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Hybrid C57BL/6J x FVB/NJ Mice Drink More Alcohol than Do C57BL/6J Mice

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Background: From several recent strain surveys (28 strains: Bachmanov et al., personal communication; 22 strains: Finn et al., unpublished), and from data in >100 other published studies of 24-hr two-bottle ethanol preference, it is known that male C57BL/6 (B6) mice self-administer about 10–14 g/kg/day and that female B6 mice self-administer about 12-18 g/kg/day. No strain has been found to consume more ethanol than B6. In one of our laboratories (Texas), we noted a markedly greater intake of ethanol in an *F*1 hybrid of B6 and FVB/NJ (FVB) mice.

Methods: To confirm and extend this finding, we repeated the study at another site (Portland) using concentrations up to 30% ethanol and also tested B6xFVB *F*1 mice in restricted access drinking procedures that produce high levels of alcohol intake.

Results: At both sites, we found that B6xFVB *F*1 mice self-administered high levels of ethanol during two-bottle preference tests (females averaging from 20 to 35 g/kg/day, males 7–25 g/kg/day, depending on concentration). *F*1 hybrids of both sexes drank significantly more 20% ethanol than both the B6 and FVB strains. Female *F*1 hybrids also drank more 30% ethanol. In the restricted access tests, ethanol consumption in the *F*1 hybrids was equivalent to that in B6 mice.

Conclusions: These data show that this new genetic model has some significant advantages when compared to existing inbred strains, and could be used to explore the genetic basis of high ethanol drinking in mice.

Key Words: Ethanol Consumption, Inbred Mouse Strains, Epistasis

UNLIKE WITH HUMAN subjects, assessment of the reinforcing effects of ethanol in rodents is necessarily a matter of inference. The two basic approaches are to allow the animal to self-administer ethanol or to use principles of Pavlovian conditioning to pair ethanol's effects with a specific cue and assess approach or avoidance of that cue in a subsequent drug-free test (Cunningham and Phillips, 2003). The earliest studies of voluntary self-administration (Richter and Campbell, 1940) were adapted from the nutrition field and offered rats two bottles, one containing an alcohol solution in tap water and the other tap water alone. These studies reported that self-administration was dependent on concentration of ethanol offered. Individual differences in ethanol preference among rats also were noted.

This basic procedure is usually termed a "two-bottle ethanol preference" test, which has many variants. The earliest attempts to document a genetic contribution to individual differences in preference drinking were successful. Mardones and Segovia–Requelme (1983) successfully bred rats to prefer or avoid ethanol-containing solutions, and McClearn and Rodgers (1959) demonstrated that C57BL/6 (B6) inbred mice had nearly absolute preference for 10% ethanol over tap water, that DBA/2 mice were near-teetotalers, and that other inbred strains showed intermediate preference. Fairly large surveys of 14 (Rodgers, 1972) or 15 (Belknap et al., 1993) inbred strains reinforced the primacy of B6 but revealed no other strains with higher preference. The genetic contribution to the trait was further evidenced by the relatively high preference of other strains (C57 L/J, C57BR/cdJ) from the C57BL lineage (Belknap et al., 1993). Subsequent analyses of even larger panels of inbred mouse strains (28 strains: Bachmanov et al., personal communication; 22 strains: Finn et al., unpublished) also have failed to reveal a more extreme preferrer of alcohol solutions than B6.

Ethanol intake is often reported using two indices. One is the "preference ratio," or the proportion of total fluid intake that is taken from the ethanol bottle in a two-bottle ethanol preference test. As already noted, preference ratio

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Table 1. Summary of Methods Performed

Method	Aim
Two-bottle choice.	Show preference for ethanol or other tastants under conditions of voluntary intake without restrictions.
Drinking in the dark.	Show ethanol acceptance during the circadian dark phase under conditions of limited access to ethanol without water deprivation.
Ethanol acceptance during scheduled fluid access.	Show ethanol acceptance under conditions of limited access to ethanol with varied duration of total fluid access (scheduled water restriction).
Ethanol metabolism (after intragastric or intraperitoneal administration of ethanol).	Show the possible differences in metabolism of ethanol or potential development of metabolic tolerance.

depends on the concentration offered, and the usual finding is that preference increases as concentration increases, up to a point, but thereafter, preference ratios decline. That is, the relationship is an inverted-U shaped curve. The second index, g of ethanol consumed per kg body weight, gives a direct measure of the amount of ethanol consumed and relates directly to blood ethanol concentration. In the studies reported below, we report both indices but base our interpretations of the data only on g/kg intake.

From the strain surveys reported above, and from the data reported in many (>100) other published studies of 24-hr two-bottle ethanol preference, it has been well-documented that male B6 mice will self administer ethanol in the range of 10–14 g/kg/day, while female B6 mice will self-administer in the range of 12–18 g/kg/day, when the choice is between a 10% ethanol solution and water.

Many null mutants and transgenics have been tested for ethanol preference. Drinking in these studies necessarily compares intake of the null mutant versus the wild-type onto which the mutation has been bred. While some of the wild-types in these studies are B6, many are not, and often the wild type is itself a hybrid of two or more inbred strains, a segregating intercross, or a partial backcross to an inbred strain. A survey of many such studies reported that about 1/3 found increased ethanol preference versus wild-type in the null mutants, 1/3 found a reduction, and about 1/3 no effect (Cunningham and Phillips, 2003). However, there are no reports of which we are aware where substantially greater g/kg doses of ethanol than those self-administered by B6 are reported.

In one of our laboratories (Texas), we have been screening null mutants for ethanol preference. The background strain on which the mutant α1 S267Q glycine receptor transgenic was held was the *F*1 hybrid of B6 and FVB/NJ (FVB). As reported in Experiment 1, we noted a very high intake of ethanol in the B6xFVB *F*1 hybrids. To substantiate this finding, and to test the hypothesis of an effect of maternal genotype, we attempted to repeat the finding in another laboratory (Oregon). As we are also exploring other methods for inducing high levels of ethanol self-administration (Finn et al., 2005; Rhodes et al., 2005), we also tested B6xFVB *F*1 mice in these newer, limited access procedures. We found that B6xFVB *F*1 mice indeed self administer extremely high levels of ethanol in two-bottle preference tests. They appeared to drink more than B6 mice in the standard two-bottle preference test, and resembled B6 in the newer assays that lead to higher brain concentrations of ethanol.

METHODS

Animals

Studies were conducted in drug-naïve C57BL/6J, FVB/NJ, and reciprocal intercross *F*1 hybrid mice derived from these two progenitors (B6xFVB *F*1 and FVBxB6 *F*1, maternal strain x paternal strain). B6 and FVB breeders were purchased from The Jackson Laboratory (Bar Harbor, ME) and mated at seven to eight weeks in the Portland Genetic Animal Models Core of the Integrative Neuroscience Initiative on Alcoholism (INIA) at Oregon Health & Science University (OHSU) or at the VA Medical Center (VAMC), and in the Texas Genetic Animal Models Core of the INIA at University of Texas at Austin. Offspring were weaned into isosexual groups of each of the four genotypes (B6, FVB, B6xFVB *F*1, FVBxB6 *F*1). Mice were housed in standard polycarbonate or polysulfone shoebox cages with Bed-o-Cob™ bedding with food (Purina 5001™) and water provided ad libitum. The colony room and testing rooms were maintained in ambient temperature of 21 ± 1°C. Colony rooms were on a 12:12 L:D light cycle (lights on at 06:00). All mice were housed and tested in the Department of Comparative Medicine at OHSU, the Veterinary Medical Unit at VAMC, or in the Animal Facility of University of Texas. All procedures were approved by the correspondent Institutional Animal Care and Use Committee and adhered to NIH Guidelines. The OHSU, VAMC, and University of Texas facilities are AAALAC accredited.

General Methods

Naïve, adult mice (between 56 and 87 days of age were used in all experiments. Experiments were conducted with conditions of lighting, food, and water like those in the colony rooms, except where stated, and animals were acclimated to testing rooms for 7-10 days before the start of each experiment. Numbers of mice/group are given in figure legends and tables. Body weights were recorded at the beginning of each experiment and at least every four days, always on a cage change day. New cages were provided every eight days. All animals were acclimated for at least two days to fluid bottles with sipper tubes containing water before introduction of an ethanol solution. In Portland, 25 ml graduated cylinder tube volumes were read to within 0.2 ml. In Texas, 50 ml water bottles with sipper tubes were weighed to within 0.01g. As spillage and evaporation controls, average weight or volume depleted from tubes in control cages without mice was subtracted from individual drinking values each day. Aaper brand (Aaper Alcohol and Chemical, Shelbyville, KY; in Texas) and Pharmco brand (Pharmco Products, Brookfield, CT; in Portland) 200 proof ethanol were used to mix solutions as v/v in tap water. Frequency of tube placement switching in 2-bottle choice experiments was daily in Texas and every 2nd day in Portland. A summary of all tests performed in this study is presented in the Table 1.

Blood Ethanol Concentration Determination

Blood samples were assayed for ethanol content by gas chromatography. Details are given in Gallaher et al. (1996).

Experiment 1: Two-bottle Ethanol Preference in F1 Hybrids

Adult male and female *F*1 hybrid mice (total $n = 20$) were tested in Texas in a two-bottle choice experiment as was described earlier (Blednov et al. 2001). The mice were weighed and then individually housed with access to two 50 ml plastic water bottles with straight sipper tubes containing tap water. Six concentrations of ethanol (3%, 6%, 9%, 12% and 15%) in tap water were offered for four days each, starting with the lowest concentration and increasing to the highest. Tube positions were switched to the opposite side daily. Before placing the next greater concentration onto each cage, all mice were weighed.

Experiment 2: Two-bottle Ethanol Preference in F1 Hybrids Plus B6 and FVB

Adult male and female B6, FVB, and B6xFVB *F*1 and FVBxB6 *F*1 mice (total $n = 65$) were moved from the main colony room into a smaller testing room in Portland. Both reciprocal crosses were included to explicitly test the hypothesis that the strain of the maternal dam would have an effect on ethanol consumption. Ten days later, the mice were weighed, and then individually housed with access to two 25 ml graduated cylinders containing tap water for two days before one tube was switched to an ethanol solution. Throughout the study, tube volumes were recorded for each squad of 8 mice once daily (beginning at 8 AM for the first squad and at 15-min intervals). Four concentrations of ethanol (3%, 10%, 20%, and 30%) in tap water were offered for four days starting with the lowest concentration and increasing to the highest, then back to 20%. Tube positions were switched for both control and ethanol groups to the opposite side after the second day of access to each concentration. Before placing the next greater concentration onto each cage, all mice were weighed. Within two minutes of the final assessment of ethanol consumption on the last day of drinking, animals were tested for ethanol-induced ataxia (vs. a similarly-treated water-only control group). Because ethanol drinking did not produce deficits in performance, these results are not discussed further. At approximately 15 min after removal of ethanol tubes (from 8:15 to 11:15 AM, depending on squad), all animals had a 20 μl blood sample drawn from the peri-orbital sinus, and then were immediately euthanized.

Experiment 3: Blood Ethanol Concentrations during Circadian Dark Phase Using Two-Bottle Ethanol Preference

*F*1 mice in Experiment 2 consumed large amounts of alcohol, but blood ethanol concentrations were very low overall. Therefore, an additional experiment was performed to measure blood ethanol concentrations after high ethanol consumption during the circadian dark phase. This study was performed in Texas. Two different groups of female *F*1 mice ($n = 28$) consumed 15% or 20% ethanol solution in a two-bottle choice paradigm for one week before the beginning of the experiment. During this week, mice had continuous access to two bottles: one with ethanol, another one with water. The positions of bottles were alternated daily. On the 8th day, the animal was weighed and at the beginning of the dark phase, two weighed bottles containing ethanol (15% or 20%) or water were placed into the cages. Amount of consumed ethanol and water was measured every 2 hrs after the beginning of the dark phase. At 9 hrs into the dark phase, the last measurement of consumption was taken and immediately thereafter, a 20 μl blood sample was taken from peri-orbital sinus to measure blood ethanol concentration.

Experiment 4: Ethanol Acceptance during The Circadian Dark Phase

Female mice of each genotype ($n = 8$–11) were moved from the main colony room into a smaller testing room in Portland at 46-65 days of age. The testing room was maintained at the colony room temperature, but the light cycle was altered to lights on at 22:00, lights off at 10:00. 19 days after moving to the room, the mice were individually housed and water bottles were replaced with one containing a sipper tube. Water was provided *ad libitum* except when ethanol was substituted for water for 2 or 4 hr per day as described by Rhodes et al. (2005). Food was always available. Testing began one week after individual housing and continued for four consecutive days. Animals were weighed an hour before lights out. Then, at 3 hr into the dark cycle each day, water bottles were replaced with 10 ml graduated cylinders containing 20% v/v ethanol in tap water. Volumes were recorded for each animal immediately after placing the ethanol-containing cylinder on the cage and then again after 2 hr (days one through three). Then the ethanol cylinders were replaced with the water bottles. On day four, the cylinders were read after 2 hr of drinking, and then left on for an additional 2 hr (4 hr total). At the end of the period of ethanol access on day four, a 20 μl blood sample was drawn from the peri-orbital sinus, and analyzed as described. Daily body weight and total consumption of ethanol (g/kg) over the testing periods were measured for each individual mouse.

Experiment 5: Ethanol Acceptance during Scheduled Fluid Access

An ethanol acceptance method using scheduled, restricted fluid access was recently found to produce high and stable intake in B6 and genetically heterogeneous mice (Finn et al., 2005), so we used this method to test *F*1 mice (mice of the inbred strains were not available) in Portland. Briefly, individually housed *F*1 mice ($n = 8$ females, $n = 15$ males) were given one 25 ml graduated cylinder with tap water *ad libitum* for two days to accustom them to the sipper tube. At 5 PM on the 2nd day of individual housing, the water tube was removed, which began the period of fluid restriction. On each subsequent day, mice had access to the water tube for a designated period. Initially, mice had access to fluid for 4 hr per day for nine days. Beginning on day 10, the period of fluid access was increased by 2 hr, and then further increased by 2 hr after each subsequent ethanol session, until fluid availability was 10 hr per day (i.e., 6 hr fluid/day for days 10-12, 8 hr fluid/day for days 13-15, and 10 hr fluid/day for days 16–21). Every 3rd day, mice had access to a 5% ethanol solution in tap water for 30 min, followed by their designated access to water to complete the access period. Thus, mice received seven sessions with the ethanol solution. A blood sample (20 μl) was taken from the peri-orbital sinus immediately following the ethanol session on day 21 and analyzed by gas chromatography for blood ethanol concentration.

Experiment 6: Ethanol Metabolism

Two groups of mice in Texas were examined for blood ethanol concentrations over time. In the first group, ethanol (4 g/kg, 20% v/v in tap water) was administrated *per os* to male and female B6, FVB, and *F*1 mice ($n = 19$). 20 μl blood samples were drawn from the peri-orbital sinus at 30, 60, 120, 180 and 240 min after administration of ethanol. The second group comprised female B6 and *F*1 mice ($n = 10$). Mice were tested as in Experiment 4 for ethanol acceptance during the circadian dark phase. At the end of the 4-hr period of ethanol access on day four, a 20 μl blood sample was drawn from the peri-orbital sinus of all mice, and analyzed as described above. Then, in half of mice ($n = 5$) additional 20 μl blood samples were drawn at 45 and 75 min. The other half of the mice were injected with ethanol (3 g/kg, IP) 50 min after ethanol acceptance session and 20 μl blood samples were collected at 15, 60, 105 and 195 min after injection of ethanol.

Experiment 7: Two-bottle Saccharin or Quinine Preference in F1 Hybrids, B6 And FVB Mice

B6, FVB and *F*1 hybrids were also tested in Texas for saccharin and quinine consumption in the two-bottle choice paradigm. Mice were offered saccharin (0.033%) or quinine hemisulfate (0.03 mM) and intakes were calculated. Each tastant was offered for four days, with bottle position changed every day. Between tastants, mice had two bottles with water for two weeks.

Data Analysis

The dependent measures were blood ethanol concentration, volume or weight of ethanol or water consumed, ethanol dose (g/kg) consumed, preference ratio, and body weight. In Oregon, the effect of genotype and/or sex on these dependent measures was analyzed by ANOVA using

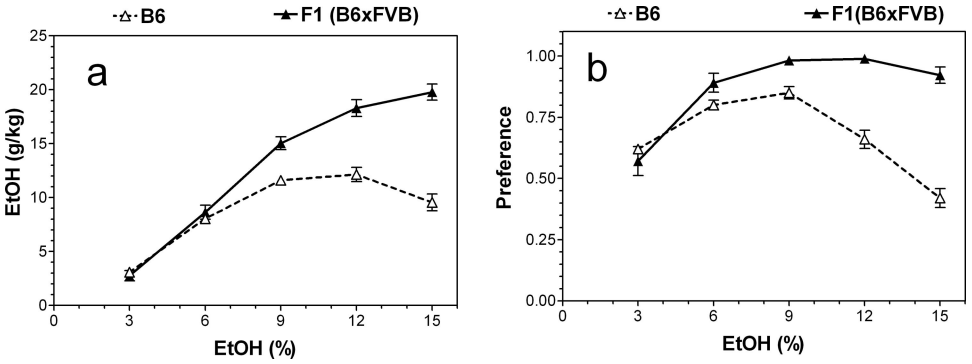

Fig. 1. Consumption of increasing concentrations of ethanol by B6 mice and B6xFVB *F*1 hybrid mice in a two-bottle preference test (Texas). Ten mice per sex were given 24 hr access to ethanol and tap water (in Texas). A) Mean ± SEM ethanol consumed (EtOH, g/kg) at each concentration, averaged across the four days of access at each concentration. B) Mean ± SEM preference ratio, calculated as the amount of ethanol consumed/total fluid consumed.

SYSTAT version 10. When appropriate, day was included as a repeated measures factor. Posthoc tests used Tukey's Honestly Significant Difference test. In Texas, the statistics software program GraphPad Prizm (Jandel Scientific, Costa Madre, CA) was used throughout. To evaluate differences between groups, analysis of variance (two-way or one-way ANOVA with posthoc Newman-Keuls Multiple Comparison Test) and Student's *t* test were carried out. Correlation and regression analyses were used to assess the relationship between ethanol dose consumed and blood ethanol concentration.

RESULTS

Experiment 1: Two-bottle Ethanol Preference in F1 Hybrids

As it was shown in one of our laboratories (Texas), *F*1 hybrid mice of both sexes increased their g/kg intake as the concentration of the ethanol solution increased. Females consumed 19.8 g/kg/day (Fig. 1A) and males 6.9 g/kg/day (data not shown) at 15% ethanol versus water. The preference data showed the expected inverted U-shaped curve (Fig. 1B). As indicated in the introduction, these data were obtained in one of our laboratories (Texas) during routine screening of mutant mice for ethanol preference. Because the background strain of the mutant α1 S267Q glycine receptor transgenic was the *F*1 hybrid of B6 and FVB, we did not use B6 mice as a control strain for this experiment. However, during other experiments we collected data from B6 mice obtained under similar conditions of standard two-bottle choice paradigm. These data indicate that B6 females consumed about 10 g/kg/day (Fig. 1 A) and showed lower preference than *F*1 hybrid mice (Fig. 1 B), while male B6 mice drank comparably to the *F*1 hybrids (data not shown).

Experiment 2: Two-bottle Ethanol Preference in F1 Hybrids Plus B6 And FVB

Intake of ethanol (g/kg/day; Fig. 2) increased with increasing ethanol concentrations for all mice, and the consumption of the FVB mice was much lower than B6 for all concentrations. The *F*1 female mice (Fig. 2A) drank even more than the B6 mice, particularly at the higher alcohol concentrations (20 and 30%). Repeated measures analysis of variance showed that the two reciprocal F1s were not different from each other, so data are presented collapsed on maternal strain (no main effect or interactions of maternal strain with either day or concentration; data not shown). To analyze the effects of concentration, genotype and sex, we calculated the average consumption over each concentration period. Thus, the 30% average concentration was the average of two days' consumption, while all others were averaged over four days (Fig. 2). Average intake of ethanol over the five periods of consumption was analyzed in a between groups (genotype, sex), repeated measures (concentration period) ANOVA. Preliminary analyses showed no significant 3-way interaction of concentration × genotype × sex ($F8,200 = 1.75$; $p = 0.09$). As female mice are well known to drink more ethanol than males, we report the data for each sex and each concentration period separately with simple one-way ANOVAs. Figure 2A shows the results for female mice. For all concentrations, there were main effects of genotype (F (2, 25–27) > 7.3; $p < 0.01$). At 3% and 10%, FVB female mice drank less ethanol than either B6 or the *F*1 hybrid ($p \leq 0.01$), which did not differ from each other. At 20%, whether offered on days 11-14 or 17-20, *F*1 hybrid females consumed more ethanol than either B6 or FVB ($p < 0.05$), while B6 drank more than FVB ($p < 0.01$). At 30%, *F*1 hybrids drank more ethanol than either progenitor strain ($p < 0.01$), which did not differ from each other. Results for male mice are shown in Fig. 2B. For all concentrations, there were main effects of genotype (F (2, 29–32) > 6.7; $p < 0.01$). At 3%, B6 male mice consumed more than the other two genotypes ($p < 0.05$). At 10% and the first 20% period, FVB males drank less than either the B6 progenitor or the *F*1 hybrid ($p \leq 0.01$). At 30%, *F*1 males drank more than FVBs ($p < 0.001$), but not significantly more than B6 males ($p = 0.09$). During the second offering of 20% ethanol, *F*1 hybrids drank more than males of both progenitor strains ($p < 0.001$), which did not differ from each other.

Blood ethanol concentrations were very low overall, with the range being 0 – 0.70 mg/ml. Sixteen of 65 animals had nonzero blood ethanol concentrations (defined as ≥0.05 mg/ml), with 13 of these being *F*1 animals. Accordingly, a significant effect of genotype was detected ($F2,59 = 3.18$; $p < 0.05$), but no effects of sex or genotype × sex interaction were found. Among those animals with nonzero blood ethanol concentrations, drinking on the last day correlated with blood ethanol concentration ($r = 0.40$, Fig. 2C).

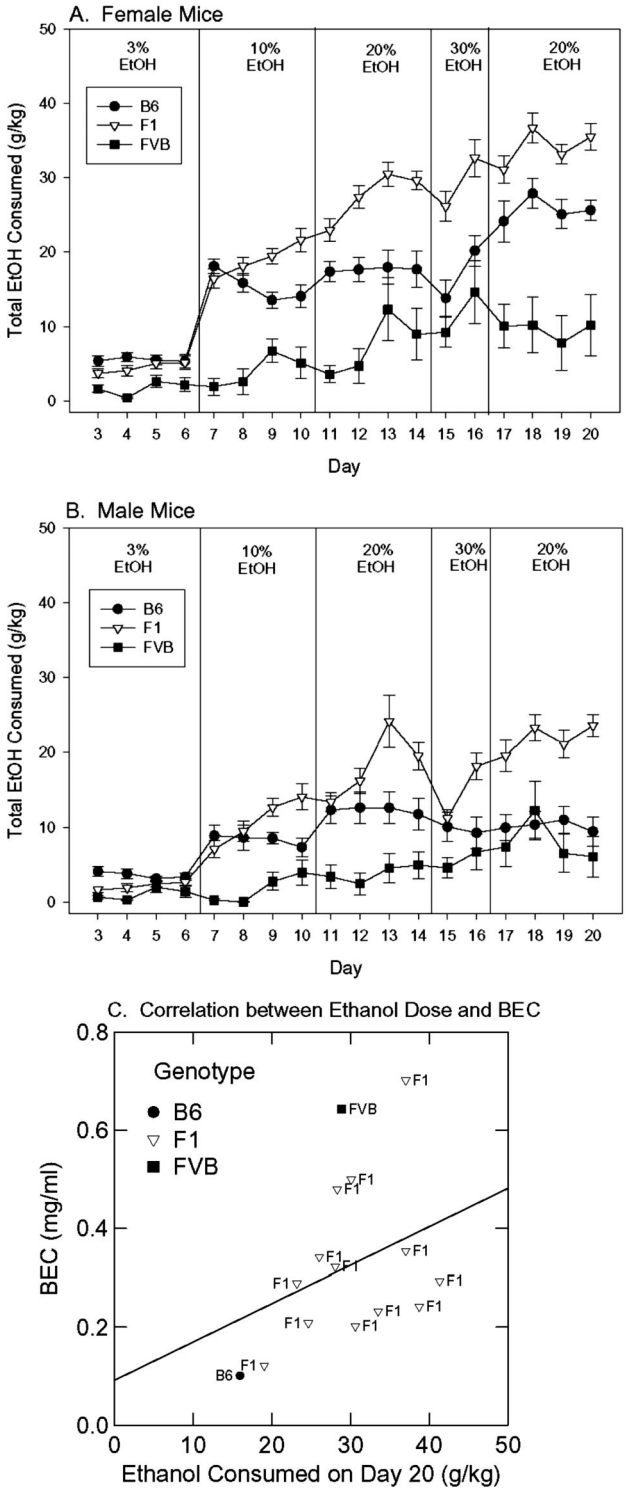

Fig. 2. Consumption of increasing concentrations of ethanol by B6, FVB, and B6xFVB *F*1 hybrid mice in a two-bottle preference test (Oregon). Mice (n = 6-10/sex/genotype; total n = 65) were given 24 hr access to ethanol and tap water in Portland. A) Mean ± SEM ethanol consumed (EtOH, g/kg) over days by female mice. B) Mean ± SEM ethanol consumed (g/kg) over days by male mice. Panel C: Relationship between ethanol consumed on day 20 (g/kg) and blood ethanol concentration (BEC, mg/ml). Only data for animals with blood ethanol concentrations ≥ 0.05 mg/ml are shown (n = 15). One additional FVB mouse had a blood ethanol concentration of 0.13 mg/ml, but is not shown because its ethanol tube leaked on day 20 (day 19 consumption was 19.03 g/kg). Blood samples were obtained between 8:15 and 11:15 AM. Solid circle: B6 mouse; Open triangle: *F*1; Solid square: FVB mouse.

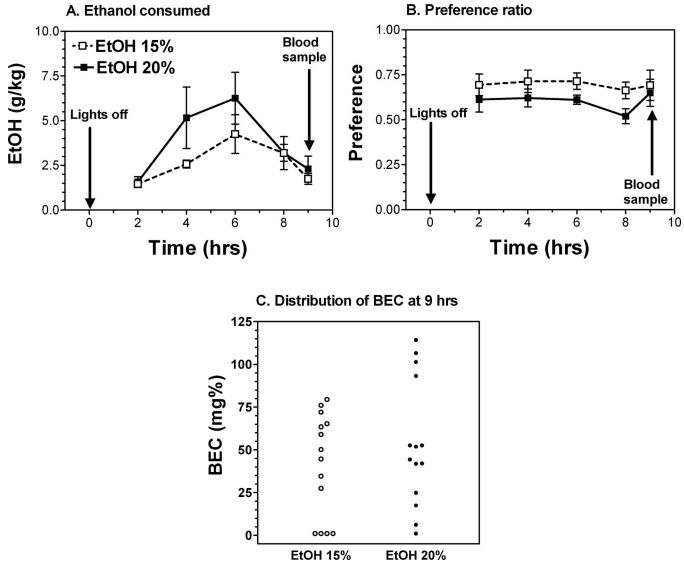

Fig. 3. Blood sampling during the circadian dark phase following two-bottle ethanol preference in B6xFVB *F*1 mice. Mice in Texas had 24 hr access to either 15 or 20% ethanol (EtOH) and water for seven days before the period shown. A and B) X-axis: Time, in hours, after onset (0; left arrow) of dark cycle. Blood sampling occurred at hour 9 of the circadian dark phase (right arrow). C) blood ethanol concentrations (BEC, mg/ml) from mice drinking 15 or 20%, respectively (scatterplot).

Experiment 3: Blood Ethanol Concentrations during Circadian Dark Phase Using Two-Bottle Ethanol Preference.

Because *F*1 mice consume substantially more alcohol than B6 when offered more concentrated alcohol solutions, we measured blood alcohol during voluntary intake in two-bottle ethanol preference after 9 hrs of consumption in the dark phase. Only *F*1 mice were tested. The reciprocal *F*1 crosses did not differ from each other (data not shown). For each concentration, main effects of time on consumption were apparent, with maximum ethanol intake between 4 and 6 hrs after lights off ($F(4, 52) > 2.8$; $p < 0.05$; Fig. 3A), but preference did not vary across the 9 hr period (Fig. 3B). Consumption of a 15% ethanol solution produced blood ethanol concentrations within the range of 0 – 0.79 mg/ml (Fig. 3C), while animals consuming 20% ethanol had blood levels of 0–1.14 mg/ml (Fig. 3C).

Experiment 4: Ethanol Acceptance during the Circadian Dark Phase

We have found that B6 mice will consume enough alcohol to produce a behaviorally significant blood ethanol concentration when given access to 20% ethanol solutions in a single bottle for only 2 or 4 hr/day during the circadian dark phase, a procedure we call drinking in the dark (Rhodes et al., 2005). Thus, we compared the B6, FVB and *F*1 genotypes with this method (Fig. 4). Only female mice were available. Analysis of variance of g/kg intake over 2-hr access periods over the four days of testing showed a significant main effect of genotype ($F3,36 = 21.16$; $p < 0.0001$) but no effects of day or interactions of day and genotype ($F < 1.40$; $p > 0.19$), indicating that

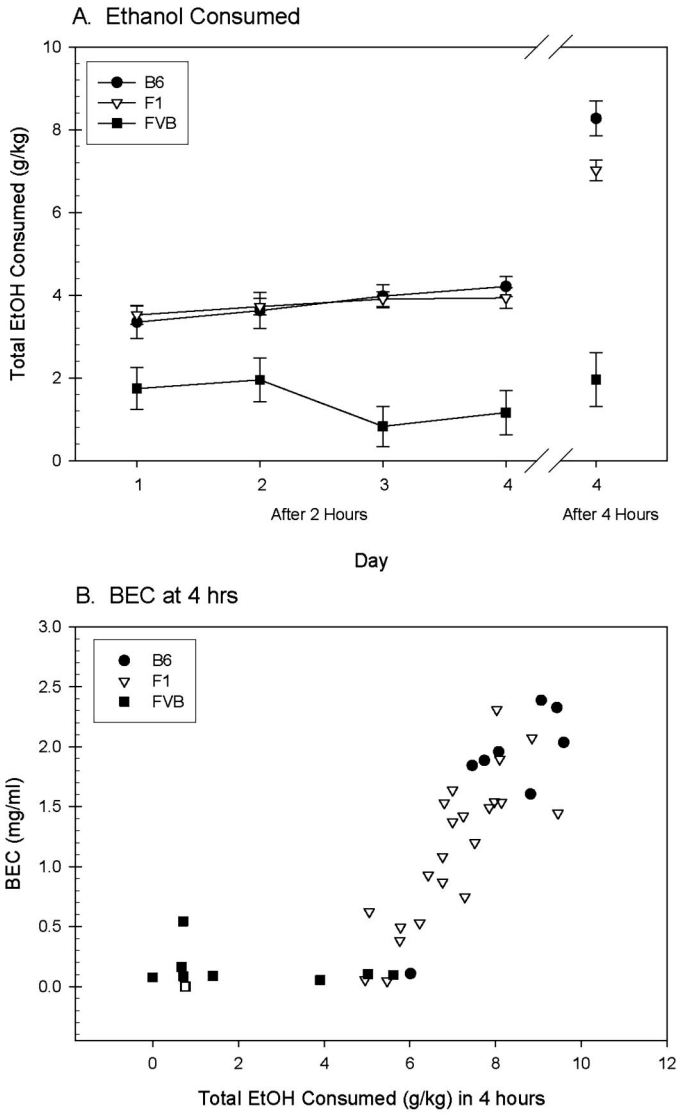

Fig. 4. The effect of drinking in the dark on amount of ethanol consumed during two (days 1–4) or four hours access (day 4 only) in B6, FVB, and B6xFVB *F*1 hybrid female mice. A) Amount of ethanol (EtOH) consumed during two (days 1–4) or four hours access. A single bottle of 20% ethanol was offered beginning 3 hr after lights off (in Portland). B) The relationship between ethanol consumed (g/kg) and blood ethanol concentration (BEC, mg/ml) measured at the end of the four-hour period on day 4. Solid circle: B6 mice; Open triangle: *F*1 hybrids; Solid square: FVB mice. n = 8–22/genotype.

the amounts consumed were stable over days. Further analyses revealed that B6 and *F*1 mice drank significantly more 20% v/v ethanol than FVB mice ($F > 5.04$; $p < 0.01$ for all one way ANOVAs on genotype, and all pairwise mean differences for FVB versus others were 1.56 g/kg or greater, $p < 0.05$). The reciprocal *F*1 crosses did not differ from each other (data not shown). The 4-hr access period on day 4 was similarly analyzed. Four-hour consumption essentially doubled that of 2-hr consumption (Fig. 4A) in the B6 and *F*1 hybrid mice, but not in FVB mice. A significant effect of genotype was detected ($F2,37 = 54.16$; $p < 0.0001$), and FVB mice drank significantly less than B6 or the *F*1 hybrid (pairwise mean differences >5.06 g/kg; $p < 0.0001$). Figure 4B shows a scatterplot of day 4 four-hour consumption (g/kg) versus blood ethanol concentration (mg/ml) for individual animals. It can be clearly seen that animals with greater consumption had greater blood ethanol concentrations; the relationship, after about 4 g/kg consumption, was nearly linear ($r = 0.88$). Blood ethanol concentrations also differed significantly among genotypes ($F2,37 = 19.96$; $p < 0.0001$), with FVB mice having significantly lower blood ethanol concentrations than the other genotypes (pairwise mean differences >1.02 mg/ml; $p \leq 0.0001$). *F*1 mice also had significantly lower blood ethanol concentrations than B6 mice (pairwise mean difference = 0.62 mg/ml; $p < 0.05$).

Experiment 5: Ethanol Acceptance during Scheduled Fluid Access

Since we have found that limited access to a 5% alcohol solution, combined with restricted access to water produces high, stable, intake of alcohol (Finn et al., 2005), we used this procedure to test *F*1 mice.

In both sexes, the average dose of ethanol consumed in the first exposure was approximately 2 g/kg. It is notable that during each subsequent 30 min ethanol session, ethanol intake was >2 g/kg in female FVBxB6 mice and 2 g/kg in the male mice (Fig. 5A). The fact that ethanol intake increased during the second ethanol session after 10 hr of fluid access provides additional evidence for the stability of the high ethanol consumption. Overall, ethanol intake was significantly higher in female than in male mice ($F1,21 = 20.32$; $p < 0.001$) and did fluctuate significantly over time ($F6,126 = 4.80$; $p < 0.001$). However, the interaction between time and sex was not significant.

The dose of ethanol consumed on day 21 produced average blood ethanol concentrations that tended to be higher in female than in male mice ($F1,20 = 3.40$; $p = 0.08$), consistent with the higher ethanol intake in the female mice (Fig. 5B). Consumption on day 21 ranged from 1.1–3.5 g/kg, producing blood ethanol concentrations ranging from 0.46-1.88 mg/ml (Fig. 5C). Blood ethanol concentration was significantly positively correlated with the dose of ethanol consumed ($r = 0.57$; $n = 22$; $p < 0.01$).

Water intake ranged from 2.4–4.65 mls over the course of the experiment, increasing with length of fluid access period. Mice generally maintained body weight over the course of the experiment (data not shown).

Experiment 6: Ethanol Metabolism

There were no significant differences between *F*1 and female mice of either progenitor strain in rate of metabolism of ethanol after intragastric administration, but FVB mice showed significantly faster clearance of ethanol than B6 mice ($p < 0.05$; Data not shown).

Consistent with results obtained in Experiment 4, female B6 and *F*1 mice consumed similar amounts of 20% alcohol during a 4-hr period of drinking in the dark phase (Fig. 6A) and showed similar blood ethanol concentrations at the end of this period (compare the 4 hr time-points in Fig. 6B,C).

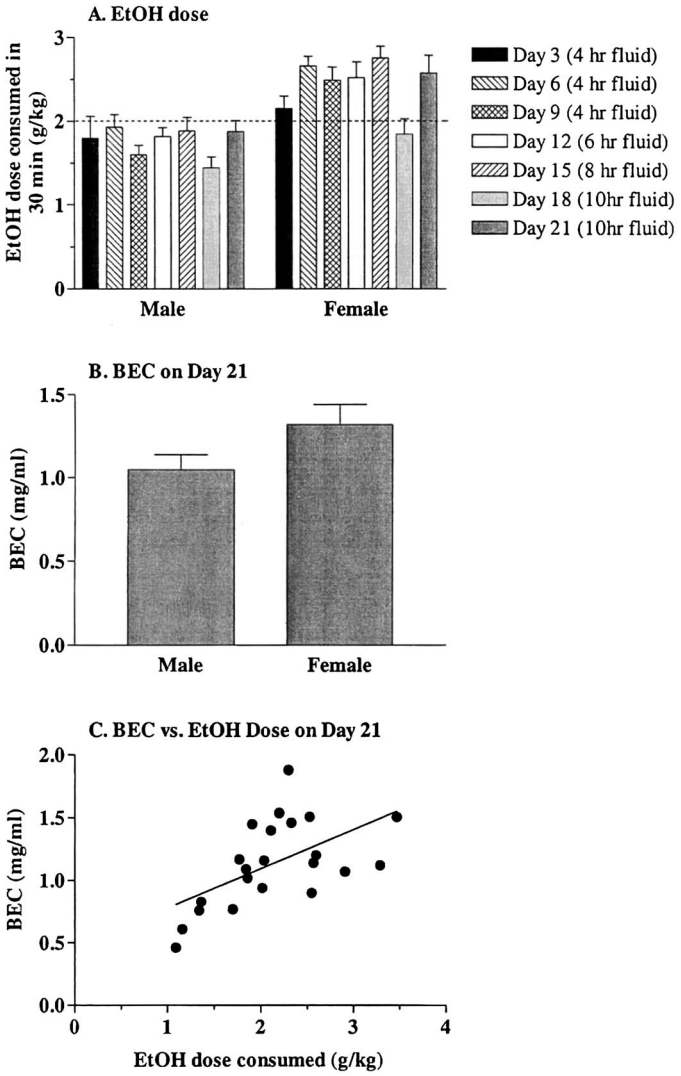

Fig. 5. The effect of scheduling fluid availability on ethanol dose consumed, blood ethanol concentration, and the correlation between blood ethanol concentration and ethanol dose consumed in male and female B6xFVB *F*1 mice. A) Ethanol (EtOH) dose consumed. B) Blood ethanol concentration (BEC). C) Correlation between blood ethanol concentration and ethanol dose consumed. Mice in Portland had 30 min access to a 5% ethanol solution every 3rd day. Values represent the mean ± SEM for 15 male and 8 female mice, except for the blood ethanol concentration data where $n = 7$ for female mice. Note the difference in y-axes. Data in panel C are collapsed across sexes, and the best-fit regression line is shown.

There were no significant differences between B6 and *F*1 mice in clearance of ethanol after its voluntary oral self-administration (Fig. 6B). The metabolism of intraperitoneally injected ethanol was similar between the genotypes after consumption of alcohol (Fig. 6C).

Experiment 7: Two-Bottle Saccharin or Quinine Preference in F1 Hybrids, B6 and Fvb Mice

The elevated ethanol consumption by *F*1 hybrids raises the question about specificity of this response to ethanol. Voluntary intake of saccharin (0.033%) solution in a two-bottle choice paradigm showed strong dependence on genotype ($F2,22 = 12.52$; $p < 0.001$, one-way ANOVA). FVB mice consumed significantly more saccharin solution than B6 mice and *F*1 hybrids ($p < 0.001$ and $p < 0.01$ respectively, Newman-Keuls Multiple Comparison Test) (Table 2). Significant dependence on genotype was also demonstrated for consumption of quinine (0.03 mM) solution ($F2,22 = 20.13$; $p < 0.001$, one-way ANOVA). However, for this tastant B6 mice showed greater intake than both FVB mice and *F*1 hybrids ($p < 0.001$ for both genotypes, Newman-Keuls Multiple Comparison Test) (Table 2).

Role of Body Weight

Differences in body weight may influence fluid and ethanol consumption. To evaluate this factor, we examined body weight data collected during all of the above studies. The difference in ethanol intakes between F1s hybrids and B6 mice was not due to differences in body weight (Table 3), as *F*1 mice were closer in body weight to B6 mice and both were lighter than FVB mice.

DISCUSSION

The present studies demonstrate that *F*1 hybrid mice from the cross of B6 and FVB drink substantially more ethanol than either progenitor strain when given a choice of ethanol solution (10, 20, or 30%) vs. water. This novel discovery was identified in one laboratory (Texas) and verified independently in another (Oregon), and marks the first report of which we are aware of any mouse genotype identified that consumes significantly more ethanol than B6 in the two-bottle choice test. The finding that *F*1 consume large quantities of ethanol appears to be robust, in that it occurs across multiple experimental designs (24 hr access to 2-bottle choice, scheduled access to ethanol, with or without water restriction, drinking during light or dark phases) and regardless of inter-laboratory differences in procedures (tube switching every day versus every other day, etc.). This difference displays selectivity for ethanol consumption and does not extend to the intake of saccharin or quinine solutions.

The phenomenon of *F*1 hybrids drinking more than either progenitor strain provides a clear demonstration of this effect or overdominance. It demonstrates that genes or alleles do not always affect alcohol drinking behavior in an additive or dominant fashion. The new *F*1 hybrid model may prove useful to explore the underlying genetic basis of epistasis in contributing to individual differences in alcohol drinking in mice. It is interesting that epistasis was only observed for the two-bottle choice test. In the drinking in the dark procedure and in the scheduled access experiment, B6 alleles were dominant over FVB with no evidence for epistasis. Thus, the epistatic interaction was only visible under certain environmental conditions (e.g., two-bottle choice).

One problem with the two-bottle preference drinking procedure is that it is not clear when or if the mice achieve behaviorally relevant blood ethanol concentrations (Dole and Gentry, 1984). In the two-bottle preference study (Ex-

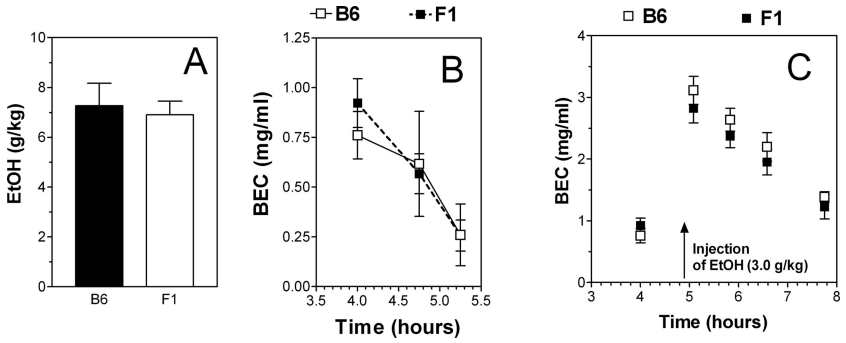

Fig. 6. The change in blood ethanol concentration (mg/ml) after oral self-administration administration of ethanol (20% in tap water) during 4-hrs drinking in the dark phase in B6 and B6xFVB *F*1 female mice. A) amount of consumed ethanol (EtOH, g/kg) (mean ± SEM) during 4 hrs drinking. $n = 10–11$/genotype. B) blood ethanol concentrations (BEC, mg/ml) over time after drinking in the dark. $n = 5$-6/genotype. C) blood ethanol concentrations (mg/ml) over time after drinking in the dark and injection of ethanol (3 g/kg, IP). $n = 5$/genotype.

Table 2. Total Intake of Saccharin and Quinine Solutions in Hybrid Female Mice

Strain	Intake (g/kg)	
	Saccharin (0.033%) solution	Quinine (0.03 mM) solution
C57BL/6J	275 + 24***	187 + 11
	n = 7	*n* = 7
FVB/NJ	582 + 57	124 + 8‡
	n = 9	*n* = 9
F1 (C57BL/6J x FVB/NJ)	364 + 39**	125 + 3‡
	n = 9	*n* = 9

Solutions: saccharin (0.033%), quinine (0.03 mM); hybrid female mice: C57BL/6J, FVB/NJ, F1 (B6xFVB).

n, Number of animals.

** $p < 0.01$.

*** $p < 0.001$, significant difference from intake of FVB mice.

‡ - $p < 0001$, significant difference from intake of B6 mice.

periment 2), *F*1 hybrids consumed about 10 g/kg per day more than B6 mice when offered 20% or 30% ethanol, with some *F*1 animals consuming more than 50 g/kg in a 24 hr period. We took a blood sample in this experiment at a time when drinking was likely to have subsided and blood ethanol concentrations were falling. Experiment 3 suggests that the highest blood ethanol concentrations will occur at approximately 6 or 7 hr into the dark cycle, in agreement with a recent study by Middaugh et al. (2003) because this is the time of peak consumption of ethanol. It should be noted that Fig. 3 presents the amount of alcohol consumed in each two-hour period, rather than cumulative alcohol consumed over time. Thus, the F1s drinking 20% ethanol in Experiment 3 had consumed about 18.5 g/kg ethanol by the time the blood sample was taken at 9 hr into the dark cycle. It is important to note that blood ethanol concentration obtained from peri-orbital sinus show close concordance with brain ethanol levels (Ponomarev and Crabbe, 2002, but see Smolen and Smolen, 1989) while ethanol concentrations from tail blood samples differ from those of brain by no more than 15-20% (Goldstein, 1983).

It is possible that the *F*1 hybrids required greater than four days exposure to ethanol before they demonstrated elevated consumption relative to B6. In the ethanol drinking in the dark study (Experiment 4), *F*1 hybrids did not differ from B6 mice in consumption of 20% ethanol during the dark phase from 3 to 7 hr into the dark cycle. Consumption of alcohol was about 7.5–8.5 g/kg in 4 hr in B6 and *F*1 mice. Thus, comparison of data in *F*1 mice across experiments 4 (4 days), 3 (8 days), and 2 (20 days) suggests the possibility that consumption by the F1s increases with increasing days of exposure.

Although mice of the progenitor strains were not available at the time of the scheduled fluid access study (Experiment 5), *F*1 mice consumed about 1.8–2.8 g/kg (females) or 1.5–2 g/kg (males) of 5% ethanol in a 30-min period, leading to blood ethanol concentrations over 1 mg/ml in all but 7 animals. In a comparable experiment that was conducted in male and female B6 mice (Finn et al., 2005), and FVB mice (Finn, unpublished) the mean ethanol dose consumed across the ethanol sessions ranged from 1.85–3.01 g/kg for B6 female, 1.80–2.84 g/kg for B6 male, 1.1–2.0 g/kg for FVB female, and from 1.1–1.95 g/kg for FVB male mice. Thus, the present findings in the *F*1 cross indicate that the ethanol intake in the female *F*1 mice was equivalent to that in the B6 females and higher than that in the FVB females. However, ethanol intake in the male B6 mice was higher than that in the *F*1 or FVB males. However, a direct comparison of these genotypes is still needed.

The difference in drinking between F1s and B6 mice in the two-bottle preference test are not likely attributed to differences in ethanol metabolism because both genotypes showed similar clearance of alcohol from blood. Furthermore, the potential development of metabolic tolerance also can be ruled out because preliminary oral self-administration of ethanol does not differentially affect clearance of intraperitoneally injected alcohol in these genotypes.

One would expect to find a large number of polymorphisms between FVB and B6, as their genealogies are quite different (Beck et al., 2000). FVB was derived from an outbred Swiss population at the National Institutes of Health, while B6 were developed from stock obtained from Miss Abbie Lathrop's mouse breeding farm (Festing, 1994; Morse, 1978). We searched several public databases for genetic polymorphisms between these strains. The Mouse Genome Database (searched December 1, 2004) found only six polymorphisms identified by polymerase chain reaction (http://www.informatics.jax.org/searches/polymorphism_form.shtml; (Blake et al., 2003). Five of them are minisatellites detected by the same probe, while the sixth is a member of the solute carrier family 12, located on mouse chromosome 8 (*Slc12a4*) near quantitative trait loci (QTLs) for blood ethanol concentrations 60 min after 2 or 3 g/kg

Table 3. Body Weight of Mice Used in Individual Experiments

	B6	FVB	B6xFVB F1
Experiment 1			
Before 3% ethanol	22.8 ± 0.8 (*n* = 44 female)		21.8 ± 0.4 (*n* = 10 female)
	27.9 ± 0.6 (*n* = 45 male)		29.3 ± 1.2 (*n* = 10 male)
After 15% ethanol	22.2 ± 0.2 (female)		23.5 ± 0.3 (female)
	28.8 ± 0.5 (male)		31.5 ± 1.4 (male)
Experiment 2			
Before 3% ethanol	20.2 ± 0.4 (*n* = 6 female)	22.3 ± 0.3 (*n* = 9 female)	20.9 ± 0.2 (*n* = 15 female)
	24.1 ± 0.4 (*n* = 7 male)	28.6 ± 0.3 (*n* = 8 male)	27.0 ± 0.6 (*n* = 20 male)
After 30% ethanol	22.0 ± 0.5 (female)	23.1 ± 0.3 (female)	22.3 ± 0.2 (female)
	24.7 ± 0.2 (male)	28.4 ± 0.3 (male)	26.5 ± 0.5 (male)
Experiment 3			
15% ethanol			20.6 ± 0.1 (*n* = 28 female)
20% ethanol			22.5 ± 0.4 (*n* = 14 female)
Experiment 4			
Day1	19.6 ± 0.5 (*n* = 8 female)	1.7 ± 0.4 (*n* = 10 female)	21.1 ± 0.3 (*n* = 22 female)
Day4	20.2 ± 0.6	21.8 ± 0.4	21.5 ± 0.3
Experiment 5			
Before 5% ethanol			18.5 ± 0.9 (*n* = 8 female)
			29.2 ± 0.5 (*n* = 15 male)
After 5% ethanol			21.0 ± 0.2 (female)
			27.4 ± 0.5 (male)
Experiment 6	21.8 ± 0.8 (*n* = 16 female)	22.6 ± 0.5 (*n* = 5 female)	23.9 ± 0.4 (*n* = 21 female)
Experiment 7	24.2 ± 0.6 (*n* = 7 female)	26.7 ± 0.7 (*n* = 9 female)	24.7 ± 0.4 (*n* = 9 female)

Body weight in g, mean+/-SEM.
n number of animals.

ethanol, IP (Grisel et al., 2002). The Center for Inherited Disease Research Mouse Microsatellite Studies website was searched February 3, 2005 (http://www.cidr.jhmi.edu/mouse/mouse_strp.html). 192 polymorphic markers between B6 and FVB were identified with a mean distance of 7.7 cM between markers. FVB and B6 are also among the strains being genotyped for single nucleotide polymorphisms (SNPs) by the Complex Trait Consortium (Wiltshire et al., 2003; Pletcher et al., 2004; http://www.well.ox.ac.uk/mouse/INBREDS/). The Mouse Phenome Database Mouse SNP site (http://aretha.jax.org/pub-cgi/phenome/mpdcgi?rtn = snps/door) was queried for SNP polymorphisms between B6 and FVB on February 2, 2005. Of the more than 8200 SNPs mapped for each of these strains, the search identified 4725 polymorphisms. Thus, the identification of the genes underlying the present findings will await further characterization of the FVB/NJ strain.

In addition to the large differences between B6 and FVB observed here in drinking in the dark and two-bottle preference, several ethanol-related behavioral differences have been observed between these strains. We have observed that FVB mice show steeper dose-response relationships than many other strains in several behavioral measures of intoxication following ethanol injection (e.g., latency to fall from a screen or splaying of the hindlimbs) (Metten et al., 2004; Crabbe et al., 2003). FVB mice also have a greater locomotor stimulant response to ethanol than B6 mice, who are known to show virtually no stimulation (Crabbe et al., 2003). Together, these and other studies using FVB mice point to the potential for previously untapped genetic differences in ethanol responses. The FVB/NJ strain used in the present studies is on the highest priority list for the Mouse Phenome Project, a consortium effort to provide basic behavioral and physiological data on a variety of mouse genotypes (Grubb et al., 2004) (http://aretha.jax.org/pub-cgi/phenome/mpdcgi?rtn = docs/pristrains). The repository of data in the Mouse Phenome Database was searched for differences between FVB and B6 on 12/01/04. Differences between FVB and B6 strains in preference for some tastants have been reported (Bachmanov et al., 2002). B6 mice display greater preference for solutions of potassium chloride and ammonium chloride, while FVB mice display greater preference for solutions of sodium chloride and sodium lactate (Bachmanov et al., 2002). While FVB mice show a moderate preference for ethanol, they are well below C57BL/6 relative to the others strains on the highest priority list of the Mouse Phenome Project (Bachmanov et al., unpublished). The Mouse Phenome Project also accepts data on *F*1 hybrids, but no B6xFVB *F*1 data have been submitted to date, and ethanol consumption has not been reported to Mouse Phenome Project in the other *F*1 hybrid data sets.

Data obtained in this study clearly show that the range of ethanol consumption in a standard two bottle preference test is not restricted to that seen in standard inbred strains but may be substantially broader. Previous studies of ethanol consumption in BXD recombinant inbred strains (Tarantino et al., 1998; Phillips et al., 1998; Gill et al., 1996) have shown that the distribution of ethanol consumption in mice whose genotypes can be traced to a hybrid of two inbred strains is usually skewed toward low consumption and falls within the range of ethanol consumption of the two parental strains. Similarly, the *F*1 hybrid cross of 129P3/J x C57BL/6ByJ (Bachmanov et al., 1996) showed less ethanol preference than C57BL/6ByJ. Other *F*1 crosses reported to date include C57BL/Crgl by DBA/NCrgl,

A/Crgl/2, C3H/Crgl/2, and BALB/cCrgl (McClearn and Rodgers, 1961) and DBA/2J × A/J, DBA/2J × C3HeB/FeJ, C57BL/6J × DBA/2J, C57BL/6J × C3HeB/FeJ, and C57BL/6J × A/J (Fuller, 1964). In these studies, preference for ethanol instead of consumption in g/kg was reported, but none of these hybrid crosses showed preference greater than or equal to B6. Use of FVB and C57BL/6 genetic backgrounds provides the first example of a genotype with ethanol consumption greater than that of B6 mice.

In conclusion, mice derived from the *F*1 hybrid cross of B6 and FVB drank higher levels of ethanol than either progenitor strain in the two bottle choice test in concentrations as high as 30%, and comparably high levels with B6 in two other self-administration tests designed to demonstrate behaviorally significant blood ethanol concentrations in limited access conditions. This is noteworthy for two reasons. First, it demonstrates the occurrence of epistasis or overdominance in two-bottle choice drinking in mice (i.e., the genetic phenomenon whereby genes or alleles interact nonadditively to affect a phenotype). Second, it identifies a mouse genotype that drinks as much or more alcohol than B6, a strain that has held the record for over 40 years for the highest ethanol consumption. The *F*1 hybrid, by virtue of this characteristic, will be a powerful addition to the group of genotypes that have been used to identify the genetic basis of high ethanol self-administration in mice.

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