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Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice

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Abstract

Because of intrinsic differences between humans and mice, no single mouse model can represent all features of a complex human trait such as alcoholism. It is therefore necessary to develop partial models. One important feature is drinking to the point where blood ethanol concentration (BEC) reaches levels that have measurable affects on physiology and/or behavior (>1.0 mg ethanol/ml blood). Most models currently in use examine relative oral self-administration from a bottle containing alcohol versus one containing water (two-bottle preference drinking), or oral operant self-administration. In these procedures, it is not clear when or if the animals drink to pharmacologically significant levels because the drinking is episodic and often occurs over a 24-h period. The aim of this study was to identify the optimal parameters and evaluate the reliability of a very simple procedure, taking advantage of a mouse genotype (C57BL/6J) that is known to drink large quantities of ethanol. We exchanged for the water bottle a solution containing ethanol in tap water for a limited period, early in the dark cycle, in the home cage. Mice regularly drank sufficient ethanol to achieve BEC>1.0 mg ethanol/ml blood. The concentration of ethanol offered (10%, 20% or 30%) did not affect consumption in g ethanol/kg body weight. The highest average BEC (~1.6 mg/ml) occurred when the water-to-ethanol switch occurred 3 h into the dark cycle, and when the ethanol was offered for 4 rather than 2 h. Ethanol consumption was consistent within individual mice, and reliably predicted BEC after the period of ethanol access. C57BL/6J mice from three sources provided equivalent data, while DBA/2J mice drank much less than C57BL/6J in this test. We discuss advantages of the model for high-throughput screening assays where the goal is to find other genotypes of mice that drink excessively, or to screen drugs for their efficacy in blocking excessive drinking.

Keywords: C57BL/6J mice; DBA/2J mice; Pharmacogenetics; Blood ethanol concentration; Drinking; Alcoholism; Self-administration

1. Introduction

Basic research into the etiology of neurological disorders is often initiated in animal models. Mice are becoming increasingly useful because of detailed knowledge of mouse genetics, which offers efficient methods for assessing genetic contributions to etiology [1]. The first step when using animals to study a mental disorder is to create a representation of the disorder. For alcoholism, Cicero [2] proposed the following criteria that he felt an animal model should satisfy: (1) ethanol should be self-administered orally; (2) the amount of ethanol consumed should elevate blood ethanol concentration (BEC) to pharmacologically significant levels; (3) ethanol should be consumed primarily for its pharmacological effects, rather than calories, taste or smell; (4) ethanol should be positively reinforcing; (5) chronic ethanol consumption should produce metabolic and functional tolerance; and (6) chronic ethanol consumption should produce signs of physical dependence. There is disagreement among alcohol researchers as to whether all these criteria are necessary or sufficient. Nonetheless, to the best of our knowledge, no

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single mouse or rat model has been able to satisfy all these criteria. A realistic and useful alternative approach is to develop multiple, partial models, each of which addresses a subset of the features [3–6].

One of the most widely used partial mouse models relevant to alcoholism is preference drinking [7–10]. In this model, animals are given two bottles, one with a dilute solution of ethanol in water, and the other with plain water. Consumption from each bottle is monitored over a 24-h period over several days to assess the subject's relative preference for the ethanol solution over water. An animal's genotype exerts a strong influence on self-administration in this model, and has been used to develop many sets of rat lines selectively bred for high ethanol self-administration [11–13] and, more recently, a set of mouse lines bred to be High (HAP) or Low (LAP) in Alcohol Preference [14]. Using this procedure, the inbred strain of mouse, C57BL/6 (closely related to the C57BL/6J used here), was identified many years ago as having a genetically influenced high preference for ethanol [7]. Other strains had intermediate preference, and one strain, DBA/2, avoided ethanol nearly completely.

Even though C57BL/6 mice take nearly all their daily fluid from a 10% ethanol solution over plain water in the two-bottle choice paradigm, it is not clear that this intake leads to a BEC that has a measurable effect on physiology and/or behavior [15]. Indeed, in a pioneering study, Dole and Gentry (1984) found that BEC fluctuated rapidly with voluntary drinking in B6 mice in the two-bottle paradigm, and was not maintained at pharmacologically significant levels for extended periods of time. This poses a problem if the model is to be used to test the efficacy of pharmacotherapies for reducing the incidence of drinking because it is not clear when in the 24-h period to apply the treatment and measure response [16].

To focus the period of alcohol self-administration, alcohol can be offered only for a short period each day. For example, a 1-h version of the two-bottle choice test has been found to increase the rate of consumption and BEC relative to continuous two-bottle choice in rats [17,18], though even with the limited access, BEC averaged only 0.50 mg/ml. Limiting access to ethanol to a 30-min period also increased self-administration in HAP mice, but again BECs averaged only about 0.60 mg/ml [19]. Lê et al. [16] described a variant of the method of Linseman [18] in which C57BL/6, BALB/c and DBA/2 mice were transferred from their home cages to a wire-mesh cage for 1 h per day during which time they were given a choice between water and ethanol. The concentration of ethanol increased from 3% (days 1–8) to 6% (days 9–20) to 12% (days 21–36). Average BEC for C57BL/6 mice was 0.59 mg/ml, while in the other strains, BECs were negligible [16]. Using a variant of this procedure, Lê et al. [20] have selectively bred rats for High (HARF) or Low (LARF) alcohol intake during a 20-min period of access. As in other studies, the highest intakes of HARF rats are after short-term access to 12% ethanol, and BECs are limited to an average of 0.60 mg/ml [20]. It is noteworthy that in the Lê et al. studies testing occurred during the light phase of the light–dark cycle. In a study of the temporal pattern of ethanol consumption, it had been previously observed that rats consume ethanol in discrete bouts mainly during the dark phase of the light–dark cycle [21], so offering ethanol for a limited period during the dark phase might have elevated BEC further.

The conclusion from these and many other studies is that even genetically predisposed animals seem to limit their voluntary drinking to amounts that can be readily metabolized, possibly in an attempt to avoid reaching intoxicating BECs [22]. Other procedures have been developed that lead to pharmacologically significant drinking in a limited access paradigm in C57BL/6 mice. However, these models tend to be quite complicated and involve substantial food and/or water restriction to motivate the mice to drink. For example, Middaugh et al. [23] described a procedure whereby C57BL/6 mice drank on average 8 g/kg ethanol in a 30-min period yielding an average BEC of 3.5 mg/ml. However, the procedure involved a 35-day acclimation period to the drinking procedures and ethanol concentrations, food restriction to 80% of baseline weight, periodic deprivation of water for 22.5 h, and the daily food allotment was given 1 h prior to the ethanol consumption test with the water bottles removed from the cage to generate "high thirst motivation" [23]. These authors have recently developed a simpler paradigm that involves 3 weeks access to two bottles, one with 12% ethanol and one with plain water but no food or water restriction. When the C57BL/6 mice were sampled 6 h into the dark phase their average BEC was just over 1.0 mg/ml [24]. Schedule-induced polydipsia [25] can produce a BEC of 3.0 mg/ml in B6 mice [26] but in this model, mice are first food deprived to 80–85% of their baseline weight 1 week before behavioral testing, and then placed into mouse operant chambers for 1 h per day for 24 h. While in the operant chambers food pellets are dispensed every 60 s, during which time the mice have access to an ethanol solution.

Another recent method adapted the "sucrose fading" procedure [27,28], in which rats were first initiated to drink ethanol in a sweetened solution. The concentration of ethanol was then gradually increased while that of sucrose was decreased, until the animals were stably self-administering only an ethanol solution. C57BL/6J (B6) mice can be trained to administer as much as 3 g/kg ethanol, achieving BECs averaging 2.5 mg/ml, after a period of sucrose fading during the dark phase [29]. These investigators have recently reported a modification of their procedure in which low concentrations of alcohol are initially offered to B6 mice that are water deprived, starting in the circadian dark phase. Over a period of 6 days, the water deprivation is reduced to zero and the alcohol concentration gradually increased. By the 11th day,

non-deprived mice are self-administering 10% ethanol during a 30 min limited access period with no water present, achieving doses of 3 g/kg [30].

Water deprivation has been used in the past to increase ethanol consumption in B6 mice [31]. Finn and her colleagues recently pursued a paradigm where B6 mice were maintained on 90-min access to water for 2–3 days, and then offered an ethanol solution for 30 min, followed immediately by access to water for the remaining hour of the session. This cycle was then repeated. B6 mice repeatedly self-administered more than 2 g/kg in 30 min, and BECs averaged more than 1.0 mg/ml. Over repeated sessions, the total period of fluid access could be extended to 10 h of fluid/day with no reduction in the amount of ethanol drunk during the initial access period [32]. This level of self-administration was shown to result in behavioral intoxication in tests of motor coordination [33].

Operant conditioning models that require the rodent to conduct an instrumental behavior (i.e., press a lever or nose poke) to gain access to ethanol have also been used. These models have high face validity and can lead to pharmacologically significant drinking in rodents [5,34–36]. However, these procedures often require that the animals be trained over a long period to learn the task, and thus a great deal of attention to each individual animal is necessary [21,36–38]. Operant conditioning procedures therefore are not feasible to implement in high-throughput screening or genetic assays.

Taken together, these and other studies suggest that when rats or mice are given a choice between alcohol and water, they tend not to drink to pharmacologically significant levels even if genetically predisposed. More complicated procedures have been successfully used to increase ethanol intake, but these usually involve periods of water and/or food restriction or a sucrose fading procedure and usually require a lengthy training period. The aim of this study was to develop a simple limited-access procedure that does not involve severe food or water restriction, and that leads to pharmacologically significant drinking (which we define as BEC>1.0 mg/ml) in B6 mice. Our intention is to use this model in high-throughput screening to find other genotypes that are predisposed to drink or to find drugs that are able to block the pharmacologically significant drinking in the B6 mice.

2. Materials and methods

2.1. Subjects and husbandry

Male and female C57BL/6J (B6) inbred mice from our own breeding colony were studied. Every three generations, we reestablished our colony with newly purchased B6 mice from the Jackson Laboratory. In Experiment 4, in addition to using B6 mice from our own colony, we included animals that were directly purchased from each of two Jackson Laboratory sources (Bar Harbor, ME, and Sacramento, CA; hereafter referred to as Jax East and Jax West, respectively). The purchased animals were acclimated to our facilities for 3 weeks prior to testing. In Experiment 6, we included DBA/2J (D2) mice purchased from Jax East. 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 photoperiod (12:12 L:D). In the colony, lights turned on at 0600 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 2200 and off at 1000 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. Sample sizes are given in the description of each individual experiment. Different animals were used in each experiment. All mice were housed and tested in the Department of Comparative Medicine at the Oregon Health and 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.

2.2. Basic paradigm: ethanol-for-water substitution

The basic paradigm we used was as follows. The standard water bottles in our colony are square, and deliver water to the mice via a pinhole in the side (<1 mm diameter), not a sipper tube. Thus, at the time when the mice were transferred to individual housing (1 week prior to the start of an experiment), the pinhole water bottles were changed to those with sipper tubes to acclimate the mice to the sipper tubes (without ball bearings). Starting at varying times after lights off (0, 1, 2, 3, or 4 h), the water bottles were replaced with 10 ml graduated cylinders containing an ethanol solution. The cylinders were fitted with a sipper tube containing a ball bearing at the end to prevent leakage. The ethanol cylinders remained in place for either 2 or 4 h and then were replaced with the water bottles. This procedure was repeated for four consecutive days. In most of the experiments, ethanol was offered at a concentration of 20% in tap water, except for the first study where we varied the concentration of ethanol. In experiments where BECs were determined, sampling was immediately at the end of the trial on day 4. A 20 μl sample of blood was taken from the periorbital sinus, and samples were processed and analyzed by gas chromatography according to previously published methods to determine BEC [39]. The descriptions of the individual experiments that follow detail the manipulations of the

parameters of the basic paradigm to determine their effect on consumption of ethanol and BEC.

2.3. Experiment 1: manipulation of ethanol concentration (n=30, B6 females)

The mice were divided into three groups (10 subjects in each) that received either 10%, 20% or 30% ethanol in place of the water bottle starting immediately at lights off. The ethanol cylinder was left in place for 2 h, and the procedure was repeated for four consecutive days. Total consumption of ethanol (g/kg) over the 2-h period was measured in each individual, each day. No blood samples were taken.

2.4. Experiment 2: examining consistency over 12 consecutive days (n=15, B6 males)

The mice were given 20% ethanol in place of the water bottle for 2 h on 12 consecutive days. The water bottle was replaced with the ethanol cylinder immediately at lights off each day except on day 12 when 9 of the 15 animals were randomly chosen to receive their ethanol cylinder starting 1 h after the lights shut off. Total consumption of ethanol (g/kg) over the 2-h period was measured each day but no blood samples were taken. On day 8, the cages were changed as a part of routine animal care maintenance, immediately following the ethanol exposure.

2.5. Experiment 3: replacing water with 20% ethanol starting 1, 2 or 3 h after lights off (n=30, B6 males and females equally represented)

The mice were divided into three groups (10 subjects in each; 5 males, 5 females) that received 2-h access to 20% ethanol in place of water starting at 1, 2 or 3 h after lights off. The procedure was repeated for four consecutive days. Consumption of ethanol (ml) was measured every 30 min over the 2-h period in each individual, each day. A blood sample was taken immediately after the end of the trial on day 4. On days 5–14, water bottles were replaced and the mice were left undisturbed. The experiment was repeated on days 15–18 (11 days after the initial blood sample was taken), offering ethanol for 2 h/day except that on day 4, the ethanol cylinder remained in place for 4 h before collecting peri-orbital blood.

2.6. Experiment 4: replacing water with 20% ethanol starting 3 or 4 h after lights off (n=22 B6 males and females)

The mice were divided into two groups (11 subjects in each; 6 males, 5 females) that received 20% ethanol in place of water starting at 3 or 4 h after lights off. The ethanol cylinder was left in place for 4 h, and the procedure was repeated for four consecutive days. Consumption of ethanol (ml) was measured every 60 min over the 4-h period in each individual, each day. Immediately at the end of the trial on day 4, a 20 μl sample of blood was taken from the periorbital sinus for measurement of BEC.

2.7. Experiment 5: comparison of B6 females from three sources (in house n=15, Jax East n=15, Jax West n=15) using a rigid attachment of the alcohol cylinder

In earlier experiments, we had noticed that an occasional animal would "play" with the light-weight, plastic alcohol cylinder, knocking it around as it lay on the cage top. Such animals would record unusually high intakes that did not match their BEC (see Results). Thus, in this experiment, the alcohol cylinders remained in the same orientation as before, except that instead of simply resting on the cage top, they were rigidly attached to the cage top to prevent all movement. Rectangular 1-cm-thick Acrylic plates (3 by 14 cm) were drilled with a hole (8 mm) that fit tightly around the sipper tubes. The plates were bolted solidly to the stainless steel bars of the cage top such that the hole in the Acrylic was flush with the hole in the cage top for passage of the sipper tube. In later experiments, we discovered that the cylinders could also be firmly attached by simply using Acco brand (Lincolnshire, IL) Medium Binder Clips (1.587 cm capacity).

The mice received 2-h access to 20% ethanol in place of water starting 3 h after lights off for three consecutive days. On the 4th day, the mice received 4-h access to the ethanol solution. Immediately at the end of the trial on day 4, a 20 μl sample of blood was taken from the periorbital sinus for measurement of BEC.

2.8. Experiment 6: comparison of B6 to D2 using rigid attachment of the alcohol cylinder (B6 males n=6, B6 females n=6, D2 males n=6, D2 females n=6)

The mice received 2-h access to 20% ethanol in place of water starting 3 h after lights shut off for three consecutive days. On the 4th day, the mice received 4-h access to the ethanol solution. Immediately at the end of the trial on day 4, a 20 μl sample of blood was taken from the periorbital sinus for measurement of BEC.

2.9. Statistics

Data were analyzed using SAS (SAS Institute). Data were generally analyzed with two-way ANOVAs. In Experiments 1, 2, and 6 when the data were analyzed across days, a repeated measures analysis was used. For the remaining experiments, only data from day 4 were analyzed, and so a simple between-subjects analysis was used. In Experiment 3, paired *t*-tests were used to examine the effect of duration of ethanol access on BEC and the ratio (BEC/ethanol consumption) as these measures were made in the same individuals. Correlations among variables measured in the same individuals were analyzed by simple linear regression. Means±S.E. are reported.

3. Results

3.1. Experiment 1: ethanol concentration

The concentration of ethanol (10%, 20% or 30%) did not significantly affect the quantity of ethanol consumed in g/kg over the 2-h period but it did affect the volume of solution consumed (Table 1). As the concentration of ethanol increased, the mice drank less volume such that g/kg remained nearly constant. Consumption of the ethanol solutions remained consistent across the 4 days (i.e., there was no trend of increased or decreased consumption over days, Table 1). For g/kg ethanol consumed, neither concentration [$F(2,25)=1.97$, $p=0.16$], day [$F(3,75)=1.82$, $p=0.15$], nor the interaction between day and concentration [$F(6,75)=0.55$, $p=0.77$] were significant. When volume of solution (ml) was used as the response variable, concentration was significant [$F(2,25)=10.67$, $p=0.0004$], but neither day [$F(3,75)=1.01$, $p=0.39$] nor the interaction between day and concentration [$F(6,75)=0.51$, $p=0.80$] were significant.

3.2. Experiment 2: consumption over 12 days

The quantity of ethanol consumed remained relatively constant over the 12 days (Fig. 1). Day was not a significant factor in the repeated measures analysis [$F(10,154)=1.57$, $p=0.12$]. The levels of drinking on day 1 tended to be slightly higher than on subsequent days and on day 9 the mice tended to drink slightly less than on other days, but neither of these changes was statistically significant. The nonsignificant decrease on day 9 may have been related to the cage change on the previous day. On day 12, ethanol consumption significantly increased in the nine mice that were given their ethanol cylinder 1 h after the lights shut off as compared to the remaining six mice and previous days responses in the repeated measures analysis (see Fig. 1) [$F(1,167)=3.78$, $p=0.05$].

3.3. Experiment 3: start time

In Experiment 2, B6 mice drank more 20% ethanol in g/kg over a 2-h period when it was substituted for water starting 1 h after lights off as compared with starting immediately at lights off. Therefore, we wished to determine whether starting 2 or 3 h after lights off would yield even greater consumption. Indeed, we found that B6 male and female mice drank approximately 90% more ethanol over a 2-h period when it was substituted for water starting 3 h after lights off as compared with starting 1 h after lights off and 50% more when starting 3 h after lights off as compared to 2 h (Fig. 2A; Table 2). For cumulative g/kg consumption of ethanol on day 4, start time was significant [$F(2,24)=8.70$, $p=0.001$], but neither sex [$F(1,24)=2.22$, $p=0.15$] nor the interaction between sex and start time [$F(2,22)=0.02$, $p=0.98$] were significant. Post hoc analysis with Tukey-adjusted p-values demonstrated that the 3-h start time differed from both the 1 h ($p=0.0009$) and 2 h ($p=0.02$) start times, but the 1 and 2 h times did not differ from each other ($p=0.44$).

When we repeated the experiment 11 days later in the same mice, we found consistent results with respect to the effect of start time on the 2-h intake data ($p=0.02$, Fig. 2B; Table 2). Sex was also a significant factor this time (data not shown) with females drinking approximately 1 g/kg more than males [$F(1,24)=7.10$, $p=0.01$], but the interaction between sex and start time was not significant [$F(2,24)=0.13$, $p=0.88$].

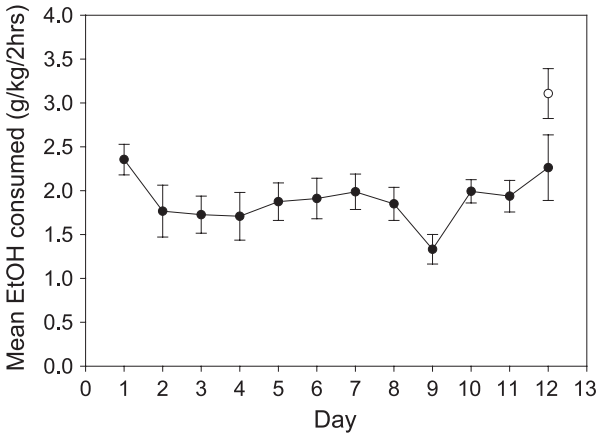

Fig. 1. Experiment 2. The quantity of ethanol consumed by male B6 mice remained relatively constant for 12 days but they consumed more when it was offered to them 1 h after lights off as compared to at lights off. Filled circles show mean quantity of 20% ethanol consumed in g/kg (±S.E.) within a 2-h period starting at lights off for 12 consecutive days. Open circle shows the same measure when alcohol was offered starting 1 h after lights off in a subset of the animals ($n=15$ males; on day 12, $n=9$ for open circle, $n=6$ for filled circle).

Table 1
Summary of results from Experiment 1

Day	Volume of ethanol solution consumed (ml)			Quantity of ethanol consumed (g/kg)		
	10%	20%	30%	10%	20%	30%
1	0.60 (0.099)	0.38 (0.062)	0.18 (0.040)	2.4 (0.38)	3.1 (0.59)	2.0 (0.43)
2	0.52 (0.100)	0.33 (0.067)	0.16 (0.029)	2.0 (0.35)	2.4 (0.43)	1.8 (0.35)
3	0.54 (0.095)	0.36 (0.056)	0.24 (0.029)	2.1 (0.34)	2.8 (0.51)	2.8 (0.35)
4	0.44 (0.083)	0.31 (0.059)	0.20 (0.058)	1.7 (0.31)	2.3 (0.41)	1.9 (0.36)

Mean volume (ml of ethanol solution) and quantity (g/kg) of ethanol consumed within a 2-h period (starting at lights off) on days 1–4 for B6 mice given 10%, 20% or 30% ethanol in tap water ($n=10$ females per group). Standard errors of the mean are shown in parentheses.

After the 2-h data were collected on day 4 of the retest, the ethanol cylinders were left in place for an additional 2 h before returning the water bottles (i.e., total of 4 h access). One mouse registered an inordinately high amount of drinking (0.8 ml solution was dispensed from the cylinder within 1/2 h) on the last reading of the 4 h access, which we believe resulted from the mouse tampering with the cylinder, so this individual was removed from analysis. Ethanol consumption continued at a steady rate over the entire 4-h period (Fig. 2B). After 4 h, the effect of start time on consumption of ethanol only approached statistical significance [$F(2,23)=3.12$, $p=0.06$], but the trend was in the same direction as before (i.e., the rank order of consumption from greatest to lowest was still the 3>2>1 h start time; see Fig. 2B and Table 2). Females continued to drink more than males [$F(1,23)=7.10$, $p=0.01$], and after 4 h the average cumulative difference was approximately 2 g/kg (data not shown). The interaction between start time and sex remained not significant [$F(2,23)=0.08$, $p=0.94$].

Because BEC was measured twice in the same individuals, once in the first test after 2 h ethanol access, and once in the retest after 4 h ethanol access, it was possible to compare BEC after 4 versus 2 h of ethanol access within individuals. BEC was significantly higher after 4 h than after 2 h as indicated by a paired t-test, $t(27)=6.1$, $p<0.0001$ (see Table 2). Fig. 3 shows the relationship between consumption and accumulated BEC. Consumption of ethanol (g/kg) was a significant linear predictor of BEC when ethanol was substituted for water for 2 h ($R^2=0.74$, $n=30$, $p<0.0001$), or 4 h ($R^2=0.50$, $n=29$, $p<0.0001$). Animals accumulated less ethanol in their blood per g/kg ethanol consumed during their 4 h retest than when ethanol was offered for only 2 h on the first test, as indicated by a paired t-test comparing the individual estimates of the slopes (BEC/consumption) between the two tests [$t(27)=3.2$, $p=0.003$; Fig. 3A,B]. On the other hand, only 1/3 of the mice reached a BEC>1.0 mg/ml after the 2 h initial test, while 23/29 mice achieved this level after the 4 h test (see Table 2).

3.4. Experiment 4: 3 versus 4 h after lights off

In Experiment 2, we found that B6 mice drank more ethanol over a 2-h period when it was substituted for water starting 3 h after lights off as compared with starting 1 or 2 h after lights off, and that ethanol consumption continued at a steady rate when the ethanol cylinders were left in place for an additional 2 h (i.e., 4 h total). Therefore, we wished to determine whether the mice would drink even more ethanol if we substituted ethanol for water starting 4 h rather than 3 h after lights off. This time, the ethanol cylinders were left in place for 4 h on all 4 days. One male was dropped from the study because he repeatedly tampered with the cylinder causing solution to be dispensed. We found that starting 3 or

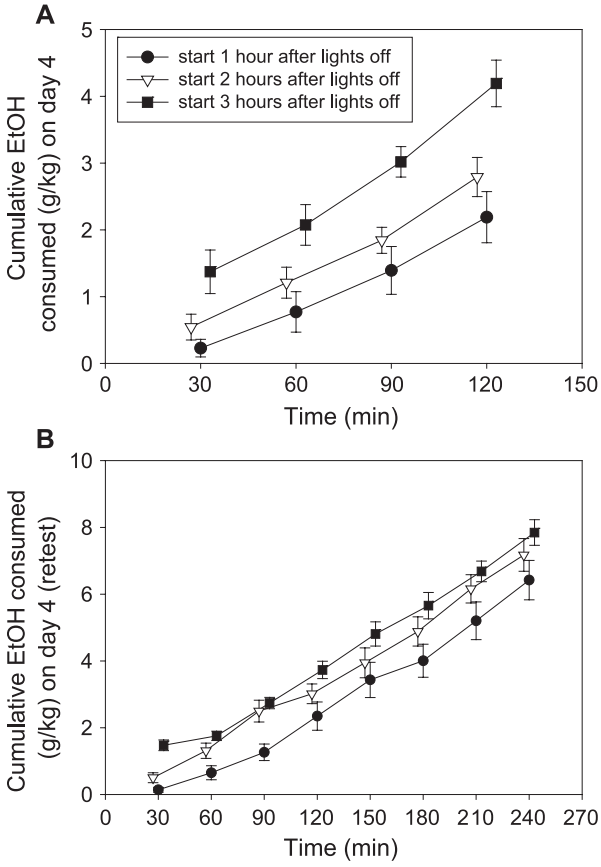

Fig. 2. Experiment 3. B6 mice consumed more ethanol when it was offered to them 3 h after lights off as compared with 1 or 2 h after lights off. Panel A shows the mean consumption of ethanol in g/kg (±S.E.) accumulated over a 2-h period in 30 min bins for mice that received ethanol starting 1, 2 or 3 h after lights off (n=5 males and 5 females per group). Only data from day 4 of the 4-day trial are shown. Panel B shows the same measure upon retest (after the mice were left undisturbed for 11 days and then subsequently given the 4-day trial again). On day 4 of the retest, ethanol access was extended to 4 h.

Table 2
Summary of results from Experiment 2

Start time after lights off	g/kg ethanol consumed in			BEC mg/ml after ethanol access	
	2 h day 4, first test	2 h day 4, retest	4 h day 4, retest	2 h Day 4, first test	4 h day 4, retest
1 h	2.19 (0.38)	2.35 (0.42)	6.42 (0.59)	0.55 (0.15), 2/10	0.92 (0.15), 5/9
2 h	2.79 (0.29)	3.02 (0.29)	7.17 (0.52)	0.74 (0.14), 3/10	1.44 (0.11), 9/10
3 h	4.19 (0.35)	3.73 (0.26)	7.85 (0.38)	1.06 (0.15), 5/10	1.59 (0.15), 9/10
Average	3.06 (0.25)	3.03 (0.21)	7.15 (0.30)	0.78 (0.09), 10/30	1.33 (0.09), 23/29

Mean ethanol consumed (g/kg) and BEC (mg/ml) collapsed across male and female B6 mice (n=5 male/5 female per group). Standard errors of the mean are shown in parentheses. For BEC, the proportion of individuals with BEC>1.0 mg/ml, are shown following the standard error.

4 h after lights off made no difference [$F(1,17)=0.52$, $p=0.48$]. In this experiment, neither the effect of sex nor the interaction between sex and start time was significant. The mean consumption of ethanol over the 4-h period on day 4 was 6.9±0.49 g/kg for the group that received ethanol 3 h after lights off versus 6.5±0.33 for the group that received ethanol 4 h after lights off. BECs were 1.24±0.18 mg/ml versus 1.17±0.19, respectively. Considering both groups together, 2/3 of the mice registered a BEC greater than 1.0 mg/ml. Consumption of ethanol (g/kg) was a significant linear predictor of BEC ($R^2=0.56$, $n=21$, $p<0.0001$). It was interesting to note that the values of ethanol consumption and BEC in this experiment were lower than in the retest of Experiment 2 when ethanol access was extended to 4 h after being maintained at 2 h per day on all previous days (see Table 2).

3.5. Experiment 5: different B6 sources using rigid cylinders

Fastening the cylinders to the cage tops effectively prevented the animals from moving the cylinders and we observed no instances of unusually high amounts of fluid being dispensed in this study as had been occasionally (~2% of the mice) observed in the previous studies (see Fig. 4). Mice from all three sources (In house, Jax East, and Jax West) drank similar quantities of ethanol and the levels were comparable, though slightly lower, than what was observed in Experiment 2 (retest) using the same procedure (2 h access on the first 3 days, and 4 h access on the 4th day, starting 3 h after lights off). In house mice drank 7.7±0.48 g/kg, Jax East drank 7.7±0.56 g/kg, and Jax West 7.5±0.30 g/kg. BECs were 1.24±0.14, 1.38±0.13, and 1.20±0.10, respectively. No significant differences in these variables were observed among groups. Considering all groups together, 78% of the animals achieved a BEC greater than 1.0 mg/ml, and consumption of ethanol over the 4-h period on the 4th day significantly predicted BEC [$R^2=0.53$, $n=45$, $p<0.0001$] (Fig. 4).

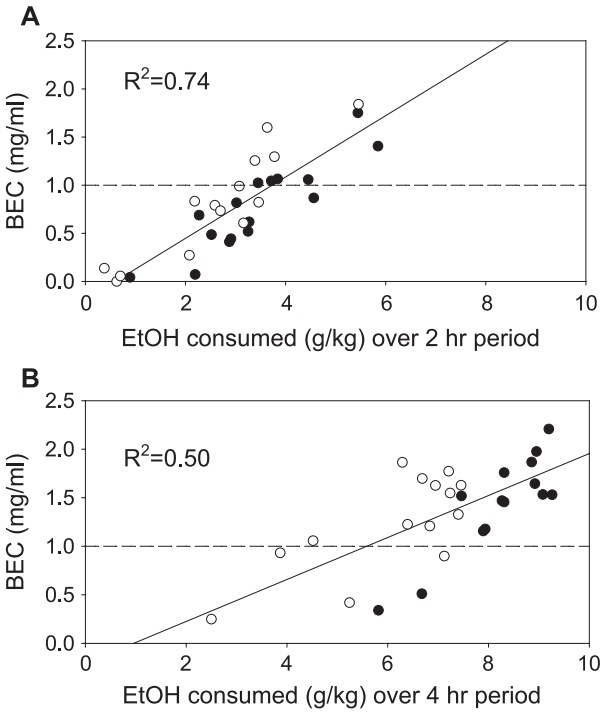

Fig. 3. Experiment 3. Ethanol drinking predicts BEC. Panel A shows the linear relationship between quantity of ethanol consumed and BEC measured immediately after the 2-h period of ethanol access on day 4 of the first test. Panel B shows the same plot for the retest when ethanol access was given for 4 h. Symbols identify sex: open=males, filled=females. Data are collapsed over the three start times.

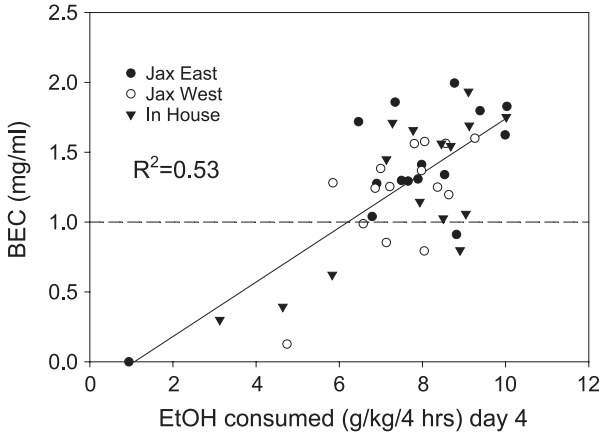

Fig. 4. Experiment 5. Ethanol drinking predicts BEC. BEC is plotted against consumption of ethanol when ethanol was substituted for water for 4 h starting 3 h after lights off. The three sources of B6 mice are shown as separate symbols. Only females were used in this study, and the correlations were calculated for data collapsed over source.

Because no differences between the sources occurred, we collapsed all three sources together to analyze the within-subject reliability of ethanol consumption among the 4 days of ethanol exposure (Fig. 5). This data set was chosen for presenting the reliability estimates over the other possible data sets because the largest number of B6 individuals within a sex ($n=45$ B6 females) could be used, allowing the greatest statistical power, as compared to the other data sets. The amount of ethanol consumed on day 1 in g/kg was unrelated to consumption on any subsequent day. However, days 2–4 were significantly correlated with each other ($p<0.05$ for each pairwise correlation) using the first 2 h of the 4-h ethanol exposure for day 4. The correlation estimates (R^2 values) can be seen in Fig. 5 and were relatively small (21–29%). One individual, in particular, drank average levels on day 1 but thereafter avoided ethanol nearly completely (see Fig. 5). When this individual was removed from the analysis, levels of drinking were still significantly correlated among days 2–4, but the R^2 values were reduced by approximately half.

3.6. Experiment 6: comparison of B6 to D2

D2 mice drank very little ethanol in this paradigm which is consistent with their behavior in many other situations.

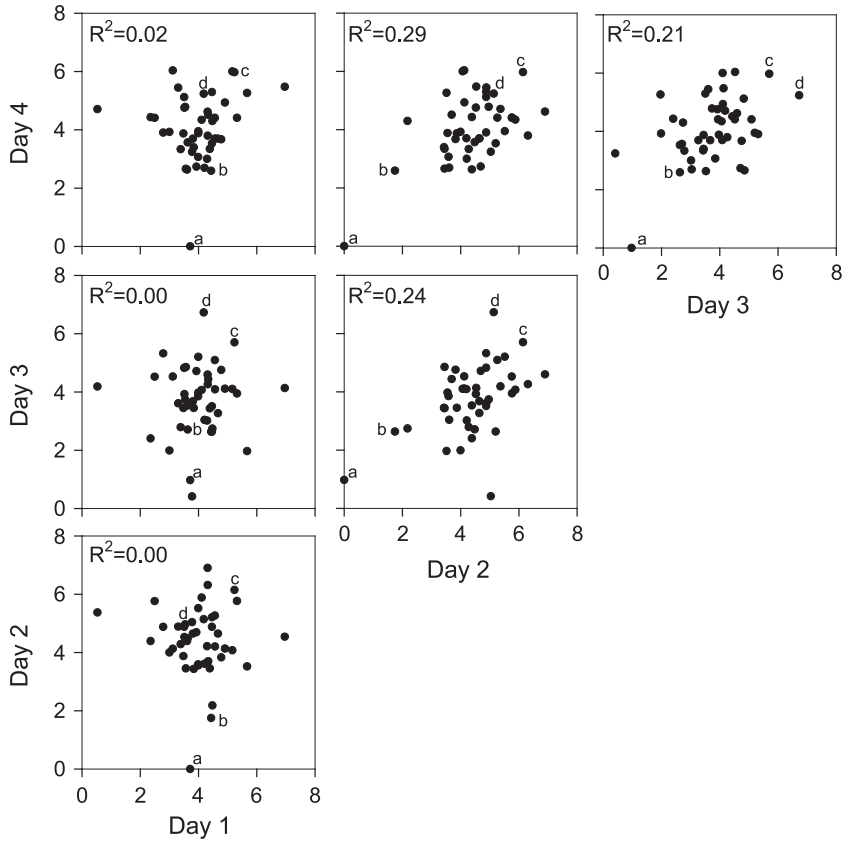

Fig. 5. Experiment 5. Ethanol drinking on day 1 is unrelated to other days but days 2–4 are significantly correlated with each other among B6 females. Consumption of ethanol in g/kg accumulated over a 2-h period on days 1–4 plotted against each other. Values for day 4 are the first 2 h of the 4-h exposure. The letters next to 4 of the data points identify individual animals; "a" and "b" consistently drank low amounts of ethanol on days 2–4 whereas "c" and "d" consistently drank high amounts of ethanol. R^2 values from simple linear regression models are shown. Note that these estimates represent the reliability attributed to environmental influences because the measurements were made in genetically identical B6 individuals.

On day 4 after 4 h of ethanol access, they consumed an average of only 1.5±0.43 g/kg, collapsed across sex, as compared to 7.5±0.24 g/kg in B6 mice [$F(1,20)=219.5$, $p<0.0001$] (Fig. 6). This level of consumption produced an average BEC of a mere 0.13±0.08 mg/ml (0/12>1.0 mg/ml) as compared to 1.4±0.12 mg/ml (10/12>1.0 mg/ml) in B6 mice [$F(1,20)=97.7$, $p<0.0001$]. Sex was a significant factor [$F(1,20)=11.6$, $p=003$], with females drinking more than males in both strains, but the interaction between sex and strain was not significant [$F(1,20)=1.17$, $p=0.29$]. For BEC, sex was not a significant factor [$F(1,20)=0.72$, $p=0.41$], but the interaction was [$F(1,20)=6.79$, $p=0.02$] with males of the B6 strain showing higher BEC than females, and males of the D2 strain showing lower BEC than females. A significant drop in consumption occurred after day 1 in D2 mice [$F(3,44)=4.69$, $p=0.006$] which did not occur in B6 mice, which is probably a reflection of conditioned taste aversion in D2 mice (Fig. 6) [31].

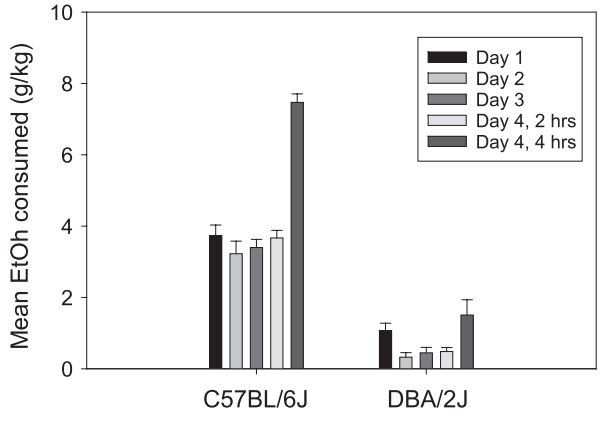

Fig. 6. Experiment 6. D2 mice drank very little as compared to B6 mice in this paradigm. Mean consumption of ethanol in g/kg (±S.E.) accumulated over a 2- or 4-h period on days 1–4 in B6 and D2 mice, collapsed across sex (n=6 males, n=6 females per strain).

4. Discussion

The procedure we describe is a very simple method to facilitate pharmacologically significant ethanol drinking in genetically predisposed mice. The water bottle is replaced with 20% ethanol for 2 or 4 h in the mouse's home cage, starting 3 h after lights shut off, and this procedure is repeated for four consecutive days. Under this condition, we demonstrate that B6 mice reliably drink ethanol to levels that produce a BEC>1.0 mg/ml (see Table 2). Other models

that have achieved a BEC>1.0 mg/ml involve food and/or water restriction and/or a lengthy training period [23,24,26]. The model appears to yield pharmacologically significant drinking only in those individuals that are predisposed to consume high amounts of ethanol because D2 mice, that are known to avoid ethanol in several other situations, drank very little (average BEC was 0.13 mg/ml) and we have preliminary data with several other genotypes, none of which thus far drink to the levels seen in B6 mice. Therefore, our simple procedure may prove useful for rapid screening of pharmaceuticals for their efficacy in limiting alcohol intake in genetically predisposed individuals. It may also prove useful for screening mutant strains, and lines could easily be established through selective breeding for high and low drinking using this model [6].

The two variables that appear to make the difference between achieving a BEC consistently above 1.0 mg/ml versus below 1.0 mg/ml in B6 mice are giving access to ethanol a few hours into the dark cycle, and depriving access to water during the limited access procedure. Middaugh et al. [24] described a paradigm where C57BL/6 mice achieved a BEC slightly above 1.0 mg/ml in an unlimited access, two-bottle choice paradigm, but these animals were acclimated to the two-bottles for 3 weeks, and ethanol was sampled 6 h into the dark phase of the light–dark cycle. In our experience, when the animals are given two-bottle choice in a limited access paradigm, BECs are substantially reduced as compared to when only ethanol is available even though consumption in g/kg is only slightly reduced (unpublished observations). Thus, we believe that having water available in some way changes the pattern of absorption, distribution and/or metabolism of the ethanol.

In our model, mice are not given access to water during the test period and so they might develop some thirst over this time which could contribute to the drinking behavior reported here. Note, however, that D2 mice avoided the ethanol nearly completely (Fig. 6). Moreover, the B6 mice in our study showed no evidence of conditioned taste aversion as they did in the Belknap et al. [31] study when the mice were fluid restricted (90 min fluid/day) prior to ethanol access. In our studies, without extreme water deprivation, the level of drinking was stable across days (see Fig. 1 and Table 1) and between tests as in Experiment 3 (see Table 2, Fig. 2). Thus, the data are consistent with the idea that B6 mice are substantially motivated to drink the ethanol solutions but the cause of the motivation is not known (i.e., whether for the taste, novelty, as part of routine fluid intake, postprandial, thirst, or for its pharmacological effects). This will be the topic of future studies.

A noteworthy finding was that the B6 mice drank more ethanol over a 2-h period when it was offered to them starting 3 h after lights off as compared to starting 1 or 2 h after lights off (see Fig. 2 and Table 2). This may be related to circadian rhythm of consumatory behavior [40–42]. It has been demonstrated that mice eat and drink according to a schedule that approximates a sinusoidal curve [40], and the peak of the curve tends to occur within the first few hours of the dark cycle [43]. It is interesting that a similar rhythm occurs for physical activity as measured by wheel running behavior [44]. Thus, it may be that B6 mice are at their peak state of arousal at approximately 3 h after lights off, and that this activated state leads to maximal expression of behavior including eating, drinking and running.

In this study, we examined several parameters for their effect on level of drinking, but many other parameters were left unexplored. Our goal was to develop a simple model and so we were looking for a paradigm with a short training or acclimation period. We demonstrated that the ethanol drinking was relatively stable if it was carried out for 12 days, and so in remaining experiments we focused on a shorter duration of 4 days. However, this number was arbitrary and we have not yet explored whether 2 days would be sufficient. Ethanol consumption on day 1 was unrelated to the other days (Fig. 6), so at least 1 day of acclimation appears to be necessary for the measurement to be reliable. Although the reliability of individual differences in intake across days 2–4 of Experiment 5 were low, it should be noted that these estimates represent the reliability attributed to environmental influences because the measurements were made in genetically identical B6 individuals. Future experiments will explore the utility of a 2-day paradigm, and the effect of varying the concentration of ethanol beyond what was explored here.

The simpler the procedure, the more amenable it is to high-throughput screening. Screening has proven useful for finding pharmaceutical treatments of complex medical disorders for which the underlying molecular pathway is unknown [45]. Armed with a simple model of pharmacologically significant drinking, it is easy to test a large number of candidate pharmaceutical treatments for their ability to block drinking. A simple model is also useful in genetic research. Mouse genetics offers promise for finding underlying causes of medical disorders but the approach requires screening many individuals and therefore is less amenable to a complex or lengthy behavioral model [6].

In addition to selecting new models of ethanol drinking for their simplicity, it is useful to consider new models because each may measure a slightly different component of the trait (e.g., ethanol drinking). For studies of the genetic influences on drinking, examination of a panel of inbred strains is a useful strategy. Within an inbred strain (such as the B6 strain reported here), all same-sex individuals are like clones, sharing the same genotype at all genes. Individual differences within a strain, therefore, are due to environmental influences. If a panel of strains is tested for multiple traits, the mean trait values for the strains can be correlated, and this yields an estimate of the extent to which the traits reflect the influence of a common set of genes [46]. The stability of such strain correlations across days offers an estimate of the reliability of the genetic differences.

The logic of assessing genetic similarity of different measures of ethanol drinking using inbred strains has been

used to compare two-bottle preference drinking with other tests. One test of self-administration is ethanol acceptance, with or without thirst motivation. Here, mice are offered only 10% ethanol in water for 24 h, during which period their acceptance of ethanol is measured. If a mouse is initially deprived of water for 24 h before the test, it is considered to be under thirst motivation. Acceptance under thirst motivation is significantly influenced by genes as shown by a successful attempt to breed mice for high or low ethanol acceptance [47]. Mice of the F2 cross of C57BL/6 and C3H/2 inbred strains were tested three times at approximately 30-day intervals, first for two-bottle preference drinking of 10% ethanol versus water, then for acceptance twice, first without and then with thirst motivation. Ethanol acceptance with and without thirst motivation were significantly, but moderately correlated (R^2=0.27), but neither acceptance trait was highly correlated with two-bottle preference (both R^2<0.09). In a study of six inbred strains, McClearn compared the standard two-bottle preference testing for 14 days with two-bottle preference for only 3 days, and alcohol acceptance under thirst motivation, using independent groups of each strain for each trait [48]. Although no statistics were presented, the raw data demonstrate that the 3- and 14-day preference tests yielded nearly identical results for the strains. Alcohol acceptance under thirst motivation clearly distinguished the highly accepting C57BL/6 strain and the avoiding DBA/2 strain, while there were some differences in the rank order of the intermediate scoring strains between the traits. For example, RIII clearly had lower two-bottle preference intakes than C3H/2, while they had slightly higher acceptance scores. Thus, these results are consistent with those reported for phenotypic correlations by Anderson and McClearn [47]. The pattern of partial but not complete genetic overlap in contributions to preference and acceptance can also be seen in studies with recombinant inbred strains studied to map specific genes [49–51].

It is interesting that among the genetically identical B6 mice some individuals reliably drank less ethanol than others (see Fig. 5). This suggests that subtle environmental differences such as those that occur during rearing can produce consistent patterns of behavior. Thus, even though the alleles were identical, and efforts were made to keep environmental variables constant, small differences such as cage location, or handling may have produced physiological changes that ultimately led to consistent differences in levels of drinking among B6 mice. Such differences could perhaps be mediated by individual differences in gene expression.

After we switched to a system that supported the ethanol delivery tubes more firmly (Experiment 5), we observed similar levels of drinking and BECs. Therefore, we do not feel that the data reported in Experiments 1–4 misrepresent the phenomena studied. In any case, this study demonstrates that B6 mice will reliably sustain pharmacologically meaningful blood levels when the water bottle is replaced with a solution containing 20% ethanol for 2 or 4 h in the home cage during the dark phase of the light–dark cycle. The highest consumption and BEC occurred when ethanol was substituted for water for 4 h starting 3 h after lights off after three previous days of 2-h ethanol access (Experiment 3, retest, see Table 2, and Experiment 4). We intend to use these parameters in future applications to screen genotypes for their predisposition to drink to intoxication, to screen drugs for their ability to block drinking, and as the basis for development of lines selectively bred to show substantial intakes in this paradigm.

Acknowledgments

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