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Oral operant ethanol self-administration in the absence of explicit cues, food restriction, water restriction and ethanol fading in C57BL/6J mice

Alexandra M. Stafford, Shawn M. Anderson, Keith L. Shelton, and Darlene H. Brunzell

Department of Pharmacology and Toxicology, Virginia Commonwealth University, 410 N 12th St., Richmond, VA 23298

Abstract

Rationale—Mouse models of EtOH self-administration are useful to identify genetic and biological underpinnings of alcohol use disorder.

Objectives—These experiments developed a novel method of oral operant EtOH self-administration in mice without explicitly paired cues, food/water restriction, or EtOH fading.

Methods—Following magazine and lever training for 0.2% saccharin (SAC), mice underwent 9 weekly overnight sessions with lever pressing maintained by dipper presentation of 0, 3, 10 or 15% EtOH in SAC or water vehicle. *Ad libitum* water was available from a bottle.

Results—Water vehicle mice ingested most fluid from the water bottle in contrast to SAC vehicle mice, which despite lever pressing demands, drank most of their fluid from the liquid dipper. Although EtOH in SAC vehicle mice showed concentration-dependent increases of g/kg EtOH intake, lever pressing decreased with increasing EtOH concentration and did not exceed that of SAC vehicle alone at any EtOH concentration. Mice reinforced with EtOH in water ingested less EtOH than mice reinforced with EtOH in SAC. EtOH in water mice, however, showed concentration-dependent increases in g/kg EtOH intake and lever presses. 15% EtOH in water mice showed significantly greater levels of lever pressing than water vehicle mice and a significant escalation of responding across weeks of exposure. Naltrexone pretreatment reduced EtOH self-administration and intake in these mice without altering responding in the vehicle control condition during the first hour of the session.

Conclusions—SAC facilitated EtOH intake but prevented observation of EtOH reinforcement. Water vehicle unmasked EtOH's reinforcing effects.

Keywords

alcohol; EtOH; operant; self-administration; mouse; reinforcement; mice; reward; naltrexone; preference

Communicating Author: Darlene H. Brunzell, Ph.D., Phone: 804-628-7584, Fax: 804-828-2117, dbrunzell@vcu.edu.

Conflicts of Interest

The authors have no conflicts of interest to disclose.

Introduction

Alcohol abuse is a pervasive problem worldwide (WHO 2011). Genetics play a major role in vulnerability to alcohol use disorder (AUD) (Schuckit and Smith 1996; Prescott and Kendler 1999; Gillespie et al. 2012), and understanding the molecular mechanisms that underlie ethanol (EtOH) use phenotype may lead to novel treatment and prevention of AUD and alcoholism. Alcohol consumption is motivated by environmental and psychosocial factors that are difficult to control in human experiments; hence, animal models are ideal for isolating biological and environmental factors which contribute to behaviors that promote EtOH use.

Mouse EtOH consumption models are commonly used to investigate the genetic and pharmacological mechanisms of EtOH endophenotypes (Tabakoff and Hoffman 2000; Rhodes and Crabbe 2003). In mice, EtOH ingestion is typically measured using bottle choice paradigms or drinking-in-the-dark (DID) (Ryabinin et al. 2003; Rhodes et al. 2005). These non-operant self-administration models assess EtOH preference compared to a vehicle solution and achieve high levels of EtOH intake, but have been criticized as being less effective at assessing EtOH reinforcement (Tabakoff and Hoffman 2000). Operant self-administration paradigms measure the ability of positive reinforcers (e.g. EtOH) to increase the likelihood that a human or animal subject will exert effort to obtain the reinforcer.

Operant self-administration measures in rats (e.g. van Erp and Miczek 2007; Cannady et al. 2013; Doyon et al. 2013; Augier et al. 2014) and mice (e.g. Elmer et al. 1986; Samson 1986; Risinger et al. 1998; Middaugh et al. 1999a; Cunningham et al. 2000; Ford 2014) often utilize strategies such as food and water restriction to promote operant EtOH self-administration, which may introduce factors other than EtOH reinforcement (e.g. thirst, caloric intake). Gradual fading of sweetener (from high to low concentrations) and EtOH (from low to high concentrations) mimics the evolution of human patterns of alcoholic drink preference (Duncan et al. 2012) and has demonstrated success in promoting EtOH self-administration in mice (e.g. Elmer et al. 1986; Risinger et al. 1998; Middaugh et al. 1999a). Other models provide EtOH in the home cages of rodents to facilitate operant EtOH self-administration (Rodd-Hendricks et al. 2002). From the perspective of understanding the biology of the progression of EtOH use, however, it would be advantageous to employ a procedure that enables independent observation of how EtOH dose and length of exposure might impact EtOH reinforcement and physiological measures. A between-subject design using vehicle controls would also be advantageous for studies assessing the effects of EtOH self-administration on neuroplasticity. Another advantage of a between-subject design is that initial sensitivity to EtOH-associated sedation and reward (i.e. “liking”), which are predictive of heavy drinking and escalation of EtOH use in humans (King et al. 2011; King et al. 2014; Schuckit and Smith 1996), may be assessed in mice during initial exposure to EtOH.

Environmental factors such as sweeteners and cues are physiologically relevant to promoting EtOH administration in humans (O'Connor and Colder 2009; Garland et al. 2012; Petit et al. 2013; Dager et al. 2013; Sjoerds et al. 2014; Dager et al. 2014) and hence are of interest to study in rodent models in a controlled fashion. Explicit cues and flavorants

become secondary reinforcers when paired with drug (Brunzell et al. 2006; Browne et al. 2014), and may have reinforcing effectiveness on their own in rodents (Olsen and Winder 2009; Regier et al. 2012; Browne et al. 2014). The development of an EtOH self-administration model in the absence of contingent sweeteners and cue presentation would facilitate isolation of biological factors which drive the primary reinforcing effects of EtOH in the absence of cues and sweeteners. The present studies controlled for sweetener that was paired with EtOH by providing EtOH in water or saccharin solution using a weekly overnight mouse model of oral operant EtOH self-administration that did not involve explicit cues, food restriction, water restriction or the gradual fading of EtOH. The availability of a water bottle in the operant conditioning chamber further enabled comparison of water bottle and liquid dipper intake in order to assess the potential rewarding properties of EtOH under these conditions when compared against vehicle control subjects.

Materials and Methods

Subjects

Fifty-four, adult, male, C57BL/6J mice (Jackson Labs, Bar Harbor, ME) aged 14-17 weeks at the initiation of training were used for this study. Mice were group-housed (4-5 per cage) in a temperature- and humidity-controlled vivarium. They were housed under a 12 h light/dark schedule (lights on at 0600 h) and had *ad libitum* access to food and water. Experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University and were in accordance with the Guidelines for the Care and Use of Laboratory Animals, as set forth by the National Institutes of Health.

Apparatus

Operant EtOH self-administration procedures were conducted in mouse operant conditioning chambers (21.6 cm × 17.8 cm × 12.7 cm; Med Associates, St. Albans, VT) housed inside sound-attenuating cabinets with a ventilation fan. Each chamber was equipped with two retractable levers placed 2.5 cm above the floor. One lever, designated active, resulted in presentation of a liquid dipper that provided 0.01 ml of fluid; the other lever, designated inactive, had no consequence when depressed. The liquid dipper was located within a magazine equidistant between the two levers and equipped with a photobeam sensor to record head entries into the magazine during the presence or absence of the liquid dipper presentation. A 100 mA house light, located 11 cm above the floor on the opposite wall, was on during the session. A water bottle with sipper tube provided *ad libitum* access to water during EtOH self-administration sessions and food pellets were placed on the floor. Med PC IV software and Med-Associates interfacing controlled liquid dipper presentations and recorded active and inactive lever pressing, liquid dipper reinforcers earned, and magazine entries during the presence (correct entry) and absence (incorrect entry) of liquid dipper presentation. Data were collected in 15 min time bins.

Drugs

EtOH was diluted in tap water or 0.2% saccharin in tap water (SAC) and provided to mice for voluntary oral intake. Naltrexone was diluted in 0.9% sterile saline and administered i.p.

at a volume of 0.1 ml per 30 g mouse immediately prior to overnight EtOH self-administration sessions.

Magazine Training

Mice received eighty liquid dipper presentations of 0.01 mL of SAC solution on a variable time 30-second schedule (13-47 s range). Mice were trained to a criterion at least 20% magazine entry during dipper presentations or for up to three training sessions. Magazine training took place during the light cycle between 1300 and 1500 h.

Acquisition of lever pressing

Mice next underwent acquisition of lever pressing in the absence of EtOH to assure that all groups of mice demonstrated reliable lever pressing and goal tracking behavior prior to their first exposure to EtOH. Mice were trained during 16 h overnight sessions to lever press for SAC dipper presentations. Sessions began between 1700 and 1800 h. Active lever pressing for SAC was maintained on FR1 for the first 20 reinforcers, FR2 for the 20 subsequent reinforcers, and FR4 for 10 subsequent reinforcers. For the remainder of the session, the FR4 schedule was shifted to a variable ratio (VR) 5 schedule (1-12 range). Training continued for 2-5 sessions until animals pressed the active lever at least 40 times and showed at least 100 s of head entry into magazine during fluid dipper presentation.

Weekly, overnight EtOH Sessions

After meeting training criteria, mice underwent 16 h overnight operant self-administration sessions once every 7 days for 9 weeks. Individual mice lever pressed on a VR5 schedule of reinforcement maintained by 0%, 3%, 10%, or 15% EtOH (v/v). Independent groups of mice were reinforced with EtOH in SAC vehicle ($n = 4-5/\text{dose}$) or tap water vehicle ($n=5-9/\text{dose}$). Due to limited availability of operant conditioning chambers, each experiment was completed in 3-4 replicates. A subset of mice reinforced with EtOH in water were subsequently administered 1.25 mg/kg i.p. naltrexone or vehicle (weeks 10-11) in a counterbalanced order prior to EtOH self-administration. These tests were subsequently repeated with 0.3 mg/kg i.p. naltrexone or vehicle (weeks 12-13). These naltrexone doses have been previously shown to decrease rodent operant self-administration of EtOH (Middaugh et al. 2000, Hay et al. 2013). Performance following the two saline sessions was averaged for analysis. Self-administration measures and EtOH intake were assessed during the first hour of the session, when peak naltrexone levels were most likely achieved (Wang et al. 2004). For all experiments, estimated drinking from the liquid dipper was calculated by multiplying the number of correct head entries (magazine head entry at the time of liquid dipper presentation) by 0.01 ml. Total EtOH dose consumed was estimated by multiplying intake volume by the EtOH concentration available for self-administration. The estimated total dose of EtOH self-administered was correlated with blood EtOH concentrations (BEC) on subsequent weeks upon completion of behavioral testing. Water bottle fluid intake was determined by measuring bottle weights immediately before and after each session. A separate dummy water bottle, located inside of the sound-attenuating cabinet, was weighed before and after each session to correct for water bottle drippage. Reinforcers earned and active lever presses provided measures of reinforcement. Response accuracy was determined by the percentage of active lever presses: $\text{active}/(\text{active} + \text{inactive lever presses})$. Bouts of

responding were evaluated during the overnight session using a skewness equation below, where x = each individual time bin value in the sample, x_i = the average of the time bin values, n = sample size, and s = standard deviation of the sample. The percentage of fluid intake from the liquid dipper (Operant:*Ad libitum* choice) was calculated by comparing estimates of liquid dipper intake to total fluid intake: liquid dipper fluid intake/(water bottle + liquid dipper fluid intake).

$$\text{Skewness} = \frac{n}{(n-1)(n-2)} \sum \left(\frac{x_i - x}{s} \right)^3$$

Blood Ethanol Concentration Analysis

For validation of the g/kg EtOH estimate, trunk blood was collected after 30 min of self-administration from a separate group of mice reinforced with 15% EtOH in SAC ($n=4$). Additionally, blood samples were collected at 4 h or 6 h into the session from a subset of mice reinforced with 0%, 3%, 10%, or 15% EtOH in water ($n=5/\text{dose}$) and 15% EtOH in SAC ($n=4$) by submandibular sampling using 5 mm Goldenrod animal lancets (MEDIpoint, Mineola, NY). Samples were collected into BD Microtainer sampling tubes containing EDTA and 50 μl whole blood aliquots were immediately pipetted into 20 ml headspace gas chromatography vials containing deionized water, 500 mg NaCl and 1-propanol internal standard. Sample vials were tightly sealed and stored at -20°C until analysis. Blood samples were tested for EtOH concentration using an Agilent model 6890 gas chromatograph (GC) equipped with a flame ionization detector (FID), 0.53mm ID Rtx BAC-1 capillary column (Restek, Bellefonte, PA) and CTC CombiPal headspace autosampler (Leap Technologies, Carrboro, NC). Samples were incubated and shaken for 10 min at 70°C prior to automated injection. The GC parameters were: 1 ml headspace injection volume, 2/1 split ratio, 5 min sample run time, injector temperature 200°C , oven temperature isothermal 50°C , detector temperature 200°C , helium carrier gas flow rate 40 ml/min, nitrogen makeup gas flow rate 18 ml/min, hydrogen flame flow rate 25 ml/min and FID air flow rate 300 ml/min. Data were collected and analyzed by Clarity GC software (Apex Data Systems, Prague, CZ) using a linear regression analysis with no weighting. EtOH concentrations were calculated by the internal standard method. A 7 point calibration curve preceded the analysis of blood EtOH concentrations. Quality control EtOH standards at concentrations similar to those found in the test samples were interspersed at regular intervals with blood samples.

Statistical analysis

Statistical analyses assessed reinforcers earned, active lever presses, percent active lever presses, EtOH consumed, skewness and Operant:*Ad libitum* choice using mixed $2 \times 4 \times 9$ (Water/SAC Vehicle x EtOH concentration x Session) ANOVA's with vehicle and EtOH concentration as between-subject factors and weekly session as a repeated measure, within-subject factor. Separate mixed 3×4 (Naltrexone dose x EtOH concentration) ANOVA's assessed reinforcers earned, active lever presses, and EtOH consumed following naltrexone administration with naltrexone dose as a within-subject factor and EtOH concentration as a between-subject factor. Significant interactions that included vehicle were followed by

independent ANOVA analysis for SAC and water vehicle mice and pairwise comparisons across vehicle groups where relevant. Significant main effects were further assessed using Dunnett's post hoc tests; significant interactions were assessed using two-tailed t-tests. Planned comparisons compared session 1 to session 9 across EtOH concentrations. Estimates of EtOH intake were compared against blood EtOH concentrations using a two-tailed Pearson correlation. SPSS software was used for all statistical analyses.

Results

Operant self-administration of EtOH in SAC versus water vehicle

There was no significant difference between groups of mice on measures of reinforcers earned, active lever pressing, response accuracy or head entries during dipper presentation for SAC prior to receiving any EtOH (Online Resource 1). For EtOH self-administration, there was a significant interaction of vehicle x EtOH concentration x session for reinforcers earned ($F_{24,336}=4.635$, $p<0.001$) and active lever presses ($F_{24,336}=4.525$, $p<0.001$). Across EtOH sessions, mice that received EtOH in SAC vehicle showed concentration-dependent changes in EtOH reinforcement and intake as measured by a significant interaction of session x EtOH concentration for reinforcers earned ($F_{24,112}=1.701$, $p<0.05$) and active lever presses ($F_{24,112}=1.768$, $p<0.05$) (Fig. 1). Animals receiving SAC vehicle and 3% EtOH in SAC, but not mice receiving higher concentrations of EtOH in SAC showed significant increases in reinforcers earned and active lever presses across sessions ($p's<0.05$). Of mice reinforced with EtOH in SAC, only mice reinforced with 15% EtOH differed on reinforcement measures from SAC vehicle controls. By the 9th week of EtOH exposure, mice receiving 15% EtOH in SAC earned significantly fewer reinforcers ($t_6=2.632$, $p<0.05$) and provided fewer lever presses ($t_6=2.544$, $p<0.05$) than animals receiving SAC vehicle. In contrast, mice reinforced with EtOH in water vehicle showed a main effect of EtOH concentration on reinforcers earned ($F_{3,28}=9.667$, $p<0.001$) and active lever presses ($F_{3,28}=9.045$, $p<0.001$), revealing that mice receiving 15% EtOH in water showed evidence of EtOH reinforcement as measured by significantly greater reinforcers earned and active lever presses compared to water vehicle control mice ($p's<0.001$) (Fig. 1). There was a significant interaction of session x EtOH concentration for reinforcers earned ($F_{24,224}=3.95$, $p<0.001$) and active lever presses ($F_{24,224}=3.79$, $p<0.001$) demonstrating that mice receiving 15% EtOH in water also showed an escalation of reinforcers earned ($t_8=-4.164$, $p<0.01$) and active lever presses ($t_8=-4.519$, $p<0.01$) across sessions. In the absence of SAC vehicle used during lever training, water, 3% and 10% EtOH mice showed significant decreases in lever pressing and reinforcers earned across sessions ($p's<0.05$). SAC vehicle mice made significantly more active lever presses and earned significantly more reinforcers than water vehicle mice ($p's<0.001$) (note y axis break for water vehicle mice), demonstrating that SAC was reinforcing and may have precluded observation of EtOH reinforcement in mice receiving SAC vehicle. Mice on average showed high levels of response accuracy, > 80%, but SAC vehicle mice achieved an overall higher level of response accuracy than water vehicle mice ($F_{1,32}=22.456$, $p<0.001$).

Intake of EtOH in SAC versus water vehicle

Estimates of g/kg EtOH intake were validated via positive correlation with BEC in a subgroup of mice reinforced with 15% EtOH in SAC ($r=0.959$; Online Resource 2). There was a significant main effect of EtOH concentration on g/kg EtOH consumed ($F_{2,31}=74.037$, $p<0.001$), indicating that higher concentrations of EtOH resulted in more g/kg EtOH consumed independent of session or vehicle. Reflective of more reinforcers earned and active lever presses, SAC vehicle mice ingested more g/kg EtOH than water vehicle mice ($F_{1,31}=113.575$, $p<0.001$), as measured by a main effect of vehicle on this measure (Fig. 2). A significant interaction of vehicle x EtOH concentration x session for EtOH intake ($F_{16,248}=4.656$, $p<0.001$) showed that SAC and water vehicle also differentially impacted EtOH intake across sessions. Consistent with significant increases in reinforcers earned and active lever presses, mice reinforced with 15% EtOH in water also showed a significant increase in g/kg EtOH consumed ($t_8=-3.383$, $p<0.05$) across EtOH sessions. In contrast, mice reinforced with 3% EtOH in water ($t_8=3.078$, $p<0.05$) and 10% EtOH in water ($t_4=6.383$, $p<0.01$) showed decreases in EtOH consumed across sessions that paralleled declines in reinforcers earned and active lever presses in these groups. To the contrary, SAC vehicle appeared to promote escalation of low dose EtOH intake as measured by increases in g/kg EtOH ingested by 3% EtOH mice on Session 9 compared to session 1. No differences of total fluid consumed or body weights were detected between groups (Table 1).

Assessing the accuracy of estimated g/kg EtOH consumption in mice

EtOH consumed was estimated from magazine head entry occurring only during liquid dipper presentation. Mice showed concentration-associated increases in BEC (Table 2). There was a significant correlation between estimates of EtOH consumption and BEC at both the 4 h ($r=0.773$, $p<0.001$) and 6 h ($r=0.652$, $p=0.001$) time points, to support that mice were drinking EtOH during magazine entries (Online Resource 2).

Bouts of responding as measured by skewness during overnight sessions

In order to assess patterns of responding, skewness of lever presses per 15 min time bin was calculated and averaged for each EtOH concentration group in SAC and water vehicle studies. As with reinforcers earned, active lever presses and g/kg EtOH intake, vehicle impacted skewness as indicated by a significant vehicle x EtOH concentration interaction ($F_{3,42}=9.547$, $p<0.001$) (Fig. 3). Skewness measures within-subject variability in responding so that a low skewness value indicates a steady pattern of lever pressing and a high skewness value captures bouts of lever pressing via identification of a pattern of responding that includes more extreme peaks and troughs (Online Resource 3). There was a main effect of EtOH concentration ($F_{3,14}=4.627$, $p<0.05$) and a significant interaction of EtOH concentration x session for skewness in mice receiving EtOH in SAC vehicle. Initial skewness scores reflected that mice showed similar patterns of lever pressing during Session 1 but 15% EtOH in SAC mice showed significantly more bouts of responding than SAC vehicle mice in the final session as measured by elevated skewness ($t_6=-3.414$, $p<0.01$) (Fig. 3). Mice receiving 3% and 10% EtOH did not differ from SAC controls.

In mice reinforced with EtOH in water, there was a main effect of EtOH concentration ($F_{3,28}=5.606$, $p<0.01$) for skewness (Fig. 3). Post hoc analysis revealed a significant

difference in skewness between mice receiving 15% EtOH and water vehicle subjects ($p<0.05$). Unlike mice receiving 15% EtOH in SAC, mice receiving 15% EtOH in water exhibited a *decrease* in skewness, or more stable responding than mice reinforced with water alone. The difference in pattern of responding between 15% EtOH in SAC and 15% EtOH in water mice may be due to the significantly greater g/kg EtOH consumed when 15% EtOH was delivered in SAC versus in water vehicle. There was an increase in skewness observed in water vehicle compared to SAC vehicle patterns of self-administration. This could be an artifact of time spent drinking from the *ad libitum* water bottle, as water vehicle mice, but not SAC vehicle mice, achieved most of their fluid intake from this alternative source.

Operant:Ad libitum fluid choice

Liquid dipper solution intake, which was dependent on lever pressing, was compared to *ad libitum* water bottle fluid intake to assess the rewarding properties of the vehicle and EtOH reinforcers in these experiments. Operant:Ad libitum choice was calculated as percentage of liquid dipper fluid intake compared to total fluid intake. There was a main effect of vehicle for this measure ($F_{1,42}=82.314$, $p<0.001$), revealing that despite having to work for liquid presentation, mice reinforced with fluids containing SAC vehicle consumed most of their total fluid intake from the liquid dipper, in contrast to mice reinforced with water vehicle, who ingested most of their fluid from the freely available water bottle (Fig. 4). There was a significant interaction of vehicle x EtOH concentration x session for Operant:Ad libitum choice ($F_{24,336}=2.503$, $p<0.001$). In animals reinforced with EtOH in SAC ($F_{24,112}=1.88$, $p<0.05$) and water ($F_{24,224}=2.881$, $p<0.001$), there was an interaction of session x EtOH concentration on preference revealing that mice receiving SAC vehicle ($t_3=-3.681$, $p<0.05$) and 3% EtOH in SAC ($t_4=-2.85$, $p<0.05$) showed significant increases in Operant:Ad libitum choice across sessions, whereas mice reinforced with 3% ($t_8=2.376$, $p<0.05$) and 10% EtOH in water ($t_4=7.909$, $p<0.01$) showed decreases in Operant:Ad libitum choice scores across sessions that were consistent with decreases in lever pressing following removal of the SAC reinforcer used during lever acquisition training. Independent of vehicle, mice reinforced with 15% EtOH showed a significantly greater percentage of liquid dipper fluid intake than vehicle mice ($p's<0.05$), suggesting that this concentration of EtOH was rewarding. As early as Session 1, mice reinforced with 15% EtOH in SAC showed a significantly greater percentage of their fluid intake from the liquid dipper compared to vehicle controls; 15% EtOH in water mice required extended training to reveal an increase in Operant:Ad libitum choice score compared to vehicle control subjects.

Effect of naltrexone on operant responding maintained by EtOH in water

To assess the ability of our operant self-administration model to detect the effects of established treatment drugs with known effectiveness in reducing human alcohol intake, vehicle, 0.3 or 1.25 mg/kg i.p. naltrexone were administered immediately before operant EtOH self-administration with reinforcers earned, active lever pressing and g/kg EtOH intake as dependent measures. During the first hour of the session, there was a significant interaction of naltrexone treatment (saline, 0.3, or 1.25 mg/kg naltrexone) x EtOH concentration on reinforcers earned ($F_{6,32}=3.788$, $p<0.01$) and EtOH consumed ($F_{4,24}=7.918$, $p<0.01$). Mice reinforced with 15% EtOH in water showed significant

reductions in reinforcers earned following 0.3 mg/kg ($t_4=2.409$, $p<0.05$) and 1.25 mg/kg ($t_4=2.94$, $p<0.05$) i.p. naltrexone treatment compared to when mice received saline vehicle injection. At the highest concentration of EtOH, 1.25 mg/kg naltrexone also reduced g/kg EtOH consumed ($t_4=3.538$, $p<0.05$) (Fig. 5). Similar trends for active lever pressing did not return a significant interaction. Planned t-test showed that 1.25 mg/kg naltrexone significantly reduced active lever pressing of mice reinforced with 15% EtOH in water compared to when they were injected with saline ($t_4=-2.169$, $p<0.05$). Naltrexone had no effect on reinforcers earned, active lever presses or EtOH consumed in water vehicle mice, demonstrating the specificity of naltrexone's effects on EtOH reinforcement. There was no significant effect of naltrexone on response accuracy during EtOH reinforcement. Consistent with reports indicating a limited 1 h bioavailability of naltrexone in mice, there was no effect of naltrexone detected by the end of the session (Online Resource 4).

Discussion

The present experiments confirm earlier reports that EtOH is reinforcing in C57BL/6J mice (Middaugh and Kelley 1999; Risinger et al. 1998; Kelly and Middaugh 1996). This observation was vehicle- and concentration-dependent. Mice ingested a range of concentrations of EtOH, but only mice receiving 15% EtOH in water vehicle showed evidence of EtOH reinforcement as measured by reinforcers earned compared to vehicle control mice. In the present study, EtOH reinforcement occurred in the absence of an added flavorant, explicit EtOH-paired cues, or food/water restriction, supporting the conclusion that lever pressing in these studies was motivated by the primary reinforcing effects of EtOH. These studies further showed an escalation of EtOH in water self-administration over weeks of exposure at this high concentration, suggesting that the overnight, weekly self-administration procedure used in these studies shows a progression of EtOH intake. Pretreatment with naltrexone attenuated EtOH reinforcers earned, lever pressing and g/kg EtOH consumed in mice reinforced with 15% EtOH in water, suggesting that the present model in C57BL/6J mice may have potential predictive validity for therapeutic effectiveness.

Mice reinforced with EtOH in a SAC vehicle solution consumed nearly twice the EtOH compared to mice reinforced with the same concentrations of EtOH in water. C57BL/6J mice in these studies showed levels of EtOH consumption that are comparable to selectively-bred "high alcohol drinking" rats (Bell et al. 2008). The SAC sweetener also supported escalation of lever pressing for 3% EtOH in SAC across weeks of exposure, an effect not observed in mice that received 3% EtOH in water vehicle. Despite this, mice reinforced with EtOH in SAC solution did not meet criteria of EtOH reinforcement as measured by significantly increased lever pressing or reinforcers earned compared to SAC vehicle mice. This appears to be due in part to a ceiling effect resulting from the reinforcing effects of SAC. Previous research shows that saccharin has primary reinforcing properties in C57BL/6J mice on its own (e.g. Messier and White 1984; Cason and Aston-Jones 2013). It is therefore interesting that mice reinforced with 15% EtOH in SAC earned significantly fewer reinforcers and active lever presses than SAC vehicle controls, suggesting that this concentration of EtOH may reduce the reinforcing effects of SAC. Previous oral operant EtOH self-administration studies implementing the sucrose fading technique using a within-

subject design in rats have similarly shown that increasing the concentration of EtOH produces concentration-dependent decreases in operant responding (Grant and Samson 1985; Samson 1986; Samson et al. 1988; Gonzales et al. 2004). Although it is possible that this high concentration of EtOH may have reduced the palatability of the SAC (Davison et al. 1976; Dudek 1982), this conclusion is not supported by Operant:*Ad Libitum* choice measures, which show that mice reinforced with 15% EtOH in SAC ingested a greater percentage of their fluid intake from the liquid dipper than mice reinforced with SAC vehicle alone. Concentration-dependent increases in g/kg EtOH in SAC consumed suggest that mice reinforced with 15% EtOH may have been titrating their dose of EtOH or that ingestion of nearly 8 g/kg of EtOH led to sedation in these mice (Sharko and Hodge 2008; Santos et al. 2013; Blednov et al. 2014). This latter interpretation is supported by BEC of 0.8 mg/ml achieved in some of these mice as well as by skewness measures, which revealed that higher concentrations of EtOH in SAC, but not water, resulted in bouts of lever pressing followed by periods of quiescence in 15% EtOH mice compared to SAC vehicle subjects (Online Resource 3). This pattern of responding would not have been predicted based on the VR schedule of reinforcement used in these studies, which typically promotes a steady state of responding (Ferster and Skinner 1957; Baum 1993). Bouts of responding in non-human primates predict the development of sustained patterns of heavy drinking (Grant et al. 2008). It is of further interest that skewness increased across weeks of exposure for 15% EtOH in SAC mice while g/kg intake remained stable, suggesting that mice sensitized to this behavioral effect of EtOH intake.

Sweetener greatly increases the palatability of EtOH and as such, encourages alcohol intake in humans (Kidorf 1990). SAC sweetener appeared to facilitate responding for low dose EtOH as demonstrated by a significant escalation of g/kg EtOH intake, lever presses and reinforcers earned in mice reinforced with 3% EtOH in SAC but not in mice reinforced with 3% EtOH in water. Mixed with SAC, mice found all EtOH doses rewarding as measured by overall higher Operant:*Ad libitum* choice scores than those achieved with EtOH in water. Although 15% EtOH in SAC mice did not earn significantly more reinforcers than SAC vehicle control mice, significantly higher Operant:*Ad libitum* choice scores reflected that they did appear to drink a greater percentage of liquid from the dipper compared to SAC controls, suggesting that the 15% EtOH concentration was rewarding. This observation was evident as early as Session 1. Longitudinal studies in human drinkers indicate that the alcohol subjective reward or “liking” is one of the best predictors for escalation of binge drinking (King et al. 2002; King and Byars 2004; King et al. 2015). It is therefore interesting that mice reinforced with 15% EtOH in SAC vehicle achieved BEC's consistent with the legal definition of intoxication in humans. Despite this, mice reinforced with 15% EtOH in SAC did not demonstrate an escalation of responding as was evidenced in mice reinforced with 3% EtOH in SAC or with 15% EtOH in water. Although reinforcement was not confirmed compared to SAC vehicle controls, this model of EtOH in SAC self-administration could be used to promote high levels of voluntary operant EtOH drinking in the C57BL/6J mouse strain that is commonly used as a background for transgenic and null mutant genetic manipulations.

Using water vehicle unmasked EtOH reinforcement in mice receiving the highest concentration of EtOH. EtOH reinforcement is a precursor to AUD and alcohol dependence (Tabakoff and Hoffman 2013). Consistent with subjective reports in humans, it is interesting that sub-intoxicating levels of EtOH intake led to reinforcement in this paradigm (McKee et al. 2009, King et al. 2002, 2011). Mice reinforced with 15% EtOH in water not only displayed a greater number of reinforcers earned and active lever presses compared to water vehicle controls, but they also showed a small but significant escalation of responding across exposure sessions. This weekly model of EtOH exposure mimics early patterns of alcohol intake observed with AUD in humans (Holdstock et al. 2000; King et al. 2002; King and Byars 2004). In rodents, intermittent EtOH exposure promotes an escalation of EtOH intake coined the “alcohol deprivation effect” (ADE) (Spanagel and Höltner 1999; Rodd et al. 2003; Khisti et al. 2006). Escalation of responding observed following abstinence from other drugs of abuse is referred to as an “incubation effect” to reflect changes in underlying brain processes that support the development of drug dependence (Grimm et al. 2001). It is not clear if chronic exposure, extended exposure sessions, protracted periods of abstinence or all these factors are required for observation of ADE/incubation. Most studies in mice involve extended periods of EtOH exposure of at least 14 days (McBride and Li 1998; Bell et al. 2006). In the present studies, where mice had weekly overnight access to EtOH, escalation of EtOH reinforcement and consumption first became evident during the 7th EtOH self-administration session for mice reinforced with 15% EtOH in water and as early as the 3rd session in mice reinforced with 3% EtOH in SAC. During EtOH self-administration, it is not clear if residual responding maintained by SAC may have promoted a threshold level of EtOH intake necessary to engender reinforcing effects and later escalation of responding in mice reinforced with 15% EtOH in water and 3% EtOH in SAC. Reductions in responding of mice reinforced with water vehicle and a significant difference between control mice reinforced with 15% EtOH in demonstrate that prior saccharin exposure alone was not sufficient to support reinforcement, however.

Reinforcing effects of EtOH in water were reduced in these studies by the EtOH treatment therapeutic, naltrexone (O'Malley et al. 2003; O'Malley et al. 2007). This opioid antagonist has previously been shown to decrease mouse operant responding maintained by EtOH (Middaugh et al. 1999b; Navarrete et al. 2013) and mouse EtOH intake during DID and two-bottle choice paradigms (Phillips et al. 1997; Kamdar et al. 2007). In the absence of any effect on water vehicle controls, naltrexone was effective at inhibiting 15% EtOH in water intake at sub-intoxicating doses consistent with its therapeutic profile in alcoholics.

In summary, these studies accomplished some, but not all, of the goals hoped to be achieved. Importantly, mice reinforced with 15% EtOH in water showed significantly greater responding than vehicle mice in the absence of food or water restriction; this finding suggests that EtOH has primary reinforcing properties that are not driven by thirst or caloric incentives. In the absence of EtOH fading, the between-subject design revealed an escalation of responding of mice reinforced with 15% EtOH in water and 3% EtOH in SAC that was not evident in other groups of mice. Together, the present data demonstrate that EtOH is reinforcing in the absence of contingent sweetener, but that contingent sweetener may facilitate responding of low dose EtOH. Although the complete removal of all cues was not possible given the noise produced by the dipper mechanism and the scent of the EtOH, these

studies were accomplished without the addition of more explicit tone and light cues that can serve as primary reinforcers and engage the dopamine system (Caggiula et al. 2001; Olsen and Winder 2009, Olsen and Winder 2012). Future studies may build upon this experimental design to test the regulation of cues on EtOH reinforcement and reward. It was hoped that the between-subject design would lend itself to detection of initial sensitivity to EtOH concentration that might predict later behaviors (Shuckit and Smith 1996; King et al. 2002; King et al. 2011; King et al., 2015). Unfortunately, prior training for SAC alone reinforcement may have overshadowed detection of differences in EtOH lever pressing and reinforcers earned between mice during the first oral operant EtOH self-administration session. The addition of a water bottle in the operant conditioning chamber enabled Operant:*Ad libitum* choice measures, however, which revealed that mice reinforced with 15% EtOH in SAC ingested more of their fluid intake from the liquid dipper compared to SAC vehicle control mice on Week 1. Interestingly, this group of mice achieved intoxicating levels of EtOH during later sessions as measured by estimates of EtOH intake and confirmed by BEC levels > 1.0 mg/ml. The high levels of BEC achieved in mice reinforced with 15% EtOH in SAC may serve as a model for heavy EtOH use, even if these mice do not show an escalation of dosing across weeks of exposure or evidence of EtOH reinforcement as compared to SAC vehicle controls.

These studies establish a mouse model of oral operant EtOH self-administration that does not employ explicit cues, EtOH fading, food deprivation or water deprivation to signal or promote ingestion of EtOH. Omission of these potentially confounding variables may be advantageous for studies designed to assess the genetic and biological mechanisms of EtOH use. As explicit cues and flavorants are important contributors to EtOH use in humans and animals alike, future studies can further manipulate these factors to explore the full biological complexities of behaviors that support EtOH use. Escalation of EtOH responding and consumption further provide a biological model to assess the neurochemical and molecular underpinnings that support elevations in EtOH reinforcement. The responsiveness of mice in these studies to naltrexone further supports the predictive validity of this model for detection of drugs with therapeutic effectiveness for treatment of AUD as well as for understanding the genetic and neurobiological underpinnings of EtOH reinforcement.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Reinforcers earned and active lever presses are shown for mice reinforced with **a-d** 0% (n=4), 3% (n=5), 10% (n=5) and 15% EtOH (n=5) in 0.2% saccharin (SAC) or **e-h** 0% (n=9), 3% (n=9), 10% (n=5) and 15% EtOH (n=9) in water vehicle across weekly sessions, highlighting differences between Sessions 1 and 9. Data are presented as means $\pm$ SEM. * $p < 0.05$ compared to 0% EtOH controls during the same session; # $p < 0.05$ compared to the same concentration on Session 1.

SAC Vehicle

Water Vehicle

Fig. 2.

EtOH consumed is shown for mice reinforced with 3%, 10% and 15% EtOH in **a-b** 0.2% saccharin (SAC, $n=4-5$ /group) or **c-d** water vehicle ($n=5-9$ /group) across weekly sessions, highlighting differences between Sessions 1 and 9. Data are presented as means $\pm$ SEM.

* $p < 0.05$ compared to 3% EtOH during the same session; # $p < 0.05$ compared to the same concentration on Session 1.

SAC Vehicle

Water Vehicle

Fig. 3. Skewness about the mean for active lever pressing are shown for mice receiving 0%, 3%, 10% and 15% EtOH in **a-b** 0.2% Saccharin (SAC, n=4-5/group) or **c-d** water vehicle (n=5-9/group). Data are presented as means $\pm$ SEM. * p <0.05 compared to 0% EtOH during the same session; # p <0.05 compared to the same concentration during Session 1.

SAC Vehicle

Water Vehicle

Fig. 4.

Operant:Ad libitum choice measures are shown for mice receiving 0%, 3%, 10% and 15% EtOH in **a-b** 0.2% saccharin (SAC, n=4-5/group) or **c-d** water vehicle (n=5-9/group). Data are presented as means $\pm$ SEM. * p <0.05 compared to 0% EtOH during the same session; # p <0.05 compared to the same concentration during Session 1.

Fig. 5.

a Reinforcers earned, **b** active lever presses and **c** g/kg EtOH intake are shown for mice reinforced with 0% (n=4), 3% (n=5), 10% (n=5) and 15% (n=5) EtOH in water during the first hour of the session following pretreatment with 0.9% saline vehicle, 0.3 or 1.25 mg/kg i.p. naltrexone. Data are presented as means $\pm$ SEM, * $p < 0.05$ compared to the same EtOH concentration following i.p. saline vehicle injection.

Table 1

Mean total fluid consumption and body weight.

Vehicle	0% ETOH		3% ETOH		10% ETOH		15% ETOH	
	Total Fluid	Weight	Total Fluid	Weight	Total Fluid	Weight	Total Fluid	Weight
SAC	3.5 (0.59)	28.3 (1.13)	2.9 (0.45)	27.8 (1.59)	2.9 (0.21)	29.9 (0.76)	1.9 (0.24)	26.7 (1.07)
Water	2.5 (0.19)	27.9 (0.69)	2.2 (0.29)	28.0 (0.94)	2.1 (0.15)	26.8 (1.11)	1.9 (0.21)	28.3 (0.76)

Mean daily total fluid intake (ml) and average body weights (g) are depicted for groups of mice receiving EtOH in 0.2% saccharin (SAC) or EtOH in water. Total fluid volume consumed was calculated as the sum of water bottle fluid consumed and liquid dipper fluid estimates for mice. SEM is shown in parentheses.

Table 2

Blood ethanol concentrations (BECs) in mg/ml at 4 and 6 h time points.

EtOH Concentration	4 hr BEC	6 hr BEC
3% EtOH in water	0.005 (0.0013)	0.002 (0.0011)
10% EtOH in water	0.006 (0.0017)	0.015 (0.0076)
15% EtOH in water	0.081 (0.0590)	0.076 (0.4680)
15% EtOH in SAC	0.793 (0.2527)	0.999 (0.3012)

Mean BECs are depicted for subsets of mice reinforced with EtOH in water or 0.2% saccharin (SAC). SEM is shown in parentheses.