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Dependence Induced Increases in Intragastric Alcohol Consumption (IGAC) in Mice

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Abstract

Three experiments used the Intragastric Alcohol Consumption (IGAC) procedure to examine effects of variations in passive ethanol exposure on withdrawal and voluntary ethanol intake in two inbred mouse strains, C57BL/6J (B6) and DBA/2J (D2). Experimental treatments were selected to induce quantitative differences in ethanol dependence and withdrawal severity by: (a) varying the periodicity of passive ethanol exposure (3, 6 or 9 infusions/day), (b) varying the dose per infusion (Low, Medium or High), and (c) varying the duration of passive exposure (3, 5 or 10 days). All experiments included control groups passively exposed to water. B6 mice generally self-infused more ethanol than D2 mice, but passive ethanol exposure increased IGAC in both strains, with D2 mice showing larger relative increases during the first few days of ethanol access. Bout data supported the characterization of B6 mice as sippers and D2 mice as gulpers. Three larger infusions per day produced a stronger effect on IGAC than six or nine smaller infusions, especially in D2 mice. Increased IGAC was strongly predicted by cumulative ethanol dose and intoxication during passive exposure in both strains. Withdrawal during the passive exposure phase was also a strong predictor of increased IGAC in D2 mice. However, B6 mice showed little withdrawal, precluding analysis of its potential role. Overall, these data support the hypothesis that dependence-induced increases in IGAC are jointly determined by two processes that might vary across genotypes: (a) tolerance to aversive post-absorptive ethanol effects, and (b) negative reinforcement (i.e., alleviation of withdrawal by self-administered ethanol).

Keywords

dependence; ethanol; inbred mice; self-administration; tolerance; withdrawal

Introduction

Ethanol dependence and withdrawal are commonly considered major criteria in the diagnosis of alcoholism (alcohol dependence) in humans (ICD-10, World Health Organization, 2007; DSM-IV-TR®, American Psychiatric Association, 2000). These neuroadaptations are not viewed merely as symptoms or consequences of chronic ethanol exposure. Rather, they are also seen as potentially important processes in the maintenance of

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Author Contributions

TLF and CLC were responsible for the study concept and design. TLF, MSP, JJR, AC, JM and PS performed surgeries and contributed to the acquisition of animal data. TLF, MSP, AC and PS assisted with data analysis and interpretation of findings. TLF and CLC drafted the manuscript. All authors critically reviewed content and approved the final version for publication.

excessive ethanol intake as suggested by the following statement, “Because Withdrawal from alcohol can be unpleasant and intense, individuals with Alcohol Dependence may continue to consume alcohol, despite adverse consequences, often to avoid or to relieve the symptoms of withdrawal” (DSM-IV-TR®, 303.90 Alcohol Dependence). Indeed, ethanol drinking relieves most of the withdrawal signs that have been reported to provoke drinking in alcoholics (Hershon, 1977).

Although early reviews found limited evidence of dependence- or withdrawal-induced ethanol self-administration in animal models (e.g., Cappell & LeBlanc, 1979, 1981, 1983), a substantial literature now shows that chronic ethanol exposure at levels known to induce dependence and withdrawal will increase later ethanol intake or preference in rats and mice, an outcome that is sometimes attributed to enhancement of ethanol reinforcement (for recent reviews, see: Becker, 2008; Heilig et al., 2010). For example, ethanol intake or operant responding for ethanol has been increased after chronic ethanol exposure via liquid diet (Schulteis et al., 1996; Brown, Jackson & Stephens, 1998), vapor chamber (Roberts, Cole & Koob, 1996; Roberts et al., 2000; Rimondini et al., 2002; Rimondini, Sommer & Heilig, 2003; Becker & Lopez, 2004; Lopez & Becker, 2005) or intragastric (IG) infusion (Deutsch & Cannis, 1980; Fidler, Clews & Cunningham, 2006; Fidler et al., 2009, 2011). Most studies have examined ethanol intake in limited-access procedures, but several studies have shown that ethanol intake is also enhanced in continuous-access procedures (Deutsch & Cannis, 1980; Rimondini et al., 2002, 2003; Fidler et al., 2006, 2009, 2011; Sommer et al., 2008). While the ability of chronic ethanol exposure to increase oral self-administration appears to depend on establishing ethanol’s reinforcing effects before dependence induction (Heilig et al., 2010), prior self-administration experience is not required to increase ethanol intakes (relative to non-dependent controls) in IG self-infusion procedures (Deutsch & Cannis, 1980; Fidler et al., 2006, 2009, 2011).

In most laboratory studies, investigators have inferred a positive relationship between magnitude of induced dependence or withdrawal and later ethanol intake on the basis of a comparison between experimental animals that have been made dependent by passive ethanol exposure and control animals exposed to an appropriate alternative treatment without ethanol. That is, the positive relationship has most often been assumed on the basis of an all-or-none manipulation rather than by examining the effects of inducing different levels of dependence and withdrawal. The strongest evidence for a positive quantitative relationship has been provided by studies in which the periodicity of passive ethanol exposure was manipulated (e.g., O’Dell et al., 2004; Lopez & Becker, 2005). More specifically, these studies showed a more rapid increase in ethanol self-administration after several intermittent ethanol vapor exposures than after a single continuous ethanol vapor exposure, a manipulation previously shown to produce corresponding differences in withdrawal severity (Becker & Hale, 1993). In other words, a treatment known to induce more severe withdrawal (i.e., intermittent ethanol vapor exposure) produced a greater increase in subsequent ethanol intake than a treatment known to induce less severe withdrawal (i.e., continuous ethanol vapor exposure).

Although the foregoing studies generally support the hypothesized positive relationship, few self-administration studies have directly assessed the severity of withdrawal induced by chronic ethanol in the same animals, and almost none have reported the correlation between induced withdrawal severity and the later enhancement of ethanol intake and preference. One notable exception is a recent study that offered a conflicting pattern of findings on the relationship between withdrawal and ethanol intake (Fidler et al., 2011). Mice selectively bred for high (HAP-2) or low (LAP-2) ethanol intake and preference were first passively exposed to ethanol or water via a surgically implanted IG catheter. Withdrawal severity (indexed by handling induced convulsions, HICs) was assessed daily at a single time point

several hours after the last of three daily ethanol infusions. Subsequently, mice were exposed to the Intragastric Alcohol Consumption (IGAC) procedure in which licks of one flavored solution (S+) were paired with IG ethanol infusion while licks of a second flavored solution (S-) were paired with IG water infusion. Overall, alcohol-preferring HAP-2 mice self-infused more ethanol than alcohol-avoiding LAP-2 mice, and ethanol-treated groups self-infused more ethanol than water control groups, regardless of genotype.

The withdrawal data were generally consistent with the hypothesized positive relationship based on the group comparison within each selected line, i.e., ethanol-treated mice showed higher HICs and self-infused more ethanol than water-treated mice within each line. However, contrary to the hypothesis, the line that showed significantly stronger withdrawal severity (LAP-2) did not show greater enhancement of IGAC. Moreover, examination of the phenotypic relationship between withdrawal severity and IGAC (collapsed across ethanol-treated mice from both selected lines) revealed a significant negative correlation. That is, mice that experienced more severe withdrawal during passive ethanol exposure tended to self-infuse less ethanol than mice experiencing less severe withdrawal. A similarly contradictory finding has been reported in a study that found greater enhancement of oral ethanol intake after intermittent ethanol vapor exposure in the HAP-2 line, which also showed (in a separate study) less severe withdrawal than the LAP-2 line (Lopez, Grahame & Becker, 2011). Since the relationship between dependence-induced withdrawal and ethanol intake has not been directly compared in other mouse genotypes, the generality of findings from the HAP/LAP studies is unknown.

The present studies were designed to further examine the relationship between withdrawal severity and ethanol intake/preference in the IGAC procedure using two different mouse genotypes, C57BL/6J (B6) and DBA/2J (D2). These inbred strains were chosen for several reasons. First, there is a substantial literature showing a large difference in voluntary oral ethanol intake, with B6 mice drinking more ethanol and showing a stronger ethanol preference than D2 mice (e.g., McClearn & Rodgers, 1959). Second, these strains differ in sensitivity to ethanol withdrawal, with D2 mice showing more severe withdrawal after ethanol vapor exposure than B6 mice (e.g., Crabbe, 1998). Thus, like the selectively bred lines, the high drinking genotype (B6) shows weaker withdrawal than the low drinking genotype (D2). Third, like the selectively bred lines, both strains showed increased IGAC after passive ethanol exposure (Fidler et al., 2011), providing general support for the positive relationship based on the all-or-none comparison of experimental vs. control groups within each strain. Moreover, in contrast to findings from the HAP/LAP study, the impact of passive ethanol exposure on IGAC during the initial days of ethanol self-infusion was stronger in the genotype with greater sensitivity to withdrawal (D2), offering additional support for the positive relationship. However, the B6/D2 study did not provide an opportunity to examine the within-subject phenotypic relationship between withdrawal severity and IGAC, a shortcoming that was addressed in the present studies.

In addition to water control groups, Experiments 1–3 each included three experimental groups that received passive ethanol treatments selected to induce quantitative differences in ethanol dependence and severity of withdrawal, which were indexed by scoring of HICs on each day of passive exposure. We then assessed effects of these manipulations on subsequent intragastric ethanol intake under no-choice (S+ only) and choice (S+ vs. S-) conditions using the IGAC procedure (Fidler et al., 2011) and determined the correlation between withdrawal severity and ethanol intake/preference within each strain. Experiment 1 examined the effect of varying the number of passive ethanol infusions (3, 6 or 9) given on each of 5 consecutive days while holding total daily dose (and cumulative dose) constant between groups. This manipulation was suggested by a previous study that showed more ethanol drinking in rats that had received 10 g/kg/day in six smaller IG infusions than in rats

that had received the same daily dose in three larger infusions, an outcome that was attributed to differences in the induction of dependence (LeMagnen et al., 1984). Experiments 2 and 3 manipulated dependence and severity of withdrawal by varying the cumulative ethanol dose during passive exposure, a variable that has been found to correlate highly with withdrawal severity in mice (Goldstein, 1972). Two different strategies for varying cumulative dose were used. In Experiment 2, groups differed in the dose (Low, Medium or High) administered during three infusions given on each of 5 consecutive days during passive exposure. In Experiment 3, we varied the duration of passive exposure (3, 5 or 10 days) while maintaining similarity across groups in the number of daily infusions and infusion dose. Higher cumulative doses were expected to induce greater dependence and more severe withdrawal, which we predicted would be positively correlated with IGAC in both strains. Finally, Experiment 4 characterized the blood ethanol concentrations (BECs) produced in each strain using a passive ethanol-dosing schedule that was common to Experiments 1–3.

Materials and Methods

Subjects

Adult male DBA/2J and C57BL/6J mice were shipped from the Jackson Laboratory (Sacramento CA) at 8–9 weeks of age and allowed to acclimate for at least 1 week before surgery. Upon arrival, mice were housed four to a cage (Thoren Caging Systems, Hazleton, PA) in a room maintained on a 12-h light/dark cycle (lights on at 0700). They had free access to Rodent Diet 5001 (Lab Diet, Richmond, IN) and water except when food was removed overnight before surgery. After surgery, mice were individually housed; wet food was provided as needed during recovery. Mice were housed in the experimental apparatus during experiments (see below), except for about 1 hr per day during chamber cleaning. The Oregon Health & Science University IACUC approved these procedures, which adhered to The National Institute of Health (NIH) “Principles of Laboratory Animal Care.”

Apparatus

Clear acrylic and aluminum chambers ($20 \times 20 \times 22.5$ cm) were enclosed in individual laminated sound-attenuating enclosures ($61 \times 40.6 \times 55.9$ cm) with ventilating fans. Each chamber was equipped with two retractable sipper tubes (ENV-252M, Med Associates, St. Albans VT) mounted 9.5 cm apart on one wall, 3.5 cm above the 18 gauge T304 stainless steel mesh floor (Western Group, Portland OR). The sipper tubes were connected to lickometers (ENV-250B, Med Associates) interfaced to a computer that stored lick totals automatically every 5 min using LabVIEW™ 6.1 software (National Instruments, Austin TX).

The IG catheter back mount (313-000BM-10/SPC, Plastics One, Roanoke VA) was attached to polyethylene tubing encased in a metal spring (C313CS, Plastics One) and connected to a 22 ga. fluid swivel (375/22, Instech Solomon, Plymouth Meeting PA) mounted above the chamber on a counterbalanced lever arm (SMCLA, Instech Solomon). A short piece of Tygon tubing (U-95609-18, Cole-Parmer Instrument Co., Vernon Hills IL) connected the swivel to a Y-connector (STCY-22-10, Small Parts, Miami Lakes FL) that received ethanol or water (via Tygon tubing) from 12-ml syringes mounted on two syringe pumps (Model A or Model R-E Razel Scientific Instruments Inc., St. Albans, VT). Each pump was driven by a 0.083 RPM (0.031 ml/min) or 0.33 RPM (0.129 ml/min) synchronous motor, depending on the experimental phase. Chambers were cleaned and food, drinking bottles and pump reservoirs were replenished every day when the mice were removed for behavioral assessments of withdrawal or manual infusions to test catheter patency.

Procedure

Due to limitations on equipment, each experiment was conducted in multiple replications of 16 mice each; both strains were included in each replication. Experiment 4 was conducted in a single replication of 24 mice. In Experiments 1–3, all mice were exposed to the following procedures: (1) surgery, (2) recovery (6–10 days), (3) habituation (3 days), (4) passive infusions of ethanol or water (3–10 days), (5) no-choice self-infusion (2 days), and (6) choice self-infusion (5 days). Procedures for all three experiments were identical in all phases except for the passive infusion phase (see Table 1). Experiment 4 ended after exposure to passive ethanol infusions. Beginning with the habituation day, animals were housed in the apparatus on a normal light-dark cycle and licking/infusion data were collected for about 23.5 h per day. Mice were removed briefly each day for behavioral assessments and apparatus cleaning. Specific details for each experiment are described below.

Surgery—The catheter was constructed from Silastic® silicone tubing, 0.51 mm i.d. × 0.94 mm o.d. (Dow Corning, Midland MI). A knob was created on one end by slipping a short piece of larger silicone tubing, 1.02 mm i.d. × 2.16 mm o.d. over the end and fixing it in place with Dow Corning Medical Adhesive A. A piece of knitted polypropylene mesh (Bard Mesh, Davol Inc., Cranston RI) was also attached to the catheter near the knob end. The catheters and back mounts were sterilized before surgery by overnight immersion in a glutaraldehyde solution (Cidex Plus 28-day solution, Johnson & Johnson, Langhorne PA) followed by flushing and rinsing with sterile water.

The surgical procedure was similar to that described elsewhere (Cunningham, Clemons & Fidler, 2002; Fidler et al., 2011). Each mouse was fully anesthetized with isoflurane gas (5% loading dose, 1.5–3% maintenance) and placed on top of several layers of paper towel on an isothermal pad (39DP, Braintree Scientific, Braintree MA). A dab of puralube was put on each eye and the mouse was injected subcutaneously with 1.0 ml saline (divided among four injection sites) before being shaved. The stomach was externalized through an incision on the animal's left side caudal to the rib cage. The knob end of the catheter was inserted through a puncture in the stomach wall and secured to the stomach by a purse-string suture and two stitches through the polypropylene mesh. A small amount of sterile water was infused to ensure that the catheter was patent and that there was no leak from the stomach. The stomach was returned to the cavity and the incision through the muscle and peritoneum was sutured. The catheter was threaded subcutaneously to a small incision on the back just posterior to the scapulae. The back mount was inserted through the same incision and made to emerge through a hole anterior to the incision. Once in place, the back mount was flushed with sterile water. The catheter was trimmed, attached to the back mount and secured with a single suture through the mesh on the back mount. The skin incisions were sutured, the back mount capped (303DCFT/1, Plastics One Inc.) and anesthesia was removed. Mice were allowed 6–10 days to recover before the experiment began. During this time, mice were infused once per day with at least 0.2 ml of sterile water. Due to a change in the IACUC protocol, mice in Experiments 3 and 4 were injected subcutaneously with the non-narcotic analgesic Meloxicam (0.2 mg/kg; M3935, Sigma, St. Louis MO) 30 min before surgery; an additional dose was given 24 h after surgery via the IG catheter. Mice were weighed and manually infused with sterile water (≥ 0.2 ml) daily before each session during all phases of the experiment.

Habituation—Mice were placed into the chamber, attached to the tether, and given free access to food and two bottles of 0.2 % w/v saccharin (Sigma-Aldrich, St. Louis MO) in tap water. The right and left bottles were available during alternate 30-min periods. This bottle alternation was intended to ensure that mice encountered both bottles and to reduce the

formation of side preferences. The bottle available first alternated over the 3 days of habituation (and was counterbalanced). No infusions were given in the chamber during this phase. All habituation sessions were about 23.5 hr long.

Passive Infusion Phase

General Procedure: All mice had access to food and two bottles of water, which were both available continuously. Passive IG ethanol (10% v/v in sterile water) or sterile water infusions were delivered at a rate of 0.031 ml/min. Infusion volume and ethanol dose were controlled by manipulating the duration of the infusion for each animal based on its body weight. The mean group infusion durations for the passive phase ranged from 35.8–46.1 min for all but one (Group Low) of the groups that received three ethanol infusions per day. Group mean infusion durations were shorter for the groups that received the smallest average dose per infusion (13.4, 19.9 and 29.3 min for Groups 9-inf, 6-inf and Low, respectively). Due to minor differences in body weight, there were also small but negligible differences in mean infusion durations for B6 and D2 mice (mean infusion duration difference ≤ 2.3 min). The ethanol doses and infusion schedules used for each group are described below in detail for each experiment (see also Table 1).

Degree of intoxication was assessed daily 2 hrs after the start of the last infusion by placing each mouse in the center of a plastic arena (28 × 28 × 13 cm) and observing its behavior for about 2 min. In Experiment 3, mice that were not scheduled to receive infusions were assessed at the same time of day. Mice were given a composite rating that was the sum of four separate scales: leg splay (0–5), wobbling (0–4), nose down (0–1) and belly down (0–1), with higher numbers indicating greater impairment. This procedure was adapted from one previously described by Metten et al. (2004). At the end of the daily session, approximately 7.5 hours after the start of the last infusion, withdrawal was assessed on a scale of 0–7 (0 = no convulsion) using handling induced convulsions (HICs; Metten and Crabbe, 2005). A previous study indicated that assessments at these times were sensitive to effects of both ethanol and genotype (Fidler et al., 2011). We assessed intoxication and withdrawal at single time points only to minimize the potential disrupting influence of repeated handling and testing (and possible induction of seizures) on the animal's ability to recover on passive exposure days and on the later initiation of IG self-infusion. If mice showed high levels of intoxication and were still impaired at the time of withdrawal assessment, the first infusion of the next day was omitted to allow additional recovery time and to avoid overdosing in the highest dose conditions. In some cases, an unusually low body weight or low level of licking was used as the basis for omitting the first infusion on the following day (see details below).

Experiment 1: Number of Daily Infusions: All mice were exposed to passive infusions on 5 consecutive days. Mice from each strain were randomly assigned to one of four groups that differed in terms of the total number of daily ethanol infusions: 0, 3, 6 or 9. The 0 (Water) group received water infusions matched in volume and timing with ethanol infusions given to the 9-inf group. The first daily infusion was given to the 3-, 6- and 9-inf groups after delays of 280, 110 and 53.6 min, respectively; subsequent infusions began at intervals of 340, 170 and 113.3 min, respectively. On these schedules, the last daily infusion began at the same time of day for each group, about 2 hrs before the assessment of intoxication. All mice received the same total daily dose, which was divided equally across the scheduled infusions. Total ethanol dose was 9.0, 10.5, 12.0, 13.5 and 15.0 g/kg on Days 1–5, respectively. These total daily doses were previously found to be effective for producing a subsequent enhancement of IGAC when given on the schedule used for the 3-inf group (Fidler et al., 2011). No adjustments in the passive dose schedule were required in this experiment.

Experiment 2: Daily Ethanol Dose: All mice were exposed to passive infusions on 5 consecutive days. Mice from each strain were randomly assigned to one of four groups that differed in terms of the daily (and cumulative) ethanol dose administered during the passive phase: Low, Medium, High or Water control. Water infusion volume for the control group was equivalent to the infusion volume for the High group on all days. These infusions occurred approximately at the onset of the dark cycle (1840–1910), in the middle of the dark cycle (0020–0050) and near the end of the dark cycle (0600–0630). Ethanol doses were 2, 3 or 4 g/kg/infusion on the first passive infusion day for the Low, Medium and High groups, respectively (i.e., total doses of 6, 9 or 12 g/kg/day). The ethanol dose per infusion was increased by 0.5 g/kg on each of the next 4 days for the Low and Medium groups. Thus, the treatment for the Medium group was identical to that given to the 3-inf group in Experiment 1. For the High group in the first two replications of this study (involving two D2 and one B6 mouse), dose per infusion was also increased by 0.5 g/kg on each subsequent day of the passive phase. However, because the High group doses produced severe behavioral impairment in several mice, dose was not increased on the final passive day in any of the remaining replications (involving 10 D2 and 11 B6 mice). In addition, adjustments were made to the planned dosing schedule for two mice in the B6 High group that showed high levels of intoxication and were still impaired at the time of withdrawal assessment. In both cases, the first infusion of the next day was omitted to allow additional recovery time. One infusion was also omitted for a D2 High group mouse that had fewer than 10 licks on the previous day. Despite these adjustments, there was no overlap in the distribution of cumulative doses received by mice in the High group with the distribution of doses received by mice in the Medium or Low groups.

Experiment 3: Number of Ethanol Exposure Days: Mice were exposed to three passive infusions per day for 3–10 days over a 10-day period. Mice from each strain were randomly assigned to one of four groups that differed in terms of the number of passive ethanol infusion days: 0, 3, 5 or 10. The Water control group received passive water infusions matched in timing and volume to those of the 10-day group. Number of days in the apparatus was held constant, as was the interval between the start of the final passive infusion and onset of the no-choice phase (see Supplementary Table 1). Mice received three infusions per day using the same temporal parameters used for Experiment 2 and the 3-inf group in Experiment 1. Ethanol doses per infusion during the first 5 days of passive exposure (5-day group) were identical to those used for the Medium group in Experiment 2 and for the 3-inf group in Experiment 1. That is, ethanol dose was 3.0 g/kg/infusion on the first passive ethanol day and the dose per infusion was increased by 0.5 g/kg/infusion on each of the next 2 (3-day group) or 4 (5- and 10-day groups) days. For the 10-day group, ethanol dose was subsequently increased by 0.25 g/kg/infusion on days 6, 8 and 10; on days 7 and 9, the dose per infusion was that used on the previous day. Adjustments were made to the planned dosing schedule for five mice in the 10-day groups due to a combination of high intoxication and withdrawal scores as well as low body weight ($\leq 80\%$ of day 1 weight) near the end of the phase. One infusion was omitted on one day (two mice in the D2 10-day group) or 2 days (two mice in the D2 10-day group and one mouse in the B6 10-day group) to allow additional recovery time. Despite these adjustments, all mice in the 10-day groups received cumulative ethanol doses that were well above those received by any mouse in the 3- or 5-day groups.

Experiment 4: Blood Ethanol Concentrations During Passive Exposure: The main purpose of this experiment was to provide an initial characterization of the blood ethanol concentrations (BECs) produced in each strain using the passive ethanol-dosing schedule that was common to Experiments 1–3 (i.e., the dose schedule used for the 3-inf, Medium and 5-day groups). This dosing schedule was also the same as that used to induce enhanced IGAC in B6, D2, HAP-2 and LAP-2 mice in previous studies (Fidler et al., 2011). Primary

interest focused on determining the maximum BEC induced by this schedule of passive exposure (which has become the “standard procedure” used in subsequent IGAC studies) and whether there was a strain difference in BEC that might complicate interpretation of genetic comparisons.

After surgery and recovery, all mice initially received habituation sessions as described earlier. Mice from each strain were then randomly assigned to two groups that differed in whether blood samples were taken at the end of the first day (Day 1 group) or fifth day (Day 5 group) of the passive infusion phase. More specifically, a 20 μ l blood sample was obtained from the tip of the tail 5 min after the end of the third (and final) infusion on Day 1 (Day 1 groups) or Day 5 (Day 5 groups). The purpose of taking this sample was to approximate the highest BEC induced by the passive infusion procedure. A second sample was taken 120 min after the start of the last infusion. The purpose of this sample was simply to characterize BEC at the time when intoxication would normally have been assessed. Since infusion duration varied across mice (due to variations in body weight) and between the first and last infusion days (due to the increase in dose over days), the time interval between the first and second sample ranged from 80.9–88.4 min for the Day 1 groups and from 63.7–70.1 min for the Day 5 groups. The experiment ended after the second sample was collected and mice were not exposed to the IGAC procedure. BECs during the IGAC procedure were determined separately in other experiments (see below). Each blood sample was added to chilled ZnSO₄ (50 μ l) and stored on ice. Next, 0.3 M Ba(OH)₂ (50 μ l) and distilled water (300 μ l) were added to each sample. Samples were then vortexed and centrifuged at 9500 g for 5 min. The supernatant was removed and analyzed by gas chromatography (Rustay & Crabbe, 2004).

Intoxication and withdrawal severity were rated on each of the first 4 days of passive exposure for the Day 5 groups as described earlier (General Procedure) and correlations between the Day 5 BECs and the 4-day mean intoxication or mean withdrawal ratings were calculated separately for each strain. Although most of these correlations were positive, none was statistically significant (all p 's > .05) and will not be discussed.

No-Choice Self-Infusion Phase—All mice were treated identically during this phase. About 8 hrs after the start of the last passive infusion, mice received access to a single drinking tube that contained 0.05% w/v grape or cherry Kool-Aid (Kraft Foods, Rye Brook, NY) and 0.2% w/v saccharin in tap water (S+). The S+ tube was in the location (right or left) that had been preferred by each mouse during the habituation and passive phases (as determined by licks and consumption). Infusions of 20% v/v ethanol in sterile water were contingent upon drinking the S+ solution. As in previous studies, ethanol concentration was increased during self-infusion to offset dilution by the orally consumed S+ solution (e.g., Fidler et al., 2006, 2009, 2011). Every 10th lick was followed by a 4-sec infusion (0.129 ml/min) of ethanol up to a maximum limit of 1.5 g/kg/30 min. Each infusion delivered an ethanol dose of 0.05 – 0.07 g/kg for mice weighing 20–30 g. When the maximum number of infusions was delivered, the S+ tube remained available, but no further infusions were made until ethanol dose during the last 30 min fell below the limit. The dose limit was imposed to minimize the likelihood that a high dose bout would induce a conditioned taste aversion to S+. The 2 no-choice days were included to ensure that all mice would encounter the contingency between the S+ and ethanol infusion. Both sessions were about 23.5 hr long.

Choice Self-Infusion Phase—The 5-day choice phase was the same as the no-choice phase except that a second drinking tube containing the other flavored Kool-Aid solution (S–) was available. Assignment of flavors as S+ and S– was counterbalanced within groups. Licks on the S– tube were paired with infusions of sterile water using the same infusion rate and limit as the S+ tube. The S+ tube remained in the same position used during the no-

choice phase. All mice were treated identically during this phase. All sessions were about 23.5 hr long.

BECs During Self-Infusion—Blood samples were taken from a subset of mice in Experiments 1 and 2 in order to characterize BECs achieved during choice self-infusion (no samples were obtained from mice in Experiment 3). To avoid disruption of our behavioral measures, blood collection occurred after the 5th day of the choice self-infusion phase. We chose not to sample all mice at an arbitrarily selected time because there was a relatively wide range in the within- and between-strain temporal patterns of ethanol intake across the 23.5-hr choice session. Thus, as in previous studies, we adopted a sampling strategy that was intended to identify only the BECs reached after periods of high drinking (Fidler et al., 2009, 2011). More specifically, a 20 μ l blood sample was taken from the tip of the tail 5 min after the end of a bout in which a mouse reached the dose limit (1.5 g/kg/30 min) during the dark part of the light/dark cycle. Blood samples were not obtained from mice that failed to reach the dose limit. Samples were prepared and analyzed as described above.

Results

The data from about 11.3% ($n = 38/336$) of the mice that began these experiments were removed due to various problems. About one-third were removed because of experimenter errors or equipment problems (e.g., infusion pump failures), one-third because of catheter problems (e.g., chewed or leaking) and one-third because of problems related to apparent overdose or impaired recovery from high-dose infusions during the passive phase. Mice in the latter category were all in the High (Experiment 2) or 10-day (Experiment 3) groups, which received the highest daily and cumulative doses. This category was about evenly split between D2 ($n = 6$) and B6 ($n = 7$) mice, indicating that there was no genetic difference. All data were removed for mice in these three categories. However, in cases where these problems did not occur until the choice self-infusion phase, data from earlier phases were included in analyses. The final number of mice per group was between 10 and 14 in Experiments 1–3; 5–7 mice were used for each group in Experiment 4. Exact group sizes are shown in Table 1. Data from each experiment were analyzed separately. However, because the designs and procedures were similar, the results from the first three experiments are presented in parallel for each dependent variable.

Separate two-way (Strain $\times$ Group) analyses of variance (ANOVAs) were used to analyze mean daily intakes during the choice and no-choice phases. Similar ANOVAs were used to analyze S+ preference ratios and ethanol intake pattern data during the choice phase (number of ethanol bouts per day, mean ethanol bout size, and percent of total intake explained by bouts of different sizes). To examine the impact of introducing a second drinking tube (S–) on ethanol intakes, we also conducted repeated measures ANOVAs that compared the no-choice and choice phases (Strain $\times$ Group $\times$ Phase). Interpretation of these secondary analyses focused on main effects of Phase or interactions involving Phase. A repeated measures ANOVA (Strain $\times$ Group $\times$ Samples) was also used to analyze BECs in Experiment 4. The alpha level for all ANOVAs was set at .05. P-values for post-hoc comparisons between strains or groups were Bonferroni-corrected for multiple comparisons.

Non-parametric statistical analyses based on rank sums were used to evaluate the intoxication and withdrawal scores because they involve ordinal scales of measurement. More specifically, Kruskal-Wallis one-way analyses were used to compare the four groups within each strain in each experiment and Mann-Whitney tests were used to compare B6 and D2 mice within each treatment condition. In order to minimize experiment-wise alpha level, we used Ryan's procedure to conduct post-hoc comparisons between groups within each strain, setting the overall alpha at .05 (Linton & Gallo, 1975).

Ethanol Intake

Passive Phase—As expected, the mean cumulative passive ethanol dose given to ethanol groups that received 3, 6 or 9 infusions in Experiment 1 was about 60 g/kg. A similar cumulative dose was given to the Medium, 5-day and Day 5 groups in Experiments 2, 3 and 4, respectively. In contrast, the Low and High groups in Experiment 2 received cumulative doses of about 45 and 74 g/kg, respectively. The 3-day and 10-day groups in Experiment 3 received cumulative doses of about 31 and 140 g/kg, respectively. The Day 1 groups in Experiment 4, which were exposed to only one day of passive exposure, received a cumulative dose of about 9 g/kg. The exact mean cumulative doses administered to each group are listed in Supplementary Table 2.

No-Choice Phase—Figure 1 (columns 1–2) depicts the mean daily ethanol intakes (g/kg/d) during the no-choice phase for all strain $\times$ group combinations. B6 mice generally self-infused more ethanol than D2 mice in all three experiments and ethanol-exposed mice generally self-infused more ethanol than water-exposed mice. Ethanol intake was inversely related to the number of daily passive ethanol infusions (Experiment 1, top panels) and positively related to cumulative passive phase ethanol dose (Experiment 2, middle panels; Experiment 3, bottom panels).

Table 2 shows the primary Strain $\times$ Group ANOVAs for each experiment during the No-Choice phase. For all three experiments both main effects (all p 's $< .0001$) and the interaction (all p 's $< .05$) were significant. Within-strain post-hoc tests (Bonferroni-adjusted) to interpret interactions are depicted in Figure 1. In Experiment 1, these tests showed that: (a) the B6 3-inf group had higher ethanol intake than the B6 water group, (b) each of the D2 ethanol groups self-infused more ethanol than the D2 water group, and (c) the D2 3-inf group self-infused more ethanol than the D2 9-inf group. Similar post-hoc tests for Experiment 2 showed that: (a) B6 water mice self-infused less ethanol than either the B6 Medium or B6 High group, and (b) D2 water mice self-infused less ethanol than any of the ethanol groups, which did not differ from each other. Post-hoc tests for Experiment 3 indicated that: (a) B6 water mice self-infused less ethanol than either the B6 5-day or B6 10-day groups, (b) the B6 3-day group self-infused less ethanol than the B6 10-day group, (c) D2 water mice self-infused less ethanol than any of the D2 ethanol groups, and (d) the D2 3-day group self-infused less ethanol than the D2 10-day group. For all three experiments, the strain comparisons within each treatment condition showed that every B6 group self-infused significantly more ethanol than the corresponding D2 group.

Choice Phase—Figure 1 (columns 3–4) also shows the mean daily ethanol intakes (g/kg/d) during choice self-infusion for all groups. The general pattern of findings was similar to that during the no-choice phase, although group effects were larger and overall ethanol intakes were generally lower than during no-choice self-infusion. The primary ANOVAs yielded significant main effects of Strain and Group in all three experiments (all p 's $< .0001$), but the interaction was significant ($p < .05$) only in Experiment 1 (Table 2). Within-strain post-hoc comparisons for Experiment 1 showed no differences among B6 groups, but the D2 3-inf group self-infused more ethanol than any of the other D2 groups. B6 mice in the water, 6- and 9-inf groups self-infused more ethanol than the corresponding D2 group, but the B6 and D2 3-inf groups did not differ. In Experiment 2, post-hoc comparisons (collapsed across strain) indicated that all of the ethanol exposed groups continued to self-infuse more ethanol than the water group. Moreover, the High group self-infused more ethanol than the Low group. Similar follow-up tests for Experiment 3 showed that all of the ethanol groups self-infused more ethanol than the water group and that the 10-day group self-infused more than the 3-day group.

No-Choice vs. Choice Phase—Ethanol intakes during the choice phase were generally lower than intakes during the no-choice phase in all three experiments. The secondary ANOVAs (Strain $\times$ Group $\times$ Phase) generally supported this conclusion as reflected by significant main effects of Phase in Experiment 1, $F(1,100) = 91.0$, $p < .0001$, Experiment 2, $F(1,83) = 57.0$, $p < .0001$, and Experiment 3, $F(1,91) = 142.3$, $p < .0001$. The Phase $\times$ Group interaction was also significant in Experiment 2, $F(3,83) = 46.2$, $p < .05$, but not in Experiments 1 or 3. Follow-up tests to interpret the interaction in Experiment 2 showed that the decrease in ethanol intakes in the choice phase relative to the no-choice phase (Water = -5.31 g/kg/d; Low = -6.27 g/kg/d; Medium = -4.34 and High = -1.88) was significant for all but the High group (Water: $F(1,21) = 25.8$, $p < .0001$; Low: $F(1,21) = 29.4$, $p < .0001$; Medium: $F(1,23) = 15.9$, $p < .002$). The secondary analyses yielded no other significant interactions involving Phase in any experiment.

Ethanol Intake Pattern

Although daily intakes are clearly sensitive to genotype and a history of passive ethanol exposure, examination of ethanol intake patterns is required to evaluate the role that might be played by ethanol's pharmacological effects (Leeman et al., 2010). To address this issue, we analyzed three additional dependent variables during the choice phase: (i) the number of bouts/day, (ii) average bout size (g/kg/bout), and (iii) the percent of total intake attributable to small (< 0.5 g/kg), medium ($0.5 - 0.99$ g/kg), and large (≥ 1.0 g/kg) bouts (for rationale, see Fidler et al., 2011). Infusion and lick data were recorded in 5-min bins during every session and a bout was defined as the ethanol intake in consecutive 5-min periods without a break greater than 5 min.

Number of Bouts/Day—Analyses of the mean number of ethanol bouts/day for all three experiments generally paralleled those for mean ethanol intake, indicating that: (a) passive ethanol exposure increased later ethanol self-infusion by increasing the number of daily bouts, and (b) B6 mice had more ethanol bouts/day than D2 mice. The group means and a summary of the statistical analyses are provided in Table 3.

Bout Size—Mean bout size was calculated by averaging the size of every bout within the choice phase, excluding mice that failed to self-infuse ethanol (Table 4). Of particular interest, D2 mice had larger bouts than B6 mice (main effect of Strain) in all three experiments, Experiment 1, $F(1,83) = 36.8$, $p < .0001$, Experiment 2, $F(1,75) = 27.4$, $p < .0001$, Experiment 3, $F(1,86) = 27.9$, $p < .0001$. Bout size was also larger in the groups that had received the highest cumulative ethanol doses during the passive phase, a conclusion supported by significant main effects of Group in Experiments 2, $F(3,75) = 2.9$, $p < .05$, and 3, $F(3,86) = 7.8$, $p < .0001$. Post-hoc tests to interpret the Group effect yielded no pair-wise comparison that reached Bonferroni-corrected criterion for significance in Experiment 2, although the water group tended to have smaller bouts than the High Group ($p = .06$). Post-hoc tests for Experiment 3 indicated that the 10-day group had larger bouts than any other group. There was no significant interaction in any experiment.

Percent of total intake explained by different sized bouts—The percentages of total choice-phase ethanol intake that occurred in small, medium or large bouts are shown in Table 5. On the basis of previous data showing that the direction of the strain difference varied as a function of bout size (Fidler et al., 2011), we performed separate Strain $\times$ Group ANOVAs on the data for small, medium, and large bouts in each experiment (Supplementary Table 3). Consistent with our earlier study, the analyses for all three experiments showed that B6 mice self-infused a higher percentage of their total ethanol intake in small bouts than D2 mice, whereas D2 mice infused a higher percentage of their total ethanol intake in large bouts than B6 mice. In support of these conclusions, the

ANOVAs for all three experiments yielded significant main effects of Strain for small (all p 's < .001) and large (all p 's < .0001) bouts. The consistently larger average bout size and the relatively greater contribution of large bout drinking to total intake in D2 mice is congruent with our previous characterization of B6 mice as “sippers” and D2 mice as “gulpers” (Fidler et al., 2011).

Passive ethanol treatment also affected the percentages of total intake attributable to small and large bouts in Experiments 2 and 3 as indicated by significant main effects of Group (all p 's < .01). Post-hoc comparisons between groups (collapsed across strain) revealed the same general pattern of findings in both experiments. That is, groups that had received more passive ethanol exposure self-infused a lower percentage of their total intake in small bouts than the water control groups. Conversely, the high ethanol exposure groups self-infused a larger percentage of their total intake in large bouts than the water control groups. The highest dose groups (High, 10-day) also differed from the lowest dose groups (Low, 3-day) within each experiment. Details of these post-hoc comparisons are shown in Table 5.

There were no differences in the percentages attributable to medium-sized bouts in Experiments 2 and 3, but there was a significant Strain $\times$ Group interaction (p < .01) in the analysis of such bouts in Experiment 1. This effect was due to a relatively high percentage of medium sized bouts in the D2 water group compared to the B6 water group and all other D2 groups (see Table 5), but interpretation of this effect is complicated by the fact that very few D2 water mice had any ethanol bouts during the choice phase (n = 4).

S+ Preference Ratio

The daily ratios of licks on the S+ tube to total licks (S+ and S− licks) were averaged across the 5 choice days for each mouse to determine mean S+ preference ratios (Figure 2). As can be seen, the B6 Water groups had higher preference ratios than the D2 Water groups. Passive ethanol exposure generally increased ratios, but this effect was much greater in D2 mice. In fact, ethanol exposure eliminated or reversed the strain difference in S+ preference. These observations were supported by significant Strain $\times$ Group interactions in the analyses of Experiments 1, $F(3,100) = 5.7$, p < .002, and 3, $F(3,91) = 6.7$, p < .0001; the interaction missed the criterion for significance in Experiment 2 (p = .10). The main effect of Group was also significant for all three experiments, Experiment 1, $F(3,100) = 8.4$, p < .001, Experiment 2, $F(3,83) = 8.2$, p < .0001, Experiment 3, $F(3,91) = 13.1$, p < .0001, but the main effect of Strain was significant only in Experiment 1, $F(1,100) = 17.1$, p < .0001. Post-hoc tests showed no differences among B6 groups in any experiment. However, one or more of the ethanol treated D2 groups showed a significantly higher ratio than the D2 water group in each experiment (see Figure 2). Post-hoc tests also showed that the B6 water group had a significantly higher ratio than D2 water group in Experiments 1 and 3, but not in Experiment 2. None of the B6 ethanol-exposed groups differed significantly from the corresponding D2 group in any experiment.

One group t-tests were also used to determine whether the S+ ratio differed from 0.50 (i.e., equal licking on the S+ and S−). These analyses indicated no significant preferences or aversions among the B6 groups in any experiment. However, all of the D2 water groups (p 's < .0001) as well as the D2 6-inf and D2 9-inf groups (p 's < .05) showed significant S+ aversions. Only the D2 10-day group showed a significant S+ preference (p < .05), although the preference shown by the D2 3-inf group approached the criterion for significance (p = .08).

Intoxication

Median daily intoxication ratings for all groups during the passive phase are shown in Table 6 together with the non-parametric statistical analyses. B6 mice generally showed greater intoxication than D2 mice in the treatment groups that produced the highest intoxication scores in each experiment, a conclusion supported by significant strain differences for the 3-inf, High and 10-day groups (all p 's < .05). Also, as would be expected, groups exposed to higher ethanol doses per infusion (e.g., 3-inf, High) or more days of ethanol infusions showed higher intoxication scores (all p 's < .05).

Withdrawal

Median daily withdrawal ratings (HICs) during the passive phase and non-parametric analyses are shown in Table 7. No B6 group showed signs of withdrawal except for the 10-day group, which was the only B6 group with a non-zero median rating. In contrast, all of the D2 ethanol-exposed groups showed significantly more withdrawal than the matched water control group (all p 's < .05). Moreover, withdrawal in the D2 ethanol groups was positively related to ethanol infusion dose in Experiments 1 and 2. Although there was a positive arithmetic relationship between median withdrawal and number of days of passive ethanol exposure in Experiment 3, none of the pair-wise comparisons between ethanol treated groups was significant (see Table 7).

HICs were also measured at about the same time of day during the no-choice phase, but the median group ratings were non-zero for only three groups: D2 3-inf (1.0), D2 High (1.0) and D2 10-day (1.3). We did not measure HICs during the choice phase to avoid any impact of this assessment on voluntary self-infusion.

Correlations

To determine whether behavioral responses during passive ethanol exposure were predictive of subsequent IGAC, separate Pearson correlations were calculated for each strain using the group medians (intoxication, withdrawal) and means (no-choice intake, choice intake, S+ preference) from the groups in all three experiments (Table 8). We focused on group scores instead of individual scores to reduce the detrimental impact of including zeros in the calculations (21% of ethanol-treated D2 mice and 86% of ethanol-treated B6 mice had withdrawal scores of 0). Because the scores used in each calculation were obtained from the same inbred strain, all variation can be attributed to environmental rather than genetic influences (i.e., these are environmental correlations). Intoxication scores were generally more predictive of no-choice intake than choice intake or S+ preference in B6 mice. However, this pattern was reversed in D2 mice, with intoxication scores more predictive of choice intake and S+ preference than no-choice intake. D2 mice showed the same pattern when median withdrawal was analyzed, yielding higher correlations with choice intake and preference than with no-choice intake. The most interesting and strongest of these correlations is depicted in Figure 3, which shows that D2 groups with the highest withdrawal severity voluntarily infused the most ethanol during the choice phase ($r = +0.9$, $p < .0001$). The high number of zero median withdrawal scores precluded calculation of meaningful correlations with that dependent variable for the B6 groups. Correlations calculated from individual scores yielded a similar overall pattern of significance, although the absolute values of the correlation coefficients were lower (see Supplementary Table 4).

Blood Ethanol Concentrations

BECs Induced by Passive Ethanol Exposure (Experiment 4)—As intended, the cumulative ethanol doses administered in Experiment 4 were similar to those given to the 3-inf, Medium and 5-day groups in earlier experiments (Supplementary Table 2). BECs

induced by passive ethanol infusion were about 50–80% higher in the Day-5 groups than in the Day-1 groups at each time point, but there were negligible differences between strains or between Samples 1 and 2. The Day-1 group mean BECs ($\pm$ SEM) for Samples 1 and 2 were 2.2 ± 0.2 and 2.0 ± 0.1 mg/ml for B6 mice and 2.0 ± 0.2 and 1.7 ± 0.1 mg/ml for D2 mice. The Day-5 group means for Samples 1 and 2 were 3.1 ± 0.5 and 3.1 ± 0.3 mg/ml for B6 mice and 3.3 ± 0.3 and 3.6 ± 0.2 mg/ml for D2 mice. The higher BECs in the Day-5 groups are most easily attributed to the higher infusion dose on Day 5 (5 g/kg) compared to Day 1 (3 g/kg). A three-way ANOVA confirmed the significant main effect of Group, $F(1,20) = 31.5$, $p < .0001$; no other effects or interactions were significant. Thus, strain differences in the effects of passive ethanol exposure on IGAC cannot be explained by differences in ethanol absorption or metabolism.

BECs Achieved by Ethanol Self-Infusion (Experiments 1 and 2)—The scatter plot in Figure 4 depicts BEC as a function of voluntary ethanol intake during the 3-h period before each sample was taken. About 43% of these BECs exceeded 1.0 mg/ml, a level that is believed to reflect intoxication in mice (Crabbe et al., 2005, 2008). After examining several time periods, the 3-h window was chosen because the overall correlation between BEC and intake was strongest for this interval, Pearson $r = +0.53$, $n = 59$, $p < .0001$. This correlation was also significant for each strain considered separately: B6, $r = +0.63$, $n = 26$, $p < .002$; D2, $r = +0.53$, $n = 33$, $p < .002$. Of interest, the five highest BECs (ranging from 2.5 to 3.4 mg/ml) were obtained from D2 mice. However, we did not make direct statistical comparisons between strains or groups because our blood sampling strategy was not designed to provide unbiased group comparisons. Rather, as indicated earlier, the purpose of these measurements was simply to characterize BECs attained after periods of relatively high ethanol intake in the IGAC procedure. Because that strategy allowed the mouse to determine when and whether a sample was taken, we did not obtain samples from all mice. Thus, these samples are not necessarily representative of other mice in the same Strain $\times$ Group conditions. An additional limitation of this sampling strategy is that it does not provide information on the lowest BECs maintained by ethanol pre-exposed mice, an issue that must be addressed in future studies.

Discussion

These studies showed that quantitative variations in passive ethanol exposure are positively associated with increases in subsequent voluntary ethanol intake and S+ preference in two inbred mouse strains, B6 and D2. In three independent experiments, we replicated and extended the principal findings from a previous study that only included an all-or-none comparison of experimental (passive ethanol infusions) vs. control (passive water infusion) groups within each strain (Fidler et al., 2011). Consistent with our previous study, the current studies showed that B6 Water mice self-infused more ethanol and were less averse to the S+ than D2 Water mice. Moreover, absolute intakes for each strain during the choice phase were similar to those in the previous IGAC study and similar to intakes measured in home-cage two-bottle choice drinking procedures (Belknap, Crabbe & Young, 1993). The low intakes in D2 Water groups confirmed that switching from the oral to IG route of administration was not sufficient to increase voluntary ethanol intake in this strain, supporting previous suggestions that aversive post-absorptive effects of ethanol normally contribute to avoidance of oral ethanol by D2 mice (Cunningham, Gremel & Groblewski, 2009; Fidler et al., 2011).

Also consistent with the previous study, passive ethanol exposure increased IG ethanol intake and preference in both strains and eliminated the strain difference in S+ preference. Moreover, the enhancing effect of passive exposure was greater in D2 mice than in B6 mice during the first few days of ethanol self-infusion (i.e., no-choice phase) in all three

experiments. Importantly, the present studies extended our previous findings by showing that the increases in intake and preference were positively correlated with cumulative passive ethanol dose (Table 9). The high voluntary ethanol intakes and S+ preferences in ethanol-exposed D2 mice are of particular interest in light of the low intakes and preferences commonly reported for this strain in home-cage two-bottle drinking procedures.

Analyses of ethanol intake patterns in all three experiments were also consistent in showing that B6 mice had more bouts per day than D2 mice, and that passive ethanol exposure increased daily intakes by increasing number of bouts per day in both strains. Additionally, all three studies showed that D2 mice had larger ethanol bouts than B6 mice and that D2 mice self-infused a higher percentage of their total ethanol intake in large bouts (≥ 1 g/kg/bout) than B6 mice. Thus, these findings strongly support our previous characterization of B6 mice as “sippers” and D2 mice as “gulpers.”

Experiment 1 showed that exposure to a high cumulative dose of ethanol each day was not sufficient to increase later IGAC. Rather, the pattern of daily ethanol exposures was critical, with three larger infusions (3-inf group) producing a stronger effect than six (6-inf) or nine (9-inf) smaller infusions. Number of infusions had an especially pronounced impact on choice phase intake and S+ preference in D2 mice. The direction of the effect was contrary to expectations based on a previous periodicity study in which rats that had received six smaller IG infusions per day later drank more ethanol than rats that had received the same total dose per day in three larger infusions (Le Magnen et al., 1984). Possible reasons for the discrepant outcomes include the use of different species, different dosing schedules and different routes of administration during self-administration testing. Although Le Magnen et al. attributed their outcome to a group difference in dependence, their study provided no independent evidence that dependence was greater in the six-infusion group than in the three-infusion group. Thus, it is possible that their group difference in ethanol drinking was unrelated to dependence or withdrawal. In contrast, Experiment 1 strongly supports the suggestion that ethanol dependence and withdrawal played a role in enhancing ethanol intake in the D2 3-inf group.

More generally, the combined data from Experiments 1–3 showed a strong positive quantitative relationship between physical withdrawal signs during the passive phase and the later increase in ethanol self-infusion in D2 mice, a relationship that was apparent in correlations based on group averages (Fig. 3) as well as in correlations based on individual subject scores (Supplementary Table 4). To our knowledge, no other model of dependence-induced intake has ever provided as comprehensive a demonstration of this relationship. Since B6 mice showed negligible signs of withdrawal across a wide range of ethanol dosing regimens, one might conclude that dependence and physical withdrawal played little or no role in mediating their later increases in IGAC. However, it is also possible that our single time point HIC assessment procedure did not provide a sufficiently sensitive index of withdrawal in B6 mice, perhaps because the assessment was not made at the time of their peak withdrawal. It is also possible that examination of a complete HIC time course and calculation of area-under-the-curve (e.g., Crabbe, 1998) or affective signs of withdrawal (Heilig et al., 2010) would provide more sensitive predictors of later IGAC in B6 mice. These issues should be addressed in future IGAC studies and in other models in which B6 mice have shown dependence-induced increases in ethanol intake (e.g., oral self-administration after intermittent vapor exposure: Becker & Lopez, 2004).

The positive relationship between withdrawal severity and subsequent IGAC in D2 mice is consistent with theories that attribute the increase in ethanol intake to negative reinforcement based on alleviation of withdrawal (Becker, 2008; Koob & Le Moal, 2008; Fidler et al., 2011). In other words, ethanol-induced alleviation of more severe withdrawal

would be expected to have a greater positive impact on subsequent increases in ethanol intake than alleviation of less severe withdrawal. It is important to distinguish the positive environmental (phenotypic) correlation observed here from the previously described negative genetic correlation between sensitivity to ethanol withdrawal and oral ethanol intake and preference (Metten et al., 1998). The negative genetic correlation is based on observations of both selectively bred and inbred mouse strains that indicate higher oral ethanol consumption and preference in genotypes with lower genetic susceptibility to ethanol withdrawal (e.g., B6, HAP) than in genotypes with higher genetic susceptibility to ethanol withdrawal (e.g., D2, LAP). In contrast to the environmental correlation, demonstration of the genetic correlation does not require measurement of ethanol intake subsequent to induction of withdrawal in the same animals. Whereas a genetic correlation suggests possible overlap in the genes influencing the related phenotypes (Crabbe et al., 1990), the environmental correlation suggests a possible causal relationship (i.e., withdrawal caused increased ethanol intake).

The positive environmental correlation observed here contrasts with the negative phenotypic correlation recently reported in HAP-2/LAP-2 mice (Fidler et al., 2011), suggesting that the direction of this relationship varies across genotypes. Nevertheless, all four of the genotypes that have been studied in the IGAC procedure (B6, D2, HAP-2, LAP-2) have shown a positive relationship between withdrawal and later ethanol intake based on comparisons between experimental and control mice within each strain (i.e., all experimental groups showed more severe withdrawal and higher intake than the same genotype control groups). If one assumes that negative reinforcement (i.e., alleviation of withdrawal) underlies the positive environmental correlation based on the group differences, the negative phenotypic correlation observed in HAP-2/LAP-2 mice might be explained by detrimental effects of withdrawal-induced physical impairment on opportunities to experience the contingency between S+ licking and ethanol infusion. In other words, the overall impact of negative reinforcement on ethanol intake might have been reduced for the most highly impaired HAP-2/LAP-2 mice simply because they experienced withdrawal alleviation less often than less impaired mice. By this account, differences in the direction of the phenotypic correlation are explained by genetic differences in the ability of acute withdrawal to interfere with approach toward and licking of the S+ tube (i.e., HAP-2/LAP-2 mice are affected more than B6/D2 mice). Similar response interference mechanisms might contribute to the negative genetic correlation between oral ethanol intake and sensitivity to ethanol withdrawal. Additional studies that examine the genetic and phenotypic relationship between withdrawal severity and IGAC in other genotypes, including other replicates of the HAP/LAP selection, are needed to evaluate the generality of these findings.

Although emphasis has been placed on the potential predictive relationship between withdrawal and later ethanol intake, consideration must also be given to the significant positive correlations between intoxication and ethanol intake (Table 8). If one interprets the intoxication measure as a general index of ethanol sensitivity, the direction of this correlation appears contrary to what would be predicted on the basis of the literature suggesting that a low level of response to ethanol is a risk factor for alcohol dependence (Crabbe, Bell & Ehlers, 2010). That literature would suggest that more highly intoxicated individuals (or genotypes) should be *less* likely (not more likely) to have high ethanol intakes. Given that prediction and the general difficulty in finding intoxication measures in rodents that model low response to alcohol (Crabbe et al., 2010), we believe that the correlations between intoxication and IGAC observed here are better interpreted as byproducts of the strong correlation between our intoxication measure and withdrawal severity. Indeed, the correlations based on group medians from all three studies were +0.61 ($n = 12$, $p < .05$) in B6 mice and +0.94 ($n = 12$, $p < .0001$) in D2 mice; correlations based on individual scores were +0.33 ($n = 12$, $p < .001$) in B6 mice and +0.48 ($n = 12$, $p < .0001$) in

D2 mice. In other words, mice that showed higher intoxication scores tended to also show more severe withdrawal, which provides the motivational substrate for enhancing ethanol intake via negative reinforcement.

Although no tolerance assessments were made, it is reasonable to suggest that passive ethanol exposure induced tolerance to one or more ethanol effects in these studies. Furthermore, tolerance would likely be influenced by variations in cumulative ethanol dose or periodicity of ethanol exposure (Kalant, LeBlanc & Gibbins, 1971). Thus, consideration must be given to the possibility that tolerance contributed to the effects of passive ethanol exposure on IGAC. For example, ethanol intake might be enhanced by development of tolerance to ethanol's motor inhibitory effects. Alternatively, tolerance to ethanol's post-absorptive aversive effects might interfere with development of conditioned taste aversion to S+ (Risinger & Cunningham, 1995). However, although tolerance to ethanol's inhibitory or aversive effects might explain increased ethanol intake or a weaker S+ aversion, tolerance cannot readily explain the development of an absolute S+ preference (i.e., preference ratio > 0.5) in the D2 10-day group or as previously reported for B6, HAP-2 and LAP-2 ethanol-treated mice (Fidler et al., 2011). Tolerance to negative effects would be expected to reduce S+ aversion, presumably leading to indifference in the selection of S+ and S- (i.e., S+ preference ratio = 0.5). However, an additional mechanism, such as negative reinforcement via alleviation of withdrawal, is needed to explain S+ preference ratios above 0.5.

Previously, we suggested that the effects of passive ethanol exposure on IGAC might be jointly determined by tolerance-induced reduction in ethanol's post-absorptive aversive effects and by negative reinforcement through alleviation of withdrawal (Fidler et al., 2011). Tolerance to post-absorptive aversive effects might be especially important in allowing ethanol-avoiding genotypes (e.g., D2) to administer sufficient ethanol to experience negative reinforcement. On the basis of the literature and current data, we further hypothesize that the relative contributions of tolerance and negative reinforcement might vary across genotypes. For example, in light of previous studies showing that B6 mice develop greater tolerance than D2 mice to ethanol's hypothermic (Crabbe et al., 1982) and taste-aversion-inducing (Risinger & Cunningham, 1995) effects, tolerance might be more importantly involved in enhancing IGAC in B6 mice than in D2 mice. Conversely, given data suggesting a stronger relationship between withdrawal severity and IGAC in D2 mice, negative reinforcement might play a more important role for D2 than for B6 mice. As noted earlier, it is also possible that D2 mice show greater negative reinforcement based on alleviation of acute physical signs of withdrawal whereas B6 mice are influenced more by alleviation of dependence-induced affective changes (e.g., anxiety) during protracted abstinence. Future studies should address these hypotheses in B6 and D2 mice as well as in other inbred and selectively bred genotypes.

When interpreting and comparing results from the IGAC model to findings from other models, it is important to note that dependence induced enhancement of ethanol intake has frequently been reported with delays of several days or weeks of abstinence between onset of withdrawal and the assessment of ethanol intake (e.g., Roberts et al., 2000; Lopez & Becker, 2005; Sommer et al., 2008). Thus, in contrast to the present studies, those studies offered no opportunity for ethanol intake to alleviate the acute physical symptoms of withdrawal that wax and wane during the first 24–48 hrs after removal of ethanol. Consequently, it has been suggested that enhanced ethanol intake after such delays are mediated by alleviation of more protracted effects of withdrawal on negative affective states (e.g., Roberts et al., 2000; Becker, 2008; Heilig et al., 2010). Currently, it is not known whether withdrawal-related increases in IGAC depend on access to ethanol and withdrawal alleviation during the initial acute phase of withdrawal. Ongoing studies in our laboratory are designed to assess this issue.

In summary, the present studies provide a more comprehensive analysis of the quantitative relationship between chronic ethanol exposure and later ethanol intake than any previous set of studies. Across all three studies, two variables were strong predictors of increased IGAC in both strains: cumulative ethanol dose and intoxication during passive exposure. However, a high cumulative dose was not sufficient to induce an increase in IGAC unless the intermittently administered unit doses were large enough to produce intoxication and withdrawal. In D2 mice, withdrawal during passive ethanol exposure was also a strong predictor of increased IGAC. However, B6 mice showed little withdrawal in these studies, precluding analysis of its potential role. Overall, these data support the hypothesis that dependence-induced increases in IG ethanol intake and S+ preference are jointly determined by two processes that might vary across genotypes: (a) tolerance to aversive post-absorptive ethanol effects, and (b) negative reinforcement (i.e., alleviation of withdrawal by self-administered ethanol).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

No-Choice Phase (columns 1 and 2): Mean (+ SEM) ethanol intake for B6 (1st column of panels) and D2 (2nd column of panels) groups of mice in Experiments 1 (top row), 2 (middle row) and 3 (bottom row). In each case, group names refer to treatment during the passive infusion phase. Separate ANOVAs for each experiment yielded significant effects of Strain, Group and their interaction. Post-hoc tests showed that each B6 group self-infused more ethanol than the corresponding D2 group. Within strain group differences for each experiment are shown on the figure. *Choice Phase* (columns 3 and 4): Mean (+ SEM) ethanol intake for B6 (3rd column of panels) and D2 (4th column of panels) groups of mice in Experiments 1 (top row), 2 (middle row) and 3 (bottom row). In each case, group names refer to treatment during the passive infusion phase. Separate ANOVAs for each experiment yielded significant effects of Strain and Group. The interaction was significant only in Experiment 1. B6 mice in the Water, 6-, and 9-inf groups self-infused more than their corresponding D2 groups. Within-strain group differences are shown on the figure.

Figure 2.

Mean (+SEM) S+ preference ratio during the choice phase for B6 (1st column of panels) and D2 (2nd column of panels) groups of mice in Experiments 1 (top row), 2 (middle row) and 3 (bottom row). In each case, group names refer to treatment during the passive infusion phase. The S+ preference ratio = S+ licks / Total licks. Experiment 1 ANOVA yielded significant effects of Strain, Group and their interaction. Experiment 2 ANOVA revealed only a group effect. Experiment 3 ANOVA revealed an effect of group and a strain by group interaction. Within-strain group differences are shown on the figure. Asterisks indicate groups that are significantly different from neutral (ratio = 0.5) in one-group t-tests.

Figure 3. Scatter plot depicting the relationship between group mean choice-phase ethanol intakes and group median withdrawal scores (HICs) in D2 mice.

Figure 4.

Scatter plot showing the relationship between BEC (mg/ml) and choice ethanol-infusion intake (g/kg) during the 180 min before each sample was taken from mice at the 30-min self-infusion dose limit. Data reflect a subset of mice in Experiments 1 and 2 (see Method).

Table 1

Passive Infusion Phase Parameters

Group (n_{B6} , n_{D2})	Days of Passive EtOH Infusions	Number of Infusions/Day	Passive Day 1 Dose/Infusion (g/kg)	Daily Dose Increase per Infusion (g/kg)	Dose/Infusion on Final Day (g/kg)
Experiment 1					
Water (13, 14)	0	9	0	0	0
3-inf (14, 14)	5	3	3.0	0.50	5
6-inf (14, 14)	5	6	1.5	0.25	2.50
9-inf (13, 12)	5	9	1.0	0.17	1.67
Experiment 2					
Water (10, 12)	0	3	0	0	0
Low (12, 10)	5	3	2	0.5	4
Medium (12, 12)	5	3	3	0.5	5
High (12, 11)	5	3	4	0.5 ^a	5.5 or 6.0
Experiment 3					
Water (13, 12)	0	3	0	0	0
3-day (13, 12)	3	3	3	0.5	4
5-day (12, 13)	5	3	3	0.5	5
10-day (12, 12)	10	3	3	D1–5 = 0.5; D6, 8, 10 = 0.25; D7, 9 = 0	5.75
Experiment 4					
Day 1 (7, 5)	1	3	3	-	3
Day 5 (5, 7)	5	3	3	0.5	5

Note. Water control group infusions were matched in volume and timing to those of the 9-inf, High and 10-day groups in Experiments 1–3, respectively. The ethanol exposure received by the 3-inf, Medium and 5-day groups was identical. The groups in Experiment 4 were exposed to the Medium treatment for either 1 (Day 1 group) or 5 (Day 5 group) days before blood samples were collected; no self-infusion data were collected.

^a Dose was increased on passive infusion days 2–5 for 3 mice and on days 2–4 for all remaining mice (see text).

Table 2

ANOVA Summaries for No-Choice and Choice Phase Ethanol Self-Infusion Intakes

	No-Choice Phase	Choice Phase
Experiment 1		
Strain	F(1,100) = 369.3 ****	F(1,100) = 101.0 ****
Group	F(3,100) = 25.5 ****	F(3,100) = 12.2 ****
Strain × Group	F(3,100) = 3.1 *	F(3,100) = 3.6 *
Experiment 2		
Strain	F(1,86) = 193.3 ****	F(1,83) = 49.3 ****
Group	F(3,86) = 22.3 ****	F(3,83) = 15.0 ****
Strain × Group	F(3,86) = 2.8 *	ns
Experiment 3		
Strain	F(1,94) = 226.0 ****	F(1,91) = 63.6 ****
Group	F(3,94) = 59.4 ****	F(3,91) = 26.4 ****
Strain × Group	F(3,94) = 2.9 *	ns

* p < .05.

**** p < .0001.

ns = p > .05.

Table 3

Mean ($\pm$ SEM) number of ethanol bouts/day during choice self-infusion

<u>Experiment 1^a</u>		
Group	B6 (n)	D2 (n)
Water	31.42 $\pm$ 3.56 ^b (13)	0.16 $\pm$ 0.11 ^b (14)
3-inf	38.67 $\pm$ 2.94 ^b (14)	23.49 $\pm$ 3.30 ^{b,c} (14)
6-inf	41.96 $\pm$ 2.52 ^b (14)	6.57 $\pm$ 2.42 ^b (14)
9-inf	40.42 $\pm$ 2.37 ^b (13)	5.97 $\pm$ 2.58 ^b (12)
<u>Experiment 2^d</u>		
Group	B6 (n)	D2 (n)
Water	31.36 $\pm$ 4.41 (10)	0.77 $\pm$ 0.38 (12)
Low	34.43 $\pm$ 3.24 (12)	14.34 $\pm$ 3.72 (10)
Medium	37.92 $\pm$ 2.74 (12)	16.33 $\pm$ 3.43 (12)
High	37.67 $\pm$ 2.74 (12)	18.51 $\pm$ 4.17 (11)
<u>Experiment 3^e</u>		
Group	B6 (n)	D2 (n)
Water	34.00 $\pm$ 1.8 ^f (13)	0.50 $\pm$ 0.27 ^f (12)
3-day	35.82 $\pm$ 1.53 ^f (13)	14.40 $\pm$ 2.65 ^{f,g} (12)
5-day	37.52 $\pm$ 1.98 ^f (12)	20.34 $\pm$ 3.01 ^{f,g} (13)
10-day	38.02 $\pm$ 2.00 ^f (12)	21.28 $\pm$ 3.60 ^{f,g} (12)

^a Significant effects of Strain, Group and interaction (p's < .0001)

^b Differs from same group for other Strain (p's < .002)

^c Differs from other groups of the same strain (p's < .0001)

^d Significant effects of Strain and Group (p's < .002), but no interaction

^e Significant effects of Strain, Group and interaction (p's < .002).

^f Differs from same group for other Strain (p's < .02)

^g Differs from water group of the same strain (p's < .02)

Table 4

Mean (± SEM) Ethanol Bout Size During Choice Self-Infusion (g/kg/bout)

Group	B6 (n)	D2 (n)
Experiment 1^a		
Water	0.35 ± 0.02 (13)	0.61 ± 0.05 (4)
3-inf	0.39 ± 0.03 (14)	0.54 ± 0.07 (13)
6-inf	0.36 ± 0.02 (14)	0.55 ± 0.06 (10)
9-inf	0.33 ± 0.02 (13)	0.61 ± 0.09 (10)
Experiment 2^{ab}		
Water	0.29 ± 0.03 (10)	0.53 ± 0.08 (5)
Low	0.36 ± 0.03 (12)	0.63 ± 0.12 (10)
Medium	0.41 ± 0.03 (12)	0.72 ± 0.07 (12)
High	0.53 ± 0.03 (12)	0.65 ± 0.07 (10)
Experiment 3^{ac}		
Water	0.27 ± 0.02 (13)	0.49 ± 0.09 (8)
3-day	0.37 ± 0.02 (13)	0.46 ± 0.04 (11)
5-day	0.39 ± 0.03 (12)	0.51 ± 0.03 (13)
10-day	0.49 ± 0.02 (12)	0.62 ± 0.05 (12)

Note. Means exclude mice with no ethanol bouts

^aMain effect of Strain, p < .0001

^bMain effect of Group, p < .05

^cMain effect of Group, p < .0001

Table 5

Mean ($\pm$ SEM) Percentage of Total Choice Phase Ethanol Intake in Different Size Bouts

Small Bouts (≤ 0.5 g/kg)			Medium Bouts (0.5–0.99 g/kg)			Large Bouts (≥ 1.0 g/kg)		
B6	D2	Experiment 1 ^a	B6	D2	Experiment 1 ^{abc}	B6	D2	Experiment 1 ^a
Water	54.2 $\pm$ 4.0	4.4 $\pm$ 4.4	Water	37.0 $\pm$ 3.2 ^d	79.9 $\pm$ 20.1 ^d	Water	8.8 $\pm$ 1.9	15.7 $\pm$ 15.7
3-inf	44.0 $\pm$ 3.9	25.4 $\pm$ 3.0	3-inf	37.3 $\pm$ 2.0	44.6 $\pm$ 4.1 ^e	3-inf	18.7 $\pm$ 3.0	30.0 $\pm$ 6.7
6-inf	53.8 $\pm$ 4.2	26.3 $\pm$ 9.3	6-inf	35.0 $\pm$ 2.9	33.2 $\pm$ 9.0 ^e	6-inf	11.3 $\pm$ 2.5	40.5 $\pm$ 9.6
9-inf	55.5 $\pm$ 3.6	24.6 $\pm$ 9.6	9-inf	34.1 $\pm$ 3.1	38.4 $\pm$ 9.9 ^e	9-inf	10.4 $\pm$ 1.6	37.0 $\pm$ 11.2
Experiment 2 ^{ab}			Experiment 2			Experiment 2 ^{ab}		
Water	62.8 $\pm$ 4.1	25.1 $\pm$ 2.2	Water	33.6 $\pm$ 2.9	35.7 $\pm$ 2.0	Water	3.6 $\pm$ 1.3	39.2 $\pm$ 3.8
Low	52.4 $\pm$ 4.0	27.2 $\pm$ 18.4	Low	35.8 $\pm$ 2.5	34.7 $\pm$ 18.1	Low	11.8 $\pm$ 1.9	38.1 $\pm$ 16.6
Medium	44.1 $\pm$ 4.6	26.8 $\pm$ 7.4	Medium	35.4 $\pm$ 1.7	31.6 $\pm$ 6.5	Medium ^f	20.5 $\pm$ 3.8	41.6 $\pm$ 9.6
High ^g	25.1 $\pm$ 2.2	15.5 $\pm$ 3.2	High	35.7 $\pm$ 2.0	23.6 $\pm$ 4.7	High ^g	39.2 $\pm$ 3.8	60.9 $\pm$ 7.8
Experiment 3 ^{ab}			Experiment 3			Experiment 3 ^{ab}		
Water	66.9 $\pm$ 4.7	44.7 $\pm$ 17.6	Water	26.2 $\pm$ 3.3	47.0 $\pm$ 16.5	Water	6.9 $\pm$ 1.9	8.3 $\pm$ 8.3
3-day	49.1 $\pm$ 4.6	34.1 $\pm$ 7.5	3-day	35.8 $\pm$ 2.5	34.9 $\pm$ 4.9	3-day ^f	15.1 $\pm$ 2.8	31.0 $\pm$ 6.8
5-day ^f	44.7 $\pm$ 4.5	27.4 $\pm$ 4.7	5-day	36.6 $\pm$ 2.8	35.5 $\pm$ 4.1	5-day ^f	18.7 $\pm$ 2.5	37.2 $\pm$ 6.9
10-day ^{fh}	30.7 $\pm$ 2.2	18.7 $\pm$ 3.7	10-day	39.9 $\pm$ 1.5	35.5 $\pm$ 3.5	10-day ^{fh}	29.4 $\pm$ 2.5	45.8 $\pm$ 6.3

Note: Group sizes are shown in Table 4. ANOVA summary tables are shown in Supplementary Table 4. Pair-wise comparisons were conducted only after a significant main effect or interaction.

^aMain effect of Strain, largest $p < .01$

^bMain effect of Group, largest $p < .01$

^cStrain $\times$ Group interaction, $p < .01$

^ddiffers from same group for other strain

^ediffers from water group of the same strain

^fdiffers from Water group

^gdiffers from Low group

h_t differs from 3-day group

Table 6

Median (IQR) Daily Intoxication Rating During the Passive Phase

Strain			
	B6	D2	Mann-Whitney U
Experiment 1			
Water	0 (0) ^a	0 (0) ^a	ns
3-inf	1.8 (2) ^b	1.1 (1) ^b	49.0 [*]
6-inf	0 (0.2) ^a	0 (0.2) ^a	ns
9-inf	0 (0) ^a	0 (0.1) ^a	ns
Kruskal-Wallis H	34.9 ^{*****}	30.2 ^{*****}	
Experiment 2			
Water	0 (0) ^c	0 (0) ^b	ns
Low	0 (0.2) ^c	0.2 (.4) ^b	39.0 [*]
Medium	1.1 (1.4) ^b	0.9 (1.1) ^b	ns
High	3.4 (2.3) ^b	2.2 (1) ^b	29.5 [*]
Kruskal-Wallis H	35.2 ^{*****}	33.8 ^{*****}	
Experiment 3			
Water	0 (0) ^b	0 (0) ^b	ns
3-day	1.7 (2) ^d	0.7 (0.8) ^b	ns
5-day	2.2 (1.4) ^d	1.8 (0.8) ^e	ns
10-day	4.0 (1.8) ^b	2.0 (1.2) ^e	13.0 ^{***}
Kruskal-Wallis H	40.9 ^{*****}	35.3 ^{*****}	

Note: Group sizes are shown in Table 3. Ratings were obtained only on days that ethanol groups were passively infused with ethanol. Values for H and U are corrected for tied scores. IQR = interquartile range.

- ^a differs from 3-inf group in the same strain
- ^b differs from all other groups in the same strain
- ^c differs from Medium and High groups in the same strain
- ^d differs from Water and 10-day groups in the same strain

^e differs from Water and 3-day groups in the same strain

* p < .05.
 *** p < .001.
 **** p < .0001.
 ns = p > .05.

Table 7
Median (IQR) Daily Withdrawal Rating (Handling Induced Convulsions) During the Passive Phase

Strain			
	B6	D2	Mann-Whitney <i>U</i>
Experiment 1			
Water	0 (0)	0 (0) ^a	ns
3-inf	0 (0)	2.5 (1.2) ^a	0.0 *****
6-inf	0 (0)	0.1 (1) ^b	49.0 **
9-inf	0 (0)	0.2 (0.6) ^b	39.0 **
Kruskal-Wallis <i>H</i>	ns	36.1 *****	
Experiment 2			
Water	0 (0)	0 (0) ^a	ns
Low	0 (0)	0.2 (0.6) ^a	36.0 **
Medium	0 (0)	1.6 (1.5) ^a	13.0 *****
High	0 (0.6)	2.7 (2) ^a	0.0 *****
Kruskal-Wallis <i>H</i>	12.0 **	30.5 *****	
Experiment 3			
Water	0 (0) ^c	0 (0) ^a	ns
3-day	0 (0) ^c	1.3 (3.2) ^d	6.5 *****
5-day	0 (0) ^c	2.0 (0.9) ^d	7.0 *****
10-day	0.1 (0.7) ^a	3.7 (1.6) ^d	0.0 *****
Kruskal-Wallis <i>H</i>	19.9 ***	29.5 *****	

Note: Group sizes are shown in Table 3. Ratings were obtained only on days that ethanol groups were passively infused with ethanol. Values for H and U are corrected for tied scores. IQR = interquartile range.

^a differs from all other groups in the same strain

^b differs from Water and 3-inf group in the same strain

^c differs from the 10-day group in the same strain

^d differs from Water group in the same strain

$p < .01$.

 $p < .001$.

 $p < .0001$.

 $ns = p > .05$.

Table 8
Correlations Between IGAC and Intoxication/Withdrawal Based on Group Means/Medians

	Median Intoxication		Median Withdrawal	
	B6	D2	B6	D2
Mean No-Choice Ethanol Intake	+0.83 *	+0.75	-	+0.78 *
Mean Choice Ethanol Intake	+0.80 *	+0.86 *	-	+0.90 *
Mean S+ Preference	+0.67	+0.83 *	-	+0.88 *

Note: Pearson r was calculated for groups from all three experiments (n = 12). Correlations with withdrawal were not calculated for B6 groups because most (8/9) group medians were zero. Alpha for each correlation was set at .005 to compensate for the large number of correlations.

* p < .005

Table 9

Correlations Between Cumulative Passive Ethanol Exposure and IGAC

	B6	D2
Mean No-Choice Ethanol Intake	+0.90*	+0.86*
Mean Choice Ethanol Intake	+0.87*	+0.79*
Mean S+ Preference	+0.75*	+0.79*

Note: Pearson r was calculated using all group means from all three experiments (n = 12). Alpha for each correlation was set at .005 to compensate for the large number of correlations.

* p < .005