and do not reflect exact timing of splits. For analyses by the method of phylogenetically independent contrasts (see text), branch lengths were set to the arbitrary values suggested by Pagel (1992).

this case, strain means) into N-1 'contrasts' that are phylogenetically independent by construction. Information on branch lengths is used to standardize each contrast, thus, in principle, rendering them 'identically distributed.' These standardized phylogenetically independent contrasts are then analyzed by ordinary statistical methods, with the constraint that all values are computed through the origin (Felsenstein 1985; Garland et al. 1992; Garland et al. 2005). Thus, degrees of freedom for hypothesis testing are the same as with conventional analyses [except in some cases where the phylogenetic tree is not fully bifurcating (Garland et al. 2005)]. Independent contrasts were calculated with the PDTREE program, available from TG on request (see Garland et al. 2005).

Phylogenetic signal in strain means

As compared with conventional statistical analyses, phylogenetic methods will necessarily yield different values for correlation estimates except in the case of using a 'star' phylogeny (Fig. 1a). In some cases, these differences may be substantial (e.g. see examples and references in Garland et al. 2005), and hence it is important to determine which statistical model (i.e. a star phylogeny vs. a hierarchical phylogeny) better fits the data under analysis. This is equivalent to determining whether related species or strains tend to resemble each other phenotypically (i.e. whether phylogenetic signal can be detected in the dataset). One criterion for model fit is the mean-squared error (MSE) of the data, with lower MSE indicating better fit [for a description of the statistical models used to generate MSE, see Blomberg et al. (2003)]. A *P* value for phylogenetic signal can be generated using a randomization test (Blomberg et al. 2003). Blomberg et al. (2003) also derived a descriptive statistic, termed *K*, which indicates the amount of phylogenetic signal in a dataset as compared with the amount expected for the specified phylogenetic tree and assuming a Brownian motion model of trait evolution. A *K* value greater than unity indicates a trait has more signal than expected, whereas a value less than one indicates less signal than expected. We used the `PHYSIG.M` MATLAB program of Blomberg et al. (2003) to calculate MSEs, *K* and to test for phylogenetic signal in the new strain mean data reported here for DID intake and BEC, collapsed across sex.

Results

Experiment 1: Microanalysis of DID in C57BL/6J mice

In the lickometer chambers, average intake \pm SEM during the 4-h ethanol drinking session (on day 4 of the 4-day test) was 6.5 g/kg \pm 0.19, collapsed across sex, and BEC was 1.0 mg/ml \pm 0.12. These levels are comparable with previous studies when animals were housed in standard shoebox cages (Rhodes et al. 2005).

A summary of the drinking pattern variables for water vs. 20% ethanol is summarized in Table 1. When 20% ethanol was substituted for water, C57BL/6J mice drank

approximately half as much fluid (Table 1, Fig. 2; $F_{1,21} = 268.3$, $P < 0.0001$). This translated into approximately half as many sipper contacts ($F_{1,21} = 64.2$, $P < 0.0001$) and half as many bouts ($F_{1,21} = 57.6$, $P < 0.0001$) for 20% ethanol as compared with water (for the definition of a bout, see *Materials and methods*). Moreover, the bouts were significantly shorter in duration for ethanol than water ($F_{1,21} = 23.6$, $P < 0.0001$). However, the number of contacts within a bout was approximately the same (Table 1, Fig. 2). In other words, when the animal was drinking (which was less often for ethanol than water), the rate of contacts was approximately twice as high for ethanol as compared with water ($F_{1,21} = 153.0$, $P < 0.0001$). These relationships were true for both males and females (Table 1).

Another distinguishing characteristic of ethanol drinking (as compared with water) was that it was episodic (Fig. 2). Inspection of the pattern of contacts on the sipper tubes shows that, in the first 30 min, the number of sipper contacts for ethanol was comparable with water, but in the second 30-min period, ethanol contacts decreased by approximately eightfold in males ($t = 4.3$, $df = 9$, $P = 0.002$) and threefold in females ($t = 6.6$, $d.f. = 12$, $P < 0.0001$), whereas water contacts remained consistent. Inspection of these patterns further suggests that contacts for ethanol tended to increase in the middle of the 4-h session for females and in both the middle and again at the end of the 4-h session for males. In contrast, water contacts tended to remain consistent throughout a majority of the 4-h period only decreasing at the end of the session for males and females (Fig. 2).

Some interesting sex differences in drinking patterns were also observed. A significant interaction between fluid type and sex was detected for the number of sipper contacts ($F_{1,21} = 5.5$, $P = 0.03$). Inspection of Table 1 shows that this interaction was caused by differences between the sexes for water (*post hoc*, $P = 0.04$, Table 1) which did not occur for ethanol. The total amount of water consumed was similar between the sexes, but females contacted the sipper approximately 30% more frequently than males to consume this amount ($P = 0.04$, Table 1). In other words, females consumed less water per contact than males and contacted more often to achieve a similar volume. This was accompanied by 88% longer drinking bouts in females than males ($P = 0.01$). Contacts were also spaced further apart for females than males resulting in 50% lower contact rate within a bout ($P = 0.0009$) (Table 1).

Experiment 2: Demonstration of intoxication in C57BL/6J mice after DID

On average, during the 4-h drinking session preceding the rotarod test, mice in the ethanol group ($n = 16$) drank 8.5 g/kg \pm 0.31 SEM. This resulted in an average BEC of 1.3 mg/ml \pm 0.16 SEM. Preceding the balance beam test, mice in the ethanol group (Batch 1 and 2 combined, $n = 37$)

Table 1: Microanalysis of the pattern of drinking 20% ethanol vs. water in C57BL/6J mice

	20% Ethanol		Water	
	Males	Females	Males	Females
Ethanol intake (g/kg)	6.41 ± 0.22	6.56 ± 0.29	–	–
Fluid volume (ml)	1.08 ± 0.04	0.87 ± 0.04	2.14 ± 0.09	2.12 ± 0.06
Sipper contacts	1090 ± 70	1050 ± 49	1755 ± 165	2307 ± 181*
Number of bouts	13.6 ± 1.2	14.8 ± 1.2	26.2 ± 2.6	30.2 ± 1.8
Number of contacts within a bout	80.8 ± 6.3	69.2 ± 5.3	62.1 ± 2.6	70.4 ± 4.8
Inter-bout interval (min)	17.7 ± 1.4	15.7 ± 1.6	8.9 ± 1.3	6.4 ± 0.6
Bout duration (min)	0.41 ± 0.07	0.54 ± 0.08	0.77 ± 0.09	1.45 ± 0.21
Contact rate (contacts/min)	430 ± 22	376 ± 25	233 ± 27	119 ± 16
Latency to 1st bout (min)	2.4 ± 0.9	5.9 ± 2.4	1.9 ± 1.5	3.9 ± 2.4

Values represent the mean ± SEM of male ($n = 10$) or female ($n = 13$) C57BL/6J mice. All variables except number of contacts within a bout and latency to 1st bout (min) showed a significant main effect of fluid type (20% ethanol vs. water).

*Denotes statistical interaction ($P < 0.05$) between fluid type and sex with *post hoc* $P < 0.01$ for effect of sex within the fluid-type water.

drank, on average, 8.5 ± 0.34 g/kg, and their average BEC was 1.1 ± 0.10 mg/ml.

Accelerating rotarod

Mice in the ethanol group on average fell from the rotarod approximately 30% sooner than mice in the water group on each of the five trials ($F_{1,30} = 12.2$, $P = 0.002$; Fig. 3a). Latency to fall increased in both groups with each subsequent trial ($F_{4,120} = 15.6$, $P < 0.0001$), and these changes occurred in parallel (i.e. no interaction between trial and group was observed; Fig. 3a).

Balance beam

On average, mice in the ethanol groups made approximately twice as many foot slips as those in the water groups ($t = 3.2$, $df = 61$, $P = 0.002$).

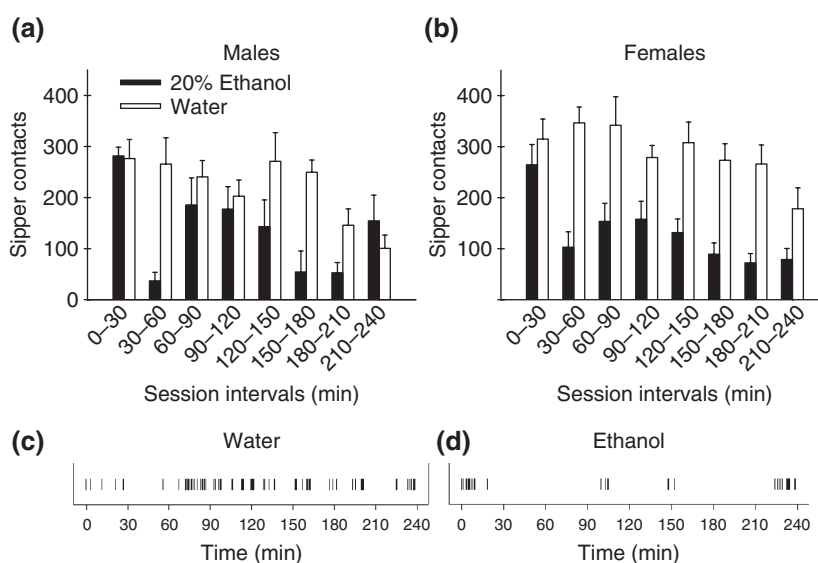
Experiment 3: DID strain differences and relationship to two-bottle choice

Batch effects

The 12 strains were tested in two separate batches (see *Materials and methods*), and C57BL/6J mice were included in each batch so that an adjustment could be made for differences between batches. On day 4 of the first test, after 4 h of ethanol exposure, C57BL/6J mice (collapsed across sex) drank 7.5 ± 0.24 g/kg in the first batch and 6.3 ± 0.51 g/kg in the second batch ($F_{1,29} = 3.1$, $P = 0.09$), and their BECs were 1.4 ± 0.12 and 0.71 ± 0.12 mg/ml ($F_{1,29} = 15.7$, $P = 0.0004$; Fig. 4b), respectively. In the retest, the batch differences were smaller and not significant. For intake, they were 7.6 ± 0.59 vs. 6.7 ± 0.34 g/kg ($F_{1,29} = 2.0$, $P = 0.16$) and for BEC, 1.2 ± 0.15 vs. 1.3 ± 0.13 ($F_{1,29} = 0.05$, $P = 0.83$), respectively. We reanalyzed all the data after making adjustments for the batch differences by multiplying

Figure 2: The pattern of drinking changes when 20% ethanol is substituted for water in C57BL/6J mice. (A)

Average number of contacts on the sipper tube in 30 min increments (\pm SEM) when the sipper contains water vs. 20% ethanol ($n = 10$ males). (B) Same graph as A for females ($n = 13$, mean \pm SEM). (C) The pattern of contacts on the sipper tube for a representative male C57BL/6J mouse drinking water. Data depicted reveal 24 bouts (see *Materials and methods* for the definition of a bout). (D) The same male mouse shown in C drinking 20% ethanol. Here, 11 bouts were detected.



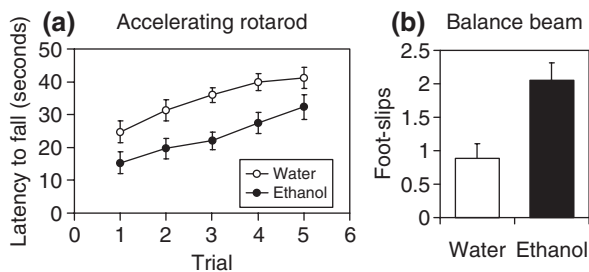


Figure 3: Drinking in the Dark causes motor impairment in female C57BL/6J mice. (A) Average latency to fall from an accelerating rotarod after 4-h exposure to 20% ethanol ($n = 16$) or water ($n = 16$). (B) Average number of foot-slips while traversing a balance beam after 4-h exposure to 20% ethanol ($n = 37$) or water ($n = 26$). Mean \pm SEM is shown.

intakes for all strains in batch 2 by the ratio 7.5/6.3 and BEC values by 1.4/0.71 for the first test and by 7.6/6.7 and 1.2/1.3 for the second test. This did not change qualitative results; therefore, only uncorrected data are shown. In all subsequent analyses of strain means and correlations of strain means, we combined the intakes and BECs from the first and second batches to represent the strain means for C57BL/6J.

Effect size for strain differences in Drinking in the Dark

Large strain differences were observed for ethanol intake (g/kg) and BEC (mg/ml) for the single bottle DID test, collapsed across sex (Fig. 4). Strain explained 53% of the variation in intake (i.e. $R^2 = 0.53$; $F_{11,151} = 15.6$, $P < 0.0001$) and 51% of the variation in BEC ($F_{11,151} = 14.4$, $P < 0.0001$) on day 4 of the first test after 4 h of ethanol exposure. In the retest, the effect sizes (R^2 values) were 46 and 55% (both $P < 0.0001$), respectively, for the same time points. C57BL/6J had a strong influence on the effect sizes. When C57BL/6J was removed from the analysis, $R^2 = 0.36$ and 0.21 for intake and BEC in the first test and 0.29 and 0.24 in the second test (all $P < 0.001$).

Pattern across days

Inspection of the means in Fig. 4a shows that the top four high-drinking strains (C57BL/6J, BALB/cJ, BALB/cByJ and FVB/NJ) and one intermediate strain (C3H/HeJ) drank a consistent quantity of ethanol (g/kg) in 2 h on each of the 4 days (repeated measures analysis, day effect, $P > 0.05$). The two lowest drinking strains (129S1/SvImJ and DBA/2J) and three intermediate strains (CBA/J, BTBR and A/J) drank significantly less alcohol on days 2–4 relative to day 1 ($P < 0.05$). AKR/J appeared to drink slightly less alcohol on days 2–4 relative to day 1, and LP/J appeared to alternate up and down each day, but these changes were not statistically significant (all $P > 0.05$).

Intake vs. BEC among strains

The relationship between ethanol intake and BEC among strains was not linear (Fig. 4b) as indicated by a significant

second-order term in a polynomial regression of the strain means ($P = 0.01$). A large difference in intake from 1.5 to 6 g/kg increased BEC from 0 to 0.5 mg/ml, whereas a small difference in intake from 6 to 7 g/kg increased BEC from 0.5 to 1 mg/ml. Without C57BL/6J, the second-order term was not significant.

Reliability

Conventional and phylogenetic methods gave similar estimates of correlations among strain means for intake and BEC (Table 2); hence, only results of conventional analyses are reported here. The strain means for intake and BEC were stable across replicate trials (Fig. 5, Table 2). The R^2 value for the linear regression of retest strain means on test strain means was 0.82 ($P < 0.0001$; Fig. 5a). The test–retest R^2 for BEC strain means was 0.83 ($P < 0.0001$; Fig. 5b). When C57BL/6J was removed from the analysis, R^2 values were 0.71 and 0.48 for intake and BEC, respectively (both $P < 0.05$).

Nine out of the 12 strains demonstrated nearly identical average intakes in the retest as compared with the first test (Fig. 5a). Two strains (129S1/SvImJ and LP/J) showed significantly larger intakes ($P < 0.05$) and slightly higher BECs ($P = 0.07$ for both) in the retest as compared with the first test. One strain (DBA/2J) showed slightly lower intakes ($P = 0.08$) in the second test as compared with the first, but this was not accompanied by a significant change in BEC ($P = 0.25$). Although the FVB/NJ strain did not show a significant increase in intake in the retest relative to the first, the BEC strain mean increased slightly ($P = 0.05$) in the retest.

Sex differences

On average, across all 12 strains, females drank approximately 1.4 g/kg more than males ($F_{1,139} = 27.0$, $P < 0.0001$) on day 4 of the first test after 4 h of ethanol exposure, and no significant strain-by-sex interaction was observed ($F_{11,139} = 1.7$, $P = 0.08$) (Table 3). It is interesting that the sex difference in intake on the first test was not large enough to affect BEC, because neither a sex effect ($F_{1,139} = 0.16$, $P = 0.69$) nor an interaction between sex and strain ($F_{11,139} = 0.45$, $P = 0.93$) occurred for BEC.

On day 4 of the retest, after 4 h of ethanol exposure, females drank, on average, 1.6 g/kg more than males ($F_{1,138} = 32.1$, $P < 0.0001$) and had 0.16-mg/ml higher BECs ($F_{1,138} = 3.9$, $P < 0.05$). No sex-by-strain interaction was observed for intake ($F_{11,138} = 1.4$, $P = 0.18$) or BEC ($F_{11,138} = 0.67$, $P = 0.77$).

Relationship to two-bottle choice

Following the retest of the DID procedure, mice from eight of the strains were tested in a limited access version of two-bottle choice (see *Methods*). In the two-bottle test, strain explained 48% of the variation in intake (i.e. $R^2 = 0.48$; $F_{7,87} = 11.6$, $P < 0.0001$) and 51% of the variation in BEC

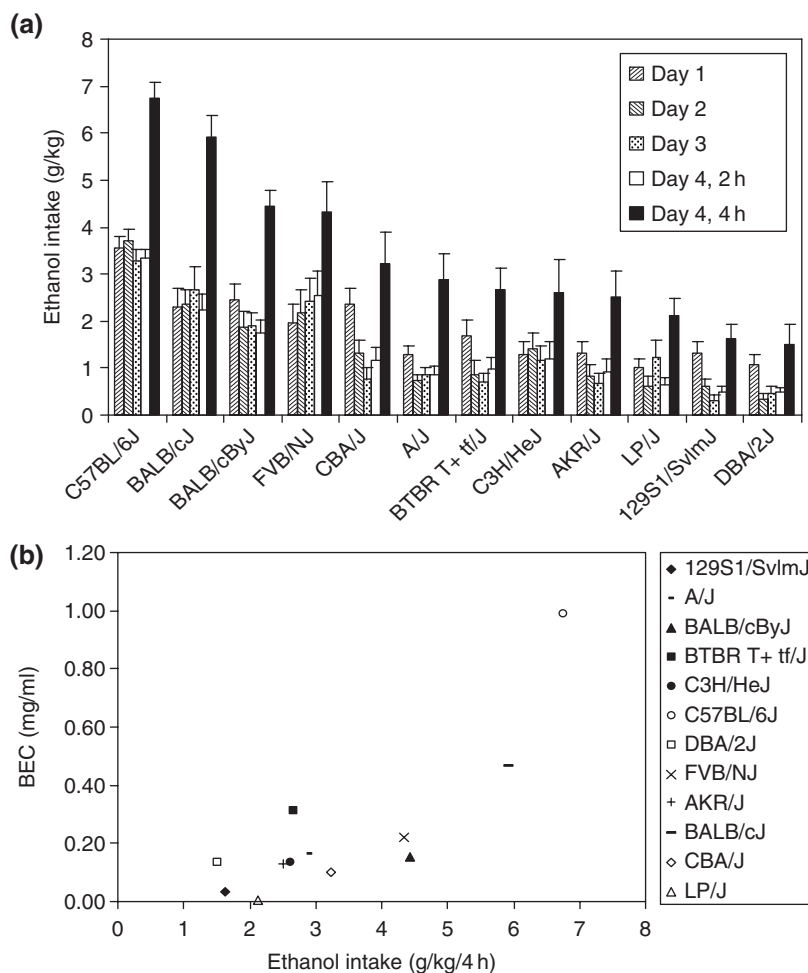


Figure 4: Strain differences in ethanol intake and blood ethanol concentration for Drinking in the Dark. (A) Mean \pm SEM consumption of ethanol in g/kg accumulated over a 2- or 4-h period on days 1–4 for 12 standard inbred strains of mice, collapsed across sex ($n = 6$ males, $n = 6$ females per strain).

(B) Strain means for blood ethanol concentration (BEC); mg/ml) plotted against the strain means for ethanol intake (g/kg) on day 4 of the first test after 4 h of ethanol exposure, collapsed across sex.

Table 2: Correlations among 12 strain means (collapsed across sex) for ethanol intake (g/kg) and blood ethanol concentration (BEC) (mg/ml) after Drinking in the Dark (DID) or limited 2-bottle choice

	DID g/kg1	DID g/kg2	2-bottle GKG	DID BEC1	DID BEC2	2-bottle BEC
DID g/kg1		0.84	0.80	0.86	0.90	0.91
DID g/kg2	0.90 (0.84)		0.92	0.54	0.72	0.90
2-bottle g/kg	0.86 (0.80)	0.92 (0.88)		0.50	0.85	0.90
DID BEC1	0.83 (0.74)	0.69 (0.39)	0.57 (0.01)		0.87	0.82
DID BEC2	0.87 (0.78)	0.83 (0.72)	0.79 (0.76)	0.91 (0.69)		0.99
2-btl BEC	0.89 (0.69)	0.86 (0.71)	0.85 (0.90)	0.90 (0.26)	0.99 (0.94)	

$n = 6$ males and 6 females per strain, except C57BL/6J where $n = 16$ males and 15 females collapsed across the two batches. DID g/kg1 and DID g/kg2 refer to cumulative intake of 20% ethanol in g/kg after 4 h of Drinking in the Dark for the first test and retest, respectively. Two-bottle g/kg refers to cumulative intake of 20% ethanol in g/kg after 4 h of two-bottle choice (20% ethanol or tap water). DID BEC1, BEC2 and 2-bottle BEC refer to the blood ethanol concentration (mg/ml) after the first and second DID tests, and limited two-bottle choice test, respectively. Pearson's correlations of the raw strain means are shown below the diagonal. Results with C57BL/6J (the high responder) removed from the analysis are shown in parentheses. Pearson's correlations of phylogenetically independent contrasts of the strain means are shown above the diagonal. Correlations among DID traits include 12 strain means ($df = 10$). Correlations with the two-bottle choice traits include eight strain means ($df = 6$). Significant ($P < 0.05$) correlations are shown in bold.

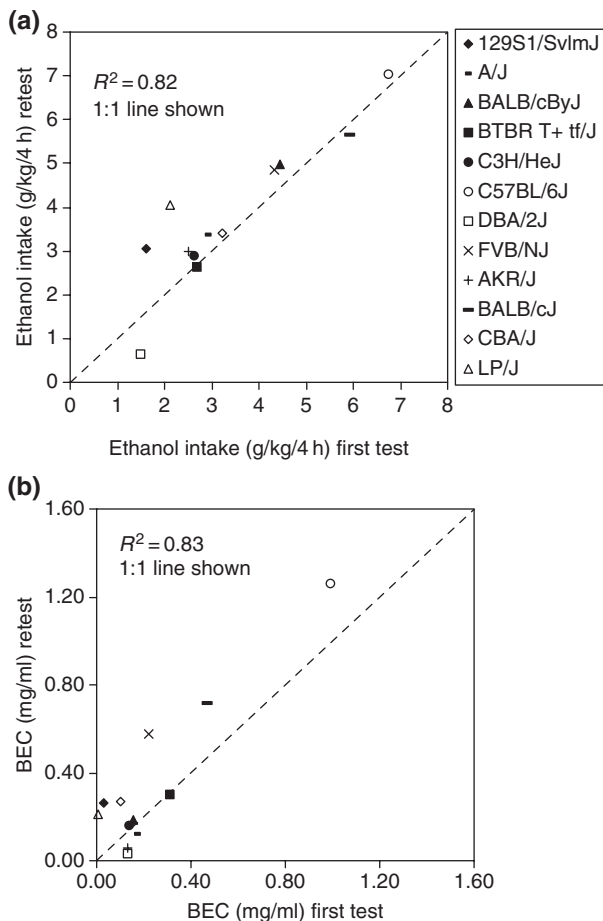


Figure 5: Drinking in the Dark (DID) strain means for intake and blood ethanol concentration (BEC) are reliable. (A) DID strain means for ethanol intake (g/kg) in the retest (Day 4) plotted against the first test (Day 4) after 4 h of ethanol exposure. (B) Same graph as A but for blood ethanol concentration (BEC) (mg/ml).

($F_{7,87} = 12.9$, $P < 0.0001$) on day 4 after 4 h of ethanol access. When C57BL/6J was removed from the analysis, $R^2 = 0.39$ and 0.21 for intake and BEC, respectively. Mean water intake \pm SEM during the two-bottle test ranged from $0.33 \text{ ml} \pm 0.13$ in FVB/NJ to $1.6 \text{ ml} \pm 0.41$ in BTBR T+tf/J ($R^2 = 0.14$; $F_{7,87} = 2.0$, $P = 0.06$) (Table 4). No strain differences were observed for total fluid intake (sum of the volume of water and ethanol solution consumed; $R^2 = 0.08$, $P = 0.39$, data not shown).

Across the eight strains, animals drank a similar quantity of ethanol in grams per kilogram in 4 h whether they were given a single bottle of 20% ethanol or a choice between 20% ethanol or water (Fig. 6a; $F_{2,406} = 2.5$, $P = 0.08$). Despite the similar intakes, BECs were approximately 40% lower for the two-bottle test as compared with the DID tests (Fig. 6b; $F_{2,406} = 12.5$, $P < 0.0001$). The P values for the *post hoc* comparisons of BEC in the two-bottle test vs. the first and second DID tests were 0.01 and <0.0001 ,

respectively. The first and second DID tests also differed from each other with the second test showing approximately 20% higher BEC than the first (Fig. 5b; $P = 0.004$). Taken together, these results suggest that, across a panel of inbred strains showing a large range in ethanol consumption, drinking from a single bottle of 20% ethanol resulted in similar intakes but higher BECs as compared with the mice which were given a choice between 20% ethanol and tap water.

Phylogenetic signal in DID intake and BEC

Based on the randomization test of Blomberg *et al.* (2003), intake in the DID test showed a non-significant tendency toward a phylogenetic signal during the first test ($P = 0.052$; $K = 0.794$) and significant signal during the second ($P = 0.024$; $K = 0.791$). For both tests, the MSE for the hierarchical phylogeny was smaller (indicating better fit) than for the star phylogeny (ratios were 1.07 and 1.06, respectively). BEC did not show significant signal at either test ($P = 0.084$ and 0.115 , respectively; $K = 0.837$ and 0.718 , respectively). Ratios of MSE on the star/hierarchical phylogeny were 1.08 and 0.95, respectively.

DID strain mean correlations with traits in the Mouse Phenome Project database (males only)

To examine the pattern of associations, we adopted an arbitrary cut off of interest of $|r| > 0.50$. Of the 609 traits included in the MPP database (combined with our own private datasets) that shared at least eight strains in common with this study, 85 showed a positive Pearson's correlation ($r > 0.50$) with DID intake (g/kg/4 h on day 4, first test) and 36 showed a negative correlation ($r < -0.50$). Note that redundancy in the database is considerable (e.g. six traits are included for adrenal mass: left, right, combined and each adjusted for body mass). For DID BEC (mg/ml on day 4 after 4 h), 67 positive correlations (greater than 0.5) and 36 negative correlations (< -0.5) were observed. Examination of the plots of the correlations for BEC showed that these were highly influenced by inclusion of the C57BL/6J strain, which displayed an extraordinarily high mean BEC relative to all other strains (Fig. 5b). Therefore, only results of correlations with DID intake are shown. Recall that strain means for DID intake are strongly correlated with BEC (Table 2) but show a more uniform distribution than BEC (Fig. 5).

Of these 121 Pearson correlations (>0.5 or <-0.5), 59 showed an R^2 value that reached the traditional level of significance ($P < 0.05$) (42 positive and 17 negative r estimates). The false discovery rate estimated for this level of significance was 30% (see *Statistical analysis* section). To control the experiment-wide false discovery rate at 5%, we set the cut off at $P < 0.000943$. Only sodium chloride preference (Fig. 7b), mass of the adrenal glands and anterior commissure length showed a positive correlation with DID intake at this level of significance (see notes in Table 5 for descriptions and sources for these traits). Only one trait,

Table 3: Strain means for Drinking in the Dark (DID) intake and blood ethanol concentration (BEC) by sex

Strain	Intake (g/kg)		BEC (mg/ml)	
	Males	Females	Males	Females
C57BL/6J	6.25 ± 0.37	7.27 ± 0.56	1.06 ± 0.15	0.92 ± 0.16
BALB/cJ	5.51 ± 0.64	6.32 ± 0.71	0.56 ± 0.21	0.37 ± 0.16
BALB/cByJ	4.05 ± 0.38	4.82 ± 0.61	0.14 ± 0.09	0.17 ± 0.10
FVB/NJ	3.59 ± 0.84	5.07 ± 0.89	0.28 ± 0.07	0.15 ± 0.10
CBA/J	1.87 ± 0.40	4.58 ± 1.01 [†]	0.19 ± 0.11	0.01 ± 0.01
A/J	1.89 ± 0.53	3.87 ± 0.82*	0.21 ± 0.13	0.12 ± 0.05
BTBR T+tf/J	2.81 ± 0.63	2.54 ± 0.67	0.27 ± 0.13	0.36 ± 0.17
C3H/HeJ	0.62 ± 0.13	4.60 ± 0.71 [‡]	0.18 ± 0.10	0.09 ± 0.07
AKR/J	1.61 ± 0.47	3.41 ± 0.89*	0.14 ± 0.06	0.12 ± 0.07
LP/J	2.33 ± 0.62	1.92 ± 0.44	0.00 ± 0.00	0.01 ± 0.01
129S1/SvImJ	1.09 ± 0.17	2.16 ± 0.54	0.02 ± 0.02	0.04 ± 0.03
DBA/2J	0.60 ± 0.19	2.41 ± 0.67*	0.25 ± 0.15	0.02 ± 0.02

Values represent mean ± SEM intake or BEC after 4 h of DID on day 4 of the first test. $n = 6$ males and 6 females per strain, except C57BL/6J where $n = 16$ males and 15 females collapsed across the two batches.

* $P < 0.0001$, [†] $P < 0.01$, [‡] $P < 0.05$ denote the effect of sex within a strain for intake. These data along with retest strain means will be posted in the Mouse Phenome Project database for public access (<http://aretha.jax.org/pub-cgi/phenome/mpdcgi?rtn=docs/home>).

percent fat mass after 8 weeks on a fatty diet, showed a significant negative correlation using this criterion (Table 5).

Conventional and phylogenetic methods gave similar estimates of correlations between DID intake and the other traits (Table 5). Of the four planned correlations with ethanol traits, DID intake showed a trend ($P = 0.08$) toward a positive correlation with ethanol intake in standard two-bottle choice taken from Belknap *et al.* (1993) (Table 5; Fig. 7a), a significant positive correlation with ethanol-induced hypothermia from Crabbe *et al.* (in press) ($P = 0.02$; note that, with the phylogenetic method, the correlation was marginally not significant, $P = 0.06$, Table 5), a significant negative correlation with chronic ethanol withdrawal severity from Metten and Crabbe (2005) (Fig. 7c, Table 5) ($P = 0.02$; note that with the phylogenetic method the correlation was

marginally not significant, $P = 0.06$, Table 5) and a significant negative correlation with conditioned taste aversion to 4 g/kg ethanol from Broadbent *et al.* (2002) ($P = 0.004$; Fig. 7d, Table 5). An example of a trait that did not reach significance by the false discovery criterion but still might be of interest is total daily activity (total breaks over 3 days) (Table 5). Activity showed a positive correlation with DID intake ($P = 0.007$) (Table 5).

Discussion

Our goals were to explore the recently described DID procedure in more detail and to evaluate its potential as a tool to study the genetic basis of pharmacologically significant ethanol drinking in mice. Many other procedures have been

Table 4: Strain means for limited access two-bottle choice

Strain	Intake (ml)		Preference ratio
	Water	20% Ethanol	
C57BL/6J	0.70 ± 0.162	1.02 ± 0.051	0.59
FVB/NJ	0.33 ± 0.134	0.99 ± 0.138	0.75
BALB/cByJ	0.84 ± 0.192	0.68 ± 0.083	0.45
129/SvImJ	1.05 ± 0.289	0.46 ± 0.082	0.31
C3H/HeJ	0.80 ± 0.235	0.40 ± 0.099	0.34
A/J	0.62 ± 0.263	0.38 ± 0.088	0.38
BTBR T+tf/J	1.55 ± 0.411	0.29 ± 0.053	0.16
DBA/2J	1.24 ± 0.340	0.15 ± 0.023	0.11

Values represent mean ± SEM intake (ml) of water vs. 20% ethanol during 4 h of limited access two-bottle choice on day 4 of the 4-day test after the animals had received two previous 4-day Drinking in the Dark tests ($n = 6$ males and 6 females/strain). Preference ratio was calculated by dividing the strain mean for intake (ml) of 20% ethanol by the sum of the strain means for water and 20% ethanol intakes (ml).

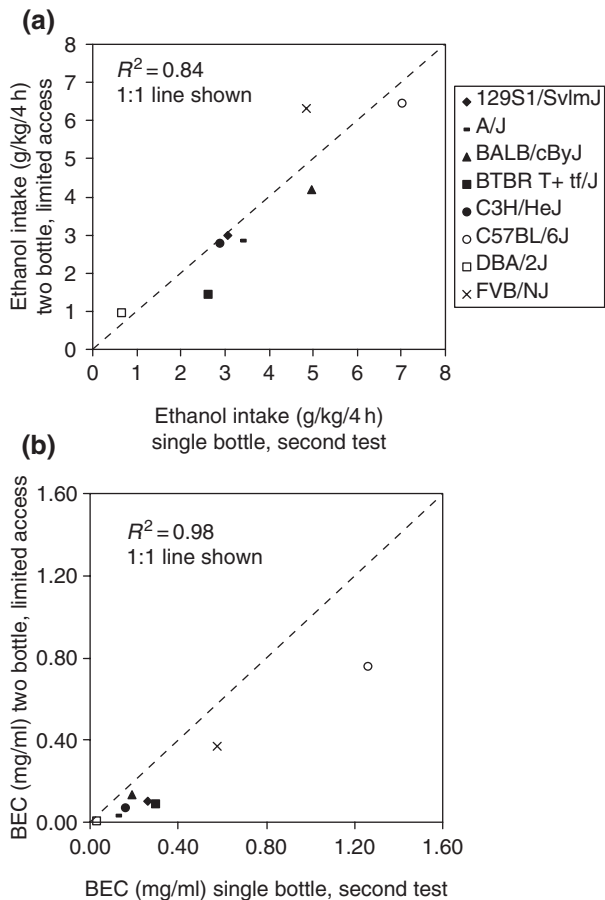


Figure 6: Similar intakes result in higher blood ethanol concentrations (BECs) in the Drinking in the Dark (DID) procedure as compared with a limited access version of two-bottle choice (see Methods). (A) DID strain means for ethanol intake (g/kg) in the retest on day 4 after 4 h of ethanol exposure plotted against limited access two-bottle choice strain means for ethanol intake (g/kg) on day 4 after 4 h of ethanol exposure. (B) Same graph as A but for BEC (mg/ml).

developed to study ethanol self-administration in rodents (e.g. Aufrere *et al.* 1997; Deutsch & Cannis 1980; Finn *et al.* 2005; Lopez & Becker 2005; Macdonall & Marcucella 1979; Marcucella & Munro 1987; McClearn & Rodgers 1959; Mittleman *et al.* 2003; Samson 1986). The main advantages of DID over these other procedures are that it often produces a BEC above 1 mg/ml in at least one inbred strain without severely restricting food or fluid access and is extremely simple to implement (Rhodes *et al.* 2005). The simplicity makes it especially useful for high-throughput genetic research (e.g. screening mutant strains, gene mapping or selective breeding) (Crabbe *et al.* 1994; Rhodes & Crabbe 2003).

The result that DID produced behavioral intoxication in the genetically predisposed strain, C57BL/6J, as measured by motor impairment on the rotarod and balance beam (Fig. 3),

demonstrates that DID can produce a pharmacologically significant effect on behavior and physiology (Crabbe *et al.* 2005; Rustay *et al.* 2003b). The large effect sizes ($R^2 \sim 50\%$) for strain differences in intake and BEC suggest that the pharmacologically significant drinking in C57BL/6J is under strong genetic control (Crabbe 2002). The finding of significant phylogenetic signal for intake (see below) is also consistent with a strong genetic effect. The fact that the effect sizes drop by approximately half when C57BL/6J is removed from the analysis is consistent with what has been known for many years: that C57BL/6J is an extraordinarily high-drinking strain relative to others (Belknap *et al.* 1993; McClearn & Rodgers 1959). Other comparisons of many inbred strains (Belknap *et al.* 1993; Rodgers 1966) confirm that the effect of C57BL genes is strong, as other strains of the C57 lineage including C57/LJ, C57BR/cdJ, C58/J and C57BL/10J are also among the highest drinking strains in such surveys.

The correlation of strain means among the DID traits and two-bottle choice tests (Table 2, Figs 6 and 7a) suggests that some of the same genes that influence ethanol drinking when animals are given a choice also influence drinking when no alternative fluid is available (i.e. pleiotropy), although linkage disequilibrium, epigenetic and/or maternal effects might also contribute to these relationships. Similarly, the correlation of the DID strain means with other ethanol- and non-ethanol-related traits suggests that all these traits share common genetic influences, either through pleiotropy or linkage disequilibrium (Broadbent *et al.* 2002; Crabbe *et al.* 1990; Crabbe *et al.* 2003b; Metten & Crabbe 1994; Metten *et al.* 1998).

The non-linear relationship between ethanol intake and BEC among strains (Fig. 4b) is important to consider when interpreting genetic studies of ethanol drinking in mice. A difference in ethanol intake between genotypes such as between a null mutant and a wildtype (Roberts *et al.* 2001; Spanagel *et al.* 2002), among inbred strains (Belknap *et al.* 1993), or between selectively bred lines (Grahame *et al.* 1999) might only result in a small change in BEC (Fig. 4b) that is not meaningful in terms of its pharmacological effect on behavior or physiology (Dole & Gentry 1984). While these results are suggestive of a threshold effect, the mechanism is currently unknown. It is possible that C57BL/6J drank at a level that exceeds the rate at which ethanol is metabolized, resulting in an accumulation of ethanol in the blood, whereas the other strains drank below this level. Alternatively, the pattern of drinking could have had a strong influence. For example, C57BL/6J consumed a large loading dose followed by smaller episodic maintenance bouts that continued up until the end of the 4-h session immediately before BEC was measured (Fig. 2), whereas the other strains might have done their drinking using a pattern less conducive to establishing a high BEC. For example, circadian rhythms are known to differ among strains (Hofstetter *et al.* 2003), and it is possible that some of the strains tested here may not have been eating and drinking at a maximum rate

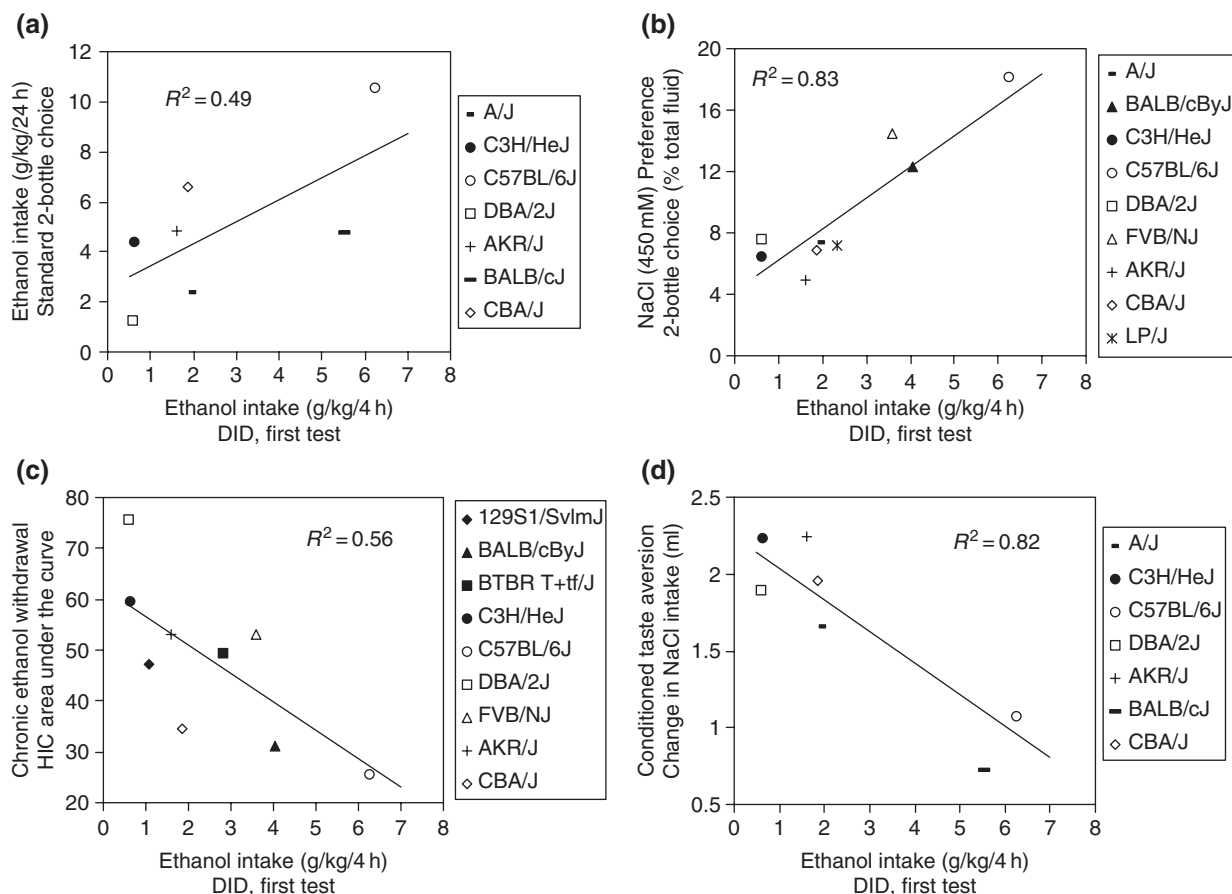


Figure 7: Ethanol intake in the Drinking in the Dark (DID) procedure is positively genetically correlated with ethanol intake in standard two-bottle choice and preference for sodium chloride solution and negatively correlated with chronic ethanol withdrawal severity and a taste aversion conditioned by ethanol injections. (A) Strain means for ethanol intake in the standard two-bottle choice test (g/kg/24 h) plotted against DID strain means for ethanol intake on day 4 of the first test (g/kg/4 h) ($n = 7$ strains; males only). The strain means for standard two-bottle choice were taken from Belknap *et al.* (1993) and represent the average quantity (g/kg/24 h) of 10% ethanol drunk on days 9 and 10 of a 10-day test. Note that Belknap *et al.* (1993) used A/HeJ instead of A/J, but these are not distinguished here due to their recent divergence. The simple linear regression line and R^2 value are shown. (B) The same graph as A except with strain means for sodium chloride preference plotted on the y -axis ($n = 9$ strains; males only). The sodium chloride preference strain means were taken from Bachmanov *et al.* (2002). They represent the percentage of 450 mM solution of sodium chloride consumed over 48 h relative to the total fluid consumed in a two-bottle choice test where the other bottle contained deionized water. (C) The same graph as A except with strain means for chronic ethanol withdrawal severity plotted on the y -axis ($n = 9$ strains; males only). The withdrawal strain means were taken from Metten and Crabbe (2005) and represent the area under the curve for handling induced convulsions following removal from 72-h exposure to ethanol vapor corrected by subtracting control group means. (D) The same graph as A except with strain means for conditioned taste aversion plotted on the y -axis ($n = 7$ strains; males only). The taste aversion strain means were taken from Broadbent *et al.* (2002) and represent the volume (ml) of .2 M NaCl drunk in 1 h minus the volume drunk after the NaCl solution was paired with a 4 g/kg i.p. injection of ethanol (a difference score). Note that y -axis data in panels A and D were from BALB/cJ rather than BALB/cByJ.

when DID was recorded. Note that the 24-h tests sample all phases of the circadian cycle and therefore do not encounter this sampling issue. Finally, drinking is often postprandial in rodents, and because the only source of fluid (20% ethanol) was likely unpalatable to many of the strains, eating may have decreased which could have subsequently caused a further reduction in fluid intake (Kotlus & Blizard 1998). We might have seen a very different pattern of strain relationships if the ethanol solution were made more palatable, for example by adding

a sweetener. Future studies are needed to sort out the contribution of drinking pattern, circadian rhythm, palatability of the ethanol solution and postprandial processes in explaining the strain differences in DID behavior observed here.

The relationship between ethanol intake and BEC across strains appears to depend on whether the mice have concomitant access to water. The same intake in g/kg resulted in an approximately 40% lower BEC when water was available (Fig. 6). This was true for all eight strains examined. One

Table 5: (Males only) Correlations of strain means for ethanol intake (Drinking in the Dark, g/kg, first test) with eight other traits from the Mouse Phenome Project

Trait	<i>n</i>	Raw Pearson's <i>r</i>	Phylogenetically independent Pearson's <i>r</i>
Ethanol intake, standard 2-bottle choice.	7	0.70 (<i>P</i> = 0.08)	0.67 (<i>P</i> = 0.10)
Conditioned taste aversion to saline after i.p. injection of 4-g/kg ethanol	7	-0.91 (<i>P</i> = 0.004)	-0.88 (<i>P</i> = 0.009)
Ethanol-induced hypothermia	8	0.80 (<i>P</i> = 0.02)	0.69 (<i>P</i> = 0.06)
Preference for 450 mM NaCl solution	9	0.91 (<i>P</i> = 0.0007)	0.89 (<i>P</i> = 0.001)
Chronic ethanol withdrawal	9	-0.75 (<i>P</i> = 0.02)	-0.65 (<i>P</i> = 0.06)
Mass of the adrenal glands	9	0.91 (<i>P</i> = 0.0007)	0.93 (<i>P</i> = 0.0003)
Anterior commissure length	9	0.93 (<i>P</i> = 0.0003)	0.92 (<i>P</i> = 0.0004)
Locomotor activity accumulated over 3 days (beam breaks)	10	0.79 (<i>P</i> = 0.007)	0.83 (<i>P</i> = 0.003)
% Body fat after 8 weeks on a fatty diet	12	-0.79 (<i>P</i> = 0.002)	-0.60 (<i>P</i> = 0.04)

Strain means for the standard two-bottle test were taken from Belknap *et al.* (1993) and represent the average quantity (g/kg/24 h) of 10% ethanol drunk on days 9 and 10 of a 10-day test. The conditioned taste aversion strain means were taken from Broadbent *et al.* (2002) and represent the volume (ml) of 0.2 M NaCl drunk in 1 h minus the volume drunk after the NaCl solution was paired with a 4 g/kg i.p. injection of ethanol (a difference score). Ethanol-induced hypothermia strain means (HT30 D1 05) were taken from one of our papers (Crabbe *et al.* in press) and represent the change in body temperature 30 min after 3 g/kg i.p. injection of ethanol. The sodium chloride preference strain means were taken from Bachmanov *et al.* (2002) and represent the percentage of 450 mM solution of sodium chloride consumed over 48 h relative to the total fluid consumed in a two-bottle choice test where the other bottle contained deionized water. The chronic ethanol withdrawal strain means were taken from Metten and Crabbe (2005) and represent the area under the curve for handling induced convulsions (HIC) following removal from 72-h exposure to ethanol vapor corrected by subtracting air-exposed control group means. Adrenal mass strain means were taken from Deschepper *et al.* (2004) and represent mass of the adrenals (mg) per gram body weight. Anterior commissure length strain means were taken from Wahlsten *et al.* (2003) and represent the length of the anterior commissure in mm at the mid-sagittal plane. Strain means for locomotor activity were taken from the Seburn project (<http://pga.jax.org/>). These data were collected at The Jackson Laboratory using a comprehensive laboratory animal monitoring system (CLAMS) (Columbus Instruments, Columbus, OH). Mice were housed individually in CLAMS chambers (without bedding) for 3 days without any treatment. Two arrays of infrared beams (2.5-cm interbeam distance) surrounded each cage to automatically record locomotor activity over the 3-day period. The strain means represent the total beam breaks over the 3 days. Means for percentage body fat were taken from the Naggert project (same URL as above). These data were also collected at The Jackson Laboratory and represent the percent tissue mass that is fat after 8 weeks on an atherogenic diet. The sample size (*n*) gives the number of strains included in the correlation. Correlations of the raw strain means are given under raw Pearson's *r*, whereas correlations of the phylogenetically independent contrasts of the strain means are given under phylogenetically independent Pearson's *r*. *P* values (two-tailed) for the correlations are shown in parentheses next to the estimates.

simple explanation for this is that the water intake dilutes the ethanol, first in the stomach and subsequently in the blood. If the mice drank more ethanol (in g/kg) when water was available, that could offset the difference, but this did not happen. Each of the eight strains surveyed drank a characteristic quantity of ethanol that was approximately the same whether water was available or not (Fig. 6a). It is possible that we would have seen a different result had we given the two-bottle test first. However, the strain mean values for intake in the first and second DID tests were remarkably similar for a majority of the strains (see *Reliability* under *Results* section) suggesting that order of testing has a negligible effect. This is certainly true for C57BL/6J where we have seen stable levels of drinking across 12 days in a 2-h DID test (Rhodes *et al.* 2005). Taken together, these results suggest that, across genotypes, restricting water access for a 4-h period during DID does not change the motivation for drinking the ethanol solution but does affect the level of alcohol reached in the blood (pharmacokinetics).

Phylogenetic signal in strain means

To the best of our knowledge, we have not previously attempted tests for phylogenetic signal, which is the tendency for related species (or strains in this context) to show similar values of a trait, for inbred strains of mice or for any other laboratory or domestic organism. Although only one of the four traits analyzed in the present study showed a statistically significant (*P* < 0.05) signal, our tests probably have relatively low power, on the order of 0.4–0.5 (Fig. 2 in Blomberg *et al.* 2003). Indeed, the *K* values we calculated for intake and for BEC in the DID tests (0.7–0.8) are well within the range of values reported for behavioral and physiological traits among species (Blomberg *et al.* 2003). Note that many factors, including error in estimating strain means and errors in phylogenetic branch lengths, will tend to lower *K* values.

One would expect the magnitude of phylogenetic signal to be greater among species than among populations within a single species, but in the only published study of this proposition, Ashton (2004) found generally similar levels of signal for

body size among populations and among species of wild animals. For comparison, we analyzed data on the mean body mass of 21 inbred strains, as obtained from the MPP database (data from Wahlsten *et al.* 2003). We created a composite phylogeny and used Pagel's (1992) arbitrary branch lengths, as for our other analyses, and found that log female body mass showed highly significant phylogenetic signal ($P = 0.001$), with a K statistic of 0.841. Blomberg *et al.* (2003) reported that for the 24 measures of adult body size, the mean K was 0.83 with a 95% CI of 0.63–1.07. Hence, K for mouse body mass is very typical of what has been found for both interspecific and intraspecific datasets (Ashton 2004; Blomberg *et al.* 2003).

Taken as a whole, our results suggest that the genetic (evolutionary) relationships of the strains likely contribute to the resemblance in ethanol-drinking behavior among groups of inbred strains (e.g. Fig. 1b). Nonetheless, accounting for this information did not change estimates of correlations among ethanol-related traits (Tables 2 and 4). This implies that previous studies of strain mean correlations (at least for these strains and alcohol-related traits) probably have not been misled by ignoring the history of strain development. However, the range of inbred strain genealogies across which these relationships can be extrapolated is unknown. For example, inclusion of phylogenetically very divergent wild-derived inbred strains in our analyses could change outcomes significantly.

Motivation for drinking in C57BL/6J

Although C57BL/6J mice drank ethanol to pharmacologically significant levels in the DID procedure, the motivation for the drinking is not known. It is possible that the animals drank the 20% ethanol because they tolerated it as a source of fluid in small quantities in spite of its aversive taste (Table 1) or because they sought the calories or novelty of the flavor rather than the pharmacological effect (Cunningham *et al.* 2000; Dole & Gentry 1984). The strong correlation of DID intake with preference for drinking a saline solution among strains ($r = 0.91$; Fig. 7b) is consistent with the hypothesis that C57BL/6J may be less sensitive to aversive or novel tastes and/or smells in their drinking water as compared with other strains. Nonetheless, the willingness of this strain to ingest ethanol under a variety of conditions, including their willingness to work to gain access to ethanol, is well-established (Risinger *et al.* 1998).

In the present study, average DID intake of 20% ethanol in C57BL/6J varied from 6.5 g/kg (in batch 2 of experiment 3) to 8.5 g/kg (in batch 1 of experiment 3) producing a range in BEC from 0.7 to 1.4 mg/ml. The source of this variation is unknown and could be a result of stochastic or subtle changes in environmental conditions that were beyond our control. This is consistent with idea that the genes that predispose ethanol drinking in C57BL/6J are permissive rather than deterministic (i.e. the effect of the genes depends on the environment) (Dole *et al.* 1988).

Pleiotropic effects of DID genes

The negative strain mean correlations of DID intake with chronic ethanol withdrawal severity and ethanol-induced conditioned taste aversion (Table 5, Fig. 7c,d) are consistent with several previous reports showing that high-drinking mouse genotypes tend to experience less-severe withdrawal and less aversion to a novel flavor paired with ethanol intoxication (Broadbent *et al.* 2002; Chester *et al.* 2003a; Chester *et al.* 2003b; Metten *et al.* 1998; Phillips *et al.* 1998; Risinger & Cunningham 1998); for a review, see Crabbe & Phillips (2004). This suggests that one reason predisposed strains may drink is they are less sensitive to the aversive effects of ethanol, a relationship that has also been supported for human alcoholics (Heath *et al.* 1999).

The positive strain mean correlation between DID intake and severity of ethanol-induced hypothermia (Table 5) is consistent with results from one replicate of a selective breeding experiment in which COLD-1 mice, selectively bred for increased hypothermic response to 3 g/kg ethanol, drank more ethanol (at concentrations above 5%) than HOT-1 mice bred for reduced hypothermic response, although this relationship was not reproduced in the second replicate of this experiment (Crabbe *et al.* 1987; Cunningham *et al.* 1991). Bachtell *et al.* (2003) suggested that the number of urocortin-containing neurons in the Edinger–Westphal nucleus might be responsible for the relationship between hypothermia and drinking in these lines, because increased number of urocortin cells in Edinger–Westphal is associated with increased ethanol drinking in several mouse genetic models (Bachtell *et al.* 2003) and COLD-1 mice have more urocortin cells in Edinger–Westphal than HOT-1 mice (Bachtell *et al.* 2003). Moreover, urocortin projections from Edinger–Westphal to the dorsal raphe nucleus appear to play a direct role in ethanol-induced hypothermia, as injections of urocortin into the dorsal raphe-induced hypothermia in C57BL/6J mice and a urocortin receptor (CRF2) antagonist injected into the dorsal raphe blocked ethanol-induced hypothermia (Turek & Ryabinin 2005).

The significant correlations between DID intake and anterior commissure length and adrenal gland mass are intriguing, but the explanation is unclear. These traits were measured in studies where animals never received ethanol; hence, correlations imply that the traits are associated with the genetic predisposition for ethanol drinking rather than effects of ethanol *per se*. The anterior commissure is a bundle of nerve fibers that connect the two hemispheres of the brain. A few studies have demonstrated that prenatal ethanol exposure can reduce the size of this structure (Cassells *et al.* 1987; Wainwright & Fritz 1985), but to the best of our knowledge, nothing is known about how size of the anterior commissure might predispose ethanol drinking. The adrenal glands release such hormones as corticosterone, aldosterone, epinephrine, testosterone, progesterone and estrogen. It is possible that the size of the adrenal glands

might be related to release or regulation of some of these hormones which subsequently could affect genetic predisposition for ethanol intake through a variety of possible mechanisms. In both humans and animal models, conditions that increase cortisol or corticosterone have been associated with enlarged adrenal glands, whereas the opposite has also been true for conditions that decrease glucocorticoid levels (e.g. Claes 2004; Coste *et al.* 2001). However, basal corticosterone levels were not reported in the MPP database, and we do not know whether they correlate with ethanol drinking in this model. Therefore, we do not wish to speculate further until more data are established. Nonetheless, these results provide clear hypotheses for future studies.

Potential as a screening tool

Finding additional high-drinking strains besides those in the C57BL/6J lineage is needed to enhance the power for finding genes that underlie high drinking. Recently, in a mutant-screening endeavor using two-bottle choice (ethanol vs. water), Blednov *et al.* (2005) discovered that the F1 hybrid of FVB/NJ and C57BL/6J drank even greater amounts of ethanol than C57BL/6J. In a follow-up experiment using DID, we found that this hybrid drank amounts of ethanol and reached BECs similar to those seen in C57BL/6J (Blednov *et al.* 2005). The results we present here suggest that the DID procedure may be useful as a screening tool to identify mutant strains that drink to intoxication such as knockouts, transgenics or strains derived through ENU mutagenesis (Augustin *et al.* 2005). Of the two traits that could be used for this purpose (BEC or intake), BEC would be better because of the greater separation between the predisposed strain (C57BL/6J) and all other strains (Fig. 4), and because it immediately provides information about whether the level of drinking is likely to have produced intoxication. The non-linear genetic relationship between intake and BEC (Fig. 4b) makes it difficult to predict whether a level of intake will lead to a BEC above 1 mg/ml. We have found that approximately this level of alcohol in the blood is needed to produce behavioral signs of intoxication (Crabbe *et al.* 2003b; Rustay *et al.* 2003a). Thus, taken together, results suggest that using BEC rather than intake or preference as the trait in a screen is a more direct way to identify genotypes that are predisposed for drinking to intoxication. We are taking advantage of this and have recently begun a selective breeding experiment using the DID procedure with high BEC as the selection criterion.

Conclusions

The DID procedure represents a simple, high-throughput self-administration procedure that can be used as a screening tool to find or develop (e.g. via selective breeding) mouse genotypes that are predisposed to drink ethanol to the point of intoxication. These models can then be used to identify

the genes and pathways that underlie predisposition to initiate drinking to intoxication. A notable advantage of DID over other procedures is that it does not require severe water or food restriction to schedule the episodes of pharmacologically significant ethanol drinking in predisposed genotypes such as C57BL/6J, but we caution that the motivation for drinking is presently not known. It also remains to be determined whether DID can produce dependence and/or tolerance, which are key features of drug addiction. These caveats aside, results suggest that the drinking behavior in DID is under strong genetic control (i.e. large differences were found across strains), hence making it suitable for gene mapping and expression analyses. Moreover, interesting patterns of strain mean correlations were observed between DID and other traits extracted from the MPP database. Planned comparisons confirmed previous reports that genotypes with low sensitivity to the aversive effects of ethanol (i.e. those showing low levels of withdrawal or taste aversion) tended to drink greater amounts of ethanol. New findings associate morphology of the anterior commissure in the brain and mass of the adrenal glands with predisposition to drink ethanol. Newly applied phylogenetically based methods validate conventional statistical approaches that ignore the history of strain development in calculating the strain mean correlation estimates. Taken together, results suggest that rudimentary differences in physiology and/or development occur between genotypes that result in a network of correlations between morphology, physiology and behavior. Cumulative data for inbred strains in Phenome and Genome databases offer a powerful tool to uncover the phenotypic and genetic architecture of such a complex trait as ethanol-drinking behavior.

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