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Effects of Acute Withdrawal on Ethanol-Induced Conditioned Place Preference in DBA/2J mice

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Abstract

Rationale—Re-exposure to ethanol during acute withdrawal might facilitate the transition to alcoholism by enhancing the rewarding effect of ethanol.

Objective—The conditioned place preference (CPP) procedure was used to test whether ethanol reward is enhanced during acute withdrawal.

Methods—DBA/2J mice were exposed to an unbiased one-compartment CPP procedure. Ethanol (0.75, 1.0 or 1.5 g/kg IP) was paired with a distinctive floor cue (CS+), whereas saline was paired with a different floor cue (CS−). The Withdrawal (W) group received CS+ trials during acute withdrawal produced by a large dose of ethanol (4 g/kg) given 8 h before each trial. The No Withdrawal (NW) group did not experience acute withdrawal during conditioning trials, but was matched for acute withdrawal experience. Floor preference was tested in the absence of ethanol or acute withdrawal.

Results—All groups eventually showed a dose-dependent preference for the ethanol-paired cue, but development of CPP was generally more rapid and stable in the W groups than in the NW groups. Acute withdrawal suppressed the normal activating effect of ethanol during CS+ trials, but there were no group differences in test activity.

Conclusions—Acute withdrawal enhanced ethanol's rewarding effect as indexed by CPP. Since this effect depended on ethanol exposure during acute withdrawal, the enhancement of ethanol reward was likely mediated by the alleviation of acute withdrawal, i.e., negative reinforcement. Enhancement of ethanol reward during acute withdrawal may be a key component in the shift from episodic to chronic ethanol consumption that characterizes alcoholism.

Keywords

Conditioned place preference; ethanol; acute withdrawal; reward; locomotor activity; DBA/2J mice

Drinking to alleviate withdrawal is included among the diagnostic criteria for alcoholism (American Psychiatric Association 2000). This phenomenon has been modeled in studies showing that experimentally induced ethanol dependence will increase later voluntary ethanol intake in both rats and mice (e.g., Becker and Lopez 2004; Brown et al. 1998; Deutsch and Cannis 1980; Fidler et al. 2006, 2009; Finn et al. 2007; Lopez and Becker 2005; O'Dell et al. 2004; Rimondini et al. 2002; Roberts et al. 1996, 2000; Schulteis et al. 1996). Although various factors influence the magnitude and duration of this increase,

substantial theoretical attention has been given to the role played by negative reinforcement based on alleviation of withdrawal (Koob 2011). Animal studies have focused primarily on ethanol's ability to alleviate the more protracted affective symptoms of withdrawal that occur several weeks or months after the onset of abstinence because of their presumed relevance for understanding relapse (Heilig et al. 2010; Koob 2011). However, studies have also shown that the ability to alleviate withdrawal during the first few days can have a substantial impact on subsequent ethanol intake, even in genotypes that ordinarily avoid ethanol (e.g., Cunningham et al. 2013; Fidler et al. 2011, 2012).

Studies of experimentally induced dependence in animals are especially useful for improving our understanding of the neurobiological processes underlying the maintenance of excessive drinking and relapse as well as for identifying treatments to eliminate such drinking and prevent relapse. However, the relevance of these post-dependence withdrawal-alleviation models for understanding the transition from initial ethanol exposure to excessive drinking is unclear. One hypothesis is that this transition is facilitated by repeated experience with the alleviation of acute withdrawal ("hangover") after episodes of binge drinking during early adulthood (i.e., 4–5 drinks within 2 h). In other words, a negative reinforcement mechanism similar to that involved in the maintenance and relapse of excessive drinking might contribute importantly to the etiology of excessive drinking. Frequent binge drinking by adolescents and young adults is one of many risk factors for future development of alcoholism, including a family history of alcoholism (Windle and Zucker 2010). Of particular interest in this context, adult children of alcoholics who have not yet developed alcoholism show greater withdrawal effects after a single drink (1 g/kg) than adult children of non-alcoholics (McCaul et al. 1991), raising the possibility that drinking during recovery from an episode of binge drinking might produce stronger negative reinforcement in individuals who are especially vulnerable for developing alcoholism. Repeated binge drinking might also enhance the magnitude of negative reinforcement produced by drinking during the post-binge period because the severity of withdrawal increases with repeated withdrawal episodes (Becker 2008).

Although negative reinforcement based on repeated alleviation of post-binge hangovers offers a plausible explanation for escalation to excessive drinking, direct evidence for this form of negative reinforcement in non-dependent animals is sparse and other processes can be invoked (e.g., sensitization to ethanol's rewarding effects, tolerance to sedative or aversive ethanol effects). The present studies were designed to provide evidence of negative reinforcement based on alleviation of acute withdrawal in non-dependent animals using an experimental design and procedure that largely eliminated alternative interpretations based on tolerance or sensitization. Acute withdrawal was induced by periodically injecting mice with a single high dose of ethanol (4 g/kg). Although not directly comparable to binge drinking in humans, this procedure is arguably similar in producing a rapid increase in blood ethanol level followed by a period of acute withdrawal. Many previous studies have shown that acute withdrawal peaks about 6–8 h after such injections using handling-induced-convulsions (HICs) to index withdrawal severity (e.g., Buck et al. 1997; Crabbe et al. 1991; Metten and Crabbe 1994). To assess negative reinforcement, we then re-exposed mice to a marginally rewarding dose of ethanol near the time of expected peak withdrawal using a conditioned place preference (CPP) training procedure.

CPP is a well-characterized model of drug-seeking behavior that has been used successfully to study the conditioned rewarding effect of ethanol in mice (Cunningham 2011; Cunningham et al. 2003, 2006). Mice typically receive several pairings of ethanol with one context (CS+) and pairings of saline with a different context (CS-). When later given a drug-free choice between the two contexts mice spend more time in the ethanol-paired context, an outcome consistent with the development of an association between the CS+ and

ethanol's rewarding effect. An important advantage of CPP over self-administration for assessing negative reinforcement during withdrawal is that the experimenter can precisely control the dose of ethanol given during the peak of acute withdrawal. In contrast, acute withdrawal might directly impair or delay ethanol self-administration, resulting in low and variable ethanol exposure during peak withdrawal. Additional advantages of CPP are that no preliminary training involving ethanol exposure is required before conditioning and that the effects of the experimental treatments can be tested in a drug-free state in the absence of withdrawal.

Previous research has shown that multiple pre-conditioning exposures to the high ethanol dose used to induce acute withdrawal does not produce an increase (i.e., sensitization) or decrease (i.e., tolerance) in ethanol-induced CPP, suggesting that pre-exposure does not alter ethanol's rewarding effect (Cunningham et al. 2002). Nevertheless, the groups conditioned during Withdrawal (W) and No-Withdrawal (NW) in the present studies were matched for overall ethanol exposure as well as for experience with acute withdrawal. Thus, group differences in CPP are not easily explained by group differences in tolerance, sensitization or history of withdrawal. We hypothesized that the rewarding effect of low ethanol doses would be enhanced when experienced during acute withdrawal. To facilitate detection of enhanced reward, we selected ethanol doses (0.75–1.5 g/kg) on the ascending (pre-asymptotic) limb of the dose-effect curve for ethanol-induced CPP (Grolewski et al. 2008). We also tested mice halfway through the conditioning phase to provide an opportunity to detect group differences in the rate at which CPP developed. Withdrawal-induced enhancement of the rewarding effect of a low ethanol dose might be expected to yield significant CPP after fewer training trials.

Materials and Methods

Animals

Adult male inbred DBA/2J mice (Jackson Laboratory, Sacramento, CA) were used. Mice were 6 weeks of age upon arrival and were allowed to acclimate to the colony room for 2 weeks before the experiment. Mice were housed in groups of 2–4 in a Thoren rack in polycarbonate shoebox cages lined with cob bedding. Food and water were continuously available. The colony room was maintained at a temperature of 21° C on a 12:12 light/dark cycle (lights on at 0700). The experiments occurred during the light phase. These procedures were conducted in accordance with The National Institute of Health (NIH) "Principles of Laboratory Animal Care" and the Oregon Health & Science University IACUC approved the protocol.

Apparatus

The CPP apparatus is described in detail in Cunningham et al. (2006). Briefly, all training and test trials were conducted in 12 identical acrylic and aluminum boxes (30 × 15 × 15 cm). Each box was enclosed in a ventilated sound- and light-attenuating chamber (Coulbourn Model E10–20) with no internal lighting. Each conditioning box contained six infrared emitter-detector pairs mounted on the acrylic walls at 5-cm intervals, 2.2 cm above the floor of the box. The detectors were interfaced to a computer that recorded activity counts (indexed as beam breaks) and time spent on each side of the box.

Two distinct interchangeable floor patterns served as the CSs. The grid floors were made of 2.3 mm rods attached to acrylic sides at 6.4 mm intervals. The hole floors were made of 16-gauge stainless steel punched with 6.4 mm round holes at 9.5 mm staggered centers. Previous studies have shown that naive DBA/2J mice spend approximately equal time on each floor during drug-free preference tests (Cunningham et al. 2003, 2006).

Ethanol Doses

Ethanol solutions (20% v/v) were prepared from a 95% stock solution with saline as the vehicle. A dose of 4 g/kg was used to induce acute withdrawal approximately 8 h later (Metten and Crabbe 1994). The doses used for place conditioning were 1.5, 1.0 and 0.75 g/kg in Experiments 1–3, respectively. These doses were selected because they are all on the ascending limb of the dose-effect curve for ethanol-induced CPP in DBA/2J mice (Groblewski et al. 2008). Dose was manipulated by varying injection volume (Linakis and Cunningham 1979). All injections were given intraperitoneally.

Procedure

The conditioned place preference procedure was divided into three phases: habituation (1 day), conditioning (four cycles of 3–7 days) and preference testing (2 days). The purpose of the habituation session was to acclimate mice to the daily procedures of handling, injection and placement in the conditioning box. During habituation, all mice were given a 25.0 ml/kg saline injection between 0700–0820. Injections were staggered so that each mouse received the second saline injection 8 h later between 1500–1620. The second injection (12.5 ml/kg) was immediately followed by placement in the conditioning box on a smooth paper floor for 5 min. Mice were returned to the home cage at the end of the habituation session.

Mice in all three experiments were divided into two groups: one that experienced acute withdrawal during CS+ conditioning trials (W) and one that experienced acute withdrawal on an Injection-Only day separate from any conditioning (NW). Thus, all mice received equal exposure to ethanol and acute withdrawal, but differed in whether CS+ trials occurred during acute withdrawal. Each group was further subdivided into conditioning subgroups such that half the mice in each group received ethanol paired with the grid floor and saline paired with the hole floor (G+ subgroups) while the other half received the opposite pairings (G– subgroups). Group sizes are indicated in the figure captions and tables. All mice received a total of four conditioning cycles. Each cycle contained three types of treatment days: CS+, CS– and Injection-Only (See Table 1). On CS+ days, mice received either 4 g/kg ethanol (W) or saline (NW) between 0700–0820. Eight h later, each mouse was injected with the conditioning dose and placed into the conditioning box on the assigned floor for 5 min. The floor of the conditioning box was either all grid or all hole during conditioning trials (i.e., one-compartment procedure). On CS– days, each mouse received a 25.0 ml/kg saline injection in the morning followed 8 h later by a 12.5 ml/kg saline injection just before placement in the conditioning box on the opposite floor for 5 min. Finally, on Injection-Only days, mice in the NW groups received a 4 g/kg ethanol injection in the morning while mice in the W groups received an equal volume saline injection. The purpose of this day was to match the W and NW groups for overall exposure to the 4-g/kg ethanol injection and the experience of acute withdrawal. All mice were then left alone in their home cages for the remainder of the day.

During each conditioning cycle, the CS+ trial preceded the CS– trial, which was the last event in the cycle. In Experiment 1, order of exposure to the CS+ trial and Injection-Only day was counterbalanced within each cycle and treatments occurred on 3 consecutive days. However, due to concern over the possibility that residual effects of the 4 g/kg dose might have influenced conditioning on the next day (i.e., 32 h later), the procedure was modified for Experiments 2 and 3 by inserting one or two no-treatment days after each treatment. The specific sequence of daily events was as follows: CS+ trial, no treatment, Injection-Only day, no treatment, no treatment, CS– trial, no treatment. Thus, each cycle lasted 7 days and at least 80 h elapsed between each 4 g/kg injection and the next conditioning trial. All mice were left alone in the home cage during No-Treatment days (Table 1).

Two preference tests were conducted. The first test was given 24 h after the end of the second conditioning cycle, and the second test was given 24 h after the end of the fourth cycle. CPP induced by doses of 1.5 g/kg or higher is generally evident after four conditioning trials in non-dependent mice (Grobowski et al. 2008), which would be expected during Test 2 for the NW group in Experiment 1. However, if acute withdrawal enhances ethanol's rewarding effect, CPP might be evident in the W group during Test 1 after only two conditioning trials. All mice were injected with saline in the home cage between 0700–0820. Eight h later, mice were injected with the appropriate saline volume and placed immediately in the conditioning box for 30 min. Half of the floor in the conditioning box was grid and the other half was hole; floor position was counterbalanced within each group. Activity and time spent on each side were recorded.

Two independent cohorts of mice were used to run Experiment 3, with each cohort containing a similar number of mice from the W and NW groups. The conditioning and test procedures for each cohort were identical, but mice in one cohort received four additional home cage exposures to 25 ml/kg saline injections during the 2 weeks before the first conditioning trial. Since preliminary analyses of the preference test data indicated there were no main effects or interactions involving the cohort factor (indicating no effect of the additional saline exposures), data from both cohorts were combined in all analyses reported for Experiment 3.

Data Analysis

The primary dependent variable was time spent on the grid floor during the preference test. Differences between the Conditioning Subgroups (G+ vs. G–) within each experimental group provide evidence for a learned preference for CS+ (Cunningham et al. 2003). That is, mice that previously received the grid floor paired with ethanol (G+) are expected to spend more time on the grid floor than mice that received the hole floor paired with ethanol (G–). A 2×2 analysis of variance (ANOVA) was used to analyze time spent on the grid floor during each preference test using Withdrawal Group (W vs. NW) and Conditioning Subgroup (G+ vs. G–) as between-group factors. A priori pair-wise comparisons between the conditioning subgroups were used to determine the presence of CPP in each treatment group using Bonferroni-corrected critical values (Cunningham et al. 2003). Test activity was analyzed using a one-way ANOVA with Withdrawal Group as the factor.

Activity during conditioning trials was initially analyzed with a 2×2 mixed ANOVA using Withdrawal Group (W vs. NW) as the between-subjects variable and Trial Type (CS+ vs. CS–) as the within-subjects variable. Post hoc tests were conducted with Bonferroni-corrected pair-wise comparisons and t-tests. Alpha level was 0.05.

Results

Preference Tests

Experiment 1—The W group showed CPP in both tests, while the NW group showed CPP only in Test 2 (Figure 1). Separate two-way ANOVAs yielded significant main effects of Conditioning Subgroup in Tests 1 and 2 [both F 's(1, 29) > 8.3, p < 0.01], but there was no significant effect of Withdrawal Group or interaction on either test. A priori Bonferroni pairwise comparisons indicated that Group W mice in the G+ conditioning subgroup spent more time on grid than Group W mice in the G– subgroup in Test 1 (p < 0.005). However, there was no significant difference between conditioning subgroups in Group NW. During Test 2, G+ mice in both withdrawal groups spent more time on the grid than G– mice (both p 's < 0.005).

Experiment 2—Both withdrawal groups showed CPP in Test 1, but only Group W maintained preference through Test 2 (Figure 2). This pattern of results suggests that the marginal CPP expressed by Group NW might have been more susceptible to the extinction produced by the first 30-min choice test. The main effect of Conditioning Subgroup was significant in both tests [both $F(1, 43) > 16.9$, $p < 0.0005$], but neither the main effect of Withdrawal Group nor interaction was significant in either test. A priori Bonferroni comparisons showed that G+ mice spent more time on the grid floor than G– mice in both groups on Test 1 (p 's < 0.05), but only Group W showed significant CPP on Test 2 ($p < 0.001$).

Experiment 3—The pattern of findings in Experiment 3 was similar to that seen in Experiment 1. That is, Group W (but not Group NW) showed CPP on Test 1, while both withdrawal groups showed CPP on Test 2 (Figure 3). There was a significant main effect of Conditioning Subgroup in both tests [both $F(1, 75) > 9.4$, $p < 0.005$], but there was no significant main effect of withdrawal or interaction. Bonferroni pairwise comparisons indicated that Group W mice in the G+ subgroup spent significantly more time on the grid floor compared to Group W mice in the G– group during Test 1 ($p < 0.002$). During Test 2, G+ mice in both withdrawal groups spent more time on the grid floor than G– mice (p 's < 0.005).

Pooled Analysis—In order to examine ethanol dose effects, the preference test data from all three experiments were pooled and subjected to a 3-way ANOVA (Dose x Withdrawal Group x Conditioning Subgroup) for each test. These analyses yielded significant main effects of Conditioning Subgroup [both $F(1, 147) > 37.2$, $p < 0.0001$] as well as significant Withdrawal Group x Conditioning Subgroup interactions [both $F(1, 147) > 4.1$, $p < 0.05$] on both tests. These interactions confirmed the development of stronger CPP in the W groups than in the NW groups. In addition, there was a marginally significant Dose x Conditioning Subgroup interaction on Test 2 [$F(2, 147) = 3.0$, $p = .05$], reflecting a dose-dependent increase in CPP that was not influenced by withdrawal. No other effects were significant in these analyses.

Test Activity—There were no significant differences in activity on either test in any experiment (Table 2). Thus, the effect of acute withdrawal on CPP cannot be attributed to group differences in the residual effects of acute withdrawal on motor activity.

Conditioning Activity

Acute withdrawal attenuated ethanol's motor activating effect during CS+ conditioning trials. Group W consistently showed lower activity on CS+ trials than Group NW, with no difference between the two groups on CS– trials (Table 3). Two-way ANOVAs showed significant main effects of Withdrawal Group in Experiments 1 [$F(1, 31) = 16.4$, $p < 0.0005$] and 3 [$F(1, 77) = 12.3$, $p < 0.001$]. A significant main effect of Trial Type in all experiments reflected the generally higher level of activity on ethanol (CS+) trials [Exp. 1: $F(1, 31) = 150.2$; Exp. 2: $F(1, 45) = 161.5$; Exp. 3: $F(1, 77) = 63.4$; all p 's < 0.0001]. Finally, the Withdrawal Group x Trial Type interaction was significant in all experiments [Exp. 1: $F(1, 31) = 31.0$; Exp. 2: $F(1, 45) = 43.0$; Exp. 3: $F(1, 77) = 63.3$; all p 's < 0.0001]. Bonferroni-corrected post-hoc comparisons for all three experiments explained the interaction by yielding a significant group difference on CS+ (but not CS–) trials, confirming that acute withdrawal suppressed ethanol's normal locomotor activating effect (p 's < 0.001).

Discussion

These experiments demonstrate that acute withdrawal during CS+ conditioning trials enhances development of ethanol-induced CPP. In all three experiments, groups that experienced withdrawal during CS+ trials (W groups) consistently showed significant preferences for the ethanol-paired floor after only two conditioning cycles and maintained that preference after additional trials, even at a dose (0.75 g/kg) near the threshold for producing ethanol CPP in DBA/2J mice (Grolewski et al. 2008). In contrast, groups not in withdrawal during CS+ trials (NW groups) showed significant CPP only after four conditioning cycles in Experiments 1 and 3. Although the NW group in Experiment 2 displayed place preference after the first two conditioning cycles, preference was not maintained during the second test after more conditioning trials had been given. It is not clear why that group showed a significant CPP in Test 1, but not in Test 2. Since this particular pattern of results was not seen in the experiments using a higher or lower ethanol dose, it might reflect sampling error. Nevertheless, all three experiments support the overall conclusion that development of ethanol-induced CPP was generally more rapid and stable in the W groups than in the NW groups. This finding can be explained by the idea that alleviation of acute withdrawal enhanced ethanol reward through a negative reinforcement mechanism. Although acute withdrawal was not directly assessed in our studies, the significant reductions in ethanol's locomotor activating effect suggest that Group W mice were experiencing withdrawal during CS+ trials.

Our data are congruent with previous studies showing increases in ethanol self-administration during withdrawal in animals previously made dependent by prolonged or repeated exposure to ethanol in liquid diet (Brown et al. 1998; Schulteis et al. 1996), air vapor (Becker and Lopez 2004; Finn et al. 2007; Lopez and Becker 2005; O'Dell et al. 2004; Rimondini et al. 2002; Roberts et al. 1996, 2000) or intragastric infusions (Cunningham et al. 2013; Deutsch & Cannis 1980; Fidler et al. 2006, 2009, 2011, 2012). However, our results extend those findings in several important ways. First, our studies demonstrate negative reinforcement by ethanol in animals that had received very little previous exposure to ethanol and would generally not be considered dependent at the time of withdrawal. Thus, this CPP model is potentially more relevant for understanding the role that negative reinforcement plays in the transition from initial to excessive drinking before the onset of dependence. Second, the mice in our experiments were not in withdrawal or exposed to ethanol at the time of testing, thereby eliminating these factors as potential confounds influencing test behavior. Third, and perhaps most importantly, the design and procedures used here largely eliminated alternative interpretations based on tolerance or sensitization to ethanol's rewarding and aversive effects because the W and NW groups were completely matched for overall exposure to ethanol and differed only in whether their CS+ trials occurred near the peak of acute withdrawal. Moreover, since both groups were also matched for the number of acute withdrawal experiences, the CPP results cannot be attributed to non-specific effects of a prior history of acute withdrawal.

We know of only one other study that has looked at the impact of ethanol withdrawal on ethanol-induced CPP in mice (Ting-a-kee et al. 2009). The primary goal of that study was to examine dopamine-dependent and independent reward pathways that are influenced by the presence or absence of withdrawal. Experimental mice were made dependent by chronic exposure to an ethanol liquid diet while control mice received a control liquid diet without ethanol. The ethanol liquid diet was replaced by the control diet during the 8 h before each CPP conditioning trial. Thus, experimental mice were dependent and withdrawn for each trial, but control mice were not. Both liquid diets were removed and replaced by standard rodent chow for 1 week after the last trial before testing. Thus, mice were not in acute withdrawal during the preference test. In contrast to the current experiments, both groups

showed equivalent CPP, regardless of their withdrawal state during conditioning. The authors suggested that two different processes might have been responsible for the similar results in both groups. More specifically, they suggested that control mice might have developed a CPP based primarily on ethanol's inherent reinforcing properties, while the dependent and withdrawn mice developed CPP based more on ethanol's ability to alleviate withdrawal.

There are several key differences between the Ting-a-kee et al. study and our studies that might explain the discrepant findings. First, they tested their mice only once after a relatively large number of conditioning trials (12 CS+ and 12 CS- trials), raising the possibility that both groups had achieved asymptotic levels of conditioning and reached a similar performance ceiling. Indeed, if we had conducted preference tests only after the final trial (or after even more trials) in our studies, our results would have been similar to their results in showing little difference between the W and NW groups. Second, Ting-a-kee et al. used a mouse strain (C57BL/6) that experiences less severe acute withdrawal (indexed by HICs) after acute (Metten and Crabbe 1994) or chronic (Crabbe 1998) ethanol exposure compared to the DBA/2J strain used in our studies. Thus, injection of ethanol on CS+ trials might have produced less negative reinforcement in their mice than in ours (Cunningham et al. 2013). Third, in contrast to our studies, their experimental mice were in withdrawal during both the CS+ and CS- conditioning trials, which allowed the complicating possibility that a conditioned place aversion developed to the CS- context. Fourth, they induced dependence and withdrawal using a chronic method (ethanol liquid diet) that involved substantially more cumulative exposure to ethanol in their experimental mice than in our Group W mice. Finally, in contrast to our studies, the experimental and control groups in their study were not matched for overall exposure to ethanol or withdrawal experience, allowing for the possibility that other processes (e.g., tolerance, sensitization) obscured effects of receiving the CS+ trials during acute withdrawal. Thus, there are many potential reasons for the null outcome reported by Ting-a-kee et al. (2009).

The negative reinforcement interpretation of our findings assumes that acute ethanol withdrawal produces an aversive motivational state with a time course similar to that indexed by handling induced convulsions, an assumption that was not directly tested in these studies. However, the suppression of ethanol-induced activation 8 h post withdrawal is consistent with the hypothesized aversive state. Moreover, a previous study has shown that a conditioned place aversion was produced in mice when a contextual cue was repeatedly paired with the aversive state produced 8 h after ethanol withdrawal in mice (Ting-a-kee et al. 2012, Supplementary Figure 1).

Although our interpretation of these data has focused on the idea that alleviation of withdrawal enhanced ethanol reward via negative reinforcement, consideration must be given to other interpretations. For example, the pattern of results observed here could also be explained by arguing that acute withdrawal did not alter ethanol reward but instead enhanced the associative learning processes that underlie CPP. This explanation predicts that other forms of learning not involving ethanol reward would also be enhanced if training occurred during acute withdrawal, a possibility that could be tested in future studies. However, facilitation of learning seems an unlikely explanation in light of previous studies showing that withdrawal after chronic ethanol exposure interferes with associative learning (e.g., Ripley et al. 2003, 2004). Another possibility is that the enhancement in CPP is somehow related to the suppressant effect of acute withdrawal on ethanol stimulated activity. While the mechanism for such an effect is not clear, there is ample evidence from previous studies that treatment-induced alterations in ethanol's activating effect on CS+ trials have little impact on development of CPP (e.g., Boyce-Rustay and Cunningham 2004; Chester and Cunningham 1999; Risinger et al. 1992).

Our assumption is that the enhancement of ethanol reward during each CS+ trial produced greater increments in the strength of the context-ethanol association, which were revealed during subsequent testing in the absence of ethanol or withdrawal. However, it is also possible that experiencing ethanol during acute withdrawal might have produced a conditioned increase in the rewarding value of the ethanol-conditioning dose that was independent of ethanol's association with context. That is, because ethanol's interoceptive cues were paired with a withdrawal-enhanced rewarding effect, subsequent injections of ethanol may have been more rewarding in Group W animals, even in the absence of withdrawal. Pairing ethanol with a new CS to see whether new learning is accelerated would test this possibility.

Differences between the W and NW groups were generally smaller on the second test than on the first, suggesting that acute withdrawal affected the rate of CPP development but not the final magnitude of CPP. This finding might indicate that acute withdrawal enhanced ethanol reward and thereby increased the learning asymptote, but that a response ceiling on CPP performance made it more difficult to detect this increase after four CS+ conditioning trials (Grolewski et al. 2008). We had hoped to avoid concerns about possible response ceiling effects by using several ethanol doses on the ascending limb of the dose-effect curve. However, the acute withdrawal effect on CPP was similar across doses, suggesting that acute withdrawal might have facilitated the rate of CPP development by enhancing the salience of the ethanol injections on conditioning trials without increasing ethanol reward.

Although Groups W and NW were matched for overall exposure to ethanol within each experiment, it is possible that their blood ethanol concentrations (BECs) after ethanol injection on CS+ trials differed either because there was residual ethanol from the morning injection or because the morning injection altered metabolism of the later injection (Thurman et al. 1982). For example, summation of residual ethanol from the morning injection with ethanol from the conditioning trial injection might be expected to enhance CPP based on the monotonic increasing dose-effect curve for ethanol-induced CPP (Grolewski et al. 2008). However, given previous data showing that a 4-g/kg ethanol dose was completely cleared within 8 h in DBA/2J mice (Gorin-Meyer et al. 2007), this interpretation seems unlikely. While there are some data suggesting acute metabolic tolerance to the second of two closely spaced bolus ethanol injections (e.g., Shimamoto et al. 2010), such tolerance would presumably be expected to reduce CPP, not enhance it. Thus, the behavioral effect observed in our studies is not adequately explained by possible group differences in BECs.

One possible limitation of the present findings is that there were no groups that did not receive large dose ethanol injections and did not experience acute withdrawal. Thus, it is not known whether there were any non-specific effects of that history of exposure to ethanol or withdrawal on CPP magnitude. However, such effects seem unlikely based on a previous study from our laboratory that showed no CPP differences between ethanol-naïve mice and mice that had previously received four exposures to a 4 g/kg ethanol dose (and acute withdrawal) at 48 h intervals before standard CPP conditioning trials (Cunningham et al. 2002).

In summary, these experiments provide what we believe to be the first demonstration that alleviation of acute withdrawal in non-dependent animals can enhance ethanol-induced CPP, an outcome consistent with enhancement of ethanol reward based on negative reinforcement. As such, this procedure offers a new behavioral model with potential for better understanding the role that negative reinforcement might play in the transition from moderate to excessive drinking. Given the high incidence of binge drinking among young adults, there would presumably be many opportunities for additional drinking during the

post-binge period to be negatively reinforced by the alleviation of acute withdrawal (hangover). Enhanced reinforcement of such drinking in combination with other risk factors for alcoholism (e.g., family history, sensitivity to ethanol) could be an important contributor to the transition from recreational to chronic ethanol consumption.

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Figure 1.

Mean time on the grid floor (sec/min + SEM) after two trials (Test 1, left panel) and four trials (Test 2, right panel) in Experiment 1. Each panel depicts data from the G+ (solid) and G- (hatched) conditioning subgroups within the NW (No Withdrawal) and W (Withdrawal) treatment groups ($n = 7-9$ /conditioning subgroup). ** $p < 0.005$; **** $p < 0.00001$.

Figure 2.

Mean time on the grid floor (sec/min + SEM) after two trials (Test 1, left panel) and four trials (Test 2, right panel) in Experiment 2. Each panel depicts data from the G+ (solid) and G- (hatched) conditioning subgroups within the NW (No Withdrawal) and W (Withdrawal) treatment groups (n = 11–12/conditioning subgroup). *p < 0.05; *** p < 0.001.

Figure 3. Mean time on the grid floor (sec/min + SEM) after two trials (Test 1, left panel) and four trials (Test 2, right panel) in Experiment 3. Each panel depicts data from the G+ (solid) and G- (hatched) conditioning subgroups within the NW (No Withdrawal) and W (Withdrawal) treatment groups (n = 19–20/conditioning subgroup). **p < 0.01; *** p < 0.001.

Table 1

Injection schedule for each conditioning cycle.

Group	Injection (time)	CS+ Day	No-Treatment Day ¹	Injection-Only Day ²	CS- Day
W	1 st (0700) 2 nd (1500)	EtOH _{4,0} EtOH _x	(Home cage)	Saline -	Saline Saline
NW	1 st (0700) 2 nd (1500)	Saline EtOH _x	(Home cage)	EtOH _{4,0} -	Saline Saline

W = Withdrawal Group; NW = No Withdrawal Group

EtOH_{4,0} = 4.0 g/kg ethanol (acute withdrawal induction dose).

EtOH_x = 1.5, 1.0 or 0.75 g/kg ethanol; conditioning doses used in Exps. 1–3, respectively.

¹ Only used in Exps. 2 and 3; mice were left undisturbed in the home cage on No-treatment days.

² Only a morning injection was given on Injection-Only days; there were no afternoon injections.

Table 2Mean test activity (counts/min $\pm$ SEM).

Experiment	Test 1		Test 2	
	NW	W	NW	
1	34.3 $\pm$ 1.6 (15)	35.8 $\pm$ 2.0 (18)	34.5 $\pm$ 1.7 (15)	33.8 $\pm$ 1.1 (18)
2	32.0 $\pm$ 1.9 (23)	34.1 $\pm$ 2.3 (24)	31.0 $\pm$ 1.2 (23)	34.7 $\pm$ 1.6 (24)
3	32.0 $\pm$ 1.1 (39)	32.6 $\pm$ 0.9 (40)	31.4 $\pm$ 1.2 (39)	29.1 $\pm$ 1.1 (40)

NW = No Withdrawal Group; W = Withdrawal Group; () = Number of mice in each group

Table 3

Mean conditioning trial activity (counts/min $\pm$ SEM).

Experiment	Group NW		Group W	
	CS- (saline)	CS+ (ethanol)	CS- (saline)	CS+ (ethanol)
1 (1.5 g/kg)	54.8 $\pm$ 2.4 (15)	118.7 $\pm$ 8.1 (15)	51.7 $\pm$ 2.0 (18)	75.6 $\pm$ 4.7 (18)
2 (1.0 g/kg)	44.7 $\pm$ 2.6 (23)	75.2 $\pm$ 3.3 (23)	48.7 $\pm$ 2.0 (24)	58.5 $\pm$ 2.8 (24)
3 (0.75 g/kg)	40.9 $\pm$ 1.6 (39)	59.5 $\pm$ 2.4 (39)	42.0 $\pm$ 1.2 (40)	42.0 $\pm$ 2.0 (40)

NW = No Withdrawal Group; W = Withdrawal Group; () = Number of mice in each group