Published in final edited form as:

Psychopharmacology (Berl). 2008 November ; 201(1): 97–106. doi:10.1007/s00213-008-1251-3.

Reference-Dose Place Conditioning with Ethanol in Mice: Empirical and Theoretical Analysis

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Abstract

Rationale—A frequently expressed criticism of the conditioned place preference (CPP) procedure is that it sometimes lacks a graded dose-response curve for many drugs.

Objective—We used a combination of standard and reference-dose CPP procedures to examine the dose-response curve for ethanol-induced CPP in DBA/2J mice.

Method—In the standard procedure, ethanol (0.5, 1.5, 2 and 4 g/kg) was paired with a distinctive floor cue whereas saline was paired with a different floor cue. In the reference-dose procedure, each cue was paired with a different dose of ethanol. All mice received four 5-min trials of each type in both procedures.

Results—Standard procedures yielded similar levels of CPP at doses of 1.5, 2 and 4 g/kg, whereas 0.5 g/kg did not produce significant CPP. However, in the reference-dose procedure, exposure to the 0.5 g/kg dose interfered with CPP normally produced by 1.5 or 2 g/kg. Moreover, mice showed significant preference for the 4 g/kg-paired cue over the 1.5 g/kg-paired cue.

Conclusions—These studies show that a reference-dose procedure can reveal effects of low doses that are sometimes difficult to detect in a standard procedure. The reference-dose procedure may also uncover differences between higher doses that normally produce similar preference. Efficacy of the reference-dose procedure may be explained by a theoretical analysis that assumes the procedure places behavior between the extremes of the performance range, offering a more sensitive method for detecting effects of manipulations that produce small changes and/or differences in the rewarding effects of ethanol.

Keywords

conditioned place preference; reference-dose procedure; reward; ethanol; locomotor activity; inbred mice (DBA/2J)

Introduction

In a standard place conditioning procedure, one set of contextual cues is paired with a drug while a second set of cues is paired with vehicle. Relative preference for the drug-paired cues during a choice test is then used to draw inferences about the drug's rewarding or aversive effects. Although widely used, the standard procedure has been criticized because it sometimes fails to show a graded dose response curve (Bardo et al. 1995; Bardo and Bevins 2000; Carr et al. 1989; Swerdlow et al. 1989). For example, the dose effect function for cocaine in rats is

often step-like, showing no effect across a range of low doses then suddenly showing a maximal effect across a range of higher doses (e.g., Bevins 2005). Similar findings have been reported in mice using ethanol, with low doses producing no effect while high doses produce a maximal effect (Cunningham et al. 1992; Risinger and Oakes 1996). With additional training trials, reliable place conditioning can be observed at lower doses, but effect magnitude is not distinguishable from that produced at higher doses (Risinger and Oakes 1996).

An alternative strategy for demonstrating dose effects in place conditioning is the “reference-dose” procedure originally described by Barr et al. (1985). Instead of comparing drug- and saline-paired cues, the reference-dose procedure involves comparing cues that have both been paired with drug. In the Barr et al. study, all groups received pairings of one cue with the same fixed (reference) dose of morphine (1 mg/kg), but differed in the comparison dose that was paired with the other cue (0.1, 0.3, 3.0 or 5.0 mg/kg). Data were analyzed by comparing group differences in preference for the cue paired with the comparison dose. This procedure yielded a graded dose effect curve inasmuch as rats preferred the 1-mg/kg-paired compartment over the 0.1-mg/kg-paired compartment and they preferred the 3- or 5-mg/kg-paired compartment over the 1-mg/kg-paired compartment (there was no preference in the group in which 0.3 mg/kg was compared to 1 mg/kg). Although the procedure produced a graded dose effect curve, there was no evidence of greater sensitivity for detecting effects of doses lower than the reference dose. More specifically, if the 0.1 mg/kg morphine dose was simply sub-threshold, the reference-dose comparison between the 1 and 0.1 mg/kg doses could be viewed as functionally equivalent to a standard procedure that compared 1 mg/kg morphine to saline. More generally, Barr et al. failed to provide evidence that the standard procedure would not have produced a similar morphine dose-effect relationship with their conditioning parameters. In fact, they only tested their highest dose in the standard procedure (i.e., 5 mg/kg vs. saline) and that group showed a preference similar to that seen in the reference-dose group given the highest dose (i.e., 5 vs. 1 mg/kg).

Bevins (2005) recently addressed the latter limitation in a cocaine dose-effect study that offered a better comparison between the dose-effect curves produced by the standard and reference-dose procedures. The standard procedure (i.e., cocaine vs. saline) yielded a step-like dose effect function, with low doses (0.1 or 0.3 mg/kg IV) producing no preference while higher doses produced equivalent preference across a wide range (0.45, 0.6, 0.9 and 1.2 mg/kg IV). In his reference-dose procedure, all groups received pairings of one cue with the cocaine reference dose (0.45 mg/kg IV), but differed in the comparison dose that was paired with the other cue (0, 0.6 or 1.2 mg/kg IV). In contrast to the standard procedure, the reference-dose procedure successfully detected a difference between the 0.6 and 1.2 mg/kg cocaine doses (i.e., the group that received 1.2 vs. 0.45 mg/kg showed a stronger preference for the comparison dose than the group that received 0.6 vs. 0.45 mg/kg). However, no attempt was made to see whether the procedure could detect a difference between the reference dose and a lower dose (e.g., 0.45 vs. 0.1 or 0.3 mg/kg). Thus, like Barr et al., Bevins provided no evidence that the reference-dose procedure had greater sensitivity for detecting effects of doses lower than the reference dose.

One significant limitation of both of the previous reference-dose studies was the use of only one reference dose, leaving open the question of how choice of the reference dose might affect sensitivity to dose differences that cannot be detected in a standard procedure. Moreover, these studies provided either no dose-effect comparison in the standard procedure (Barr et al., 1985) or comparison to only the upper portion of the standard dose-effect curve (Bevins, 2005). Additionally, neither of these reports offered a conceptual or theoretical framework for understanding why the reference-dose procedure might, in some circumstances, be more sensitive than the standard CPP procedure. The present studies were designed to address empirical shortcomings in previous reference-dose studies by providing a more comprehensive analysis across multiple reference doses and across a broad range of doses in the standard

procedure. Furthermore, these studies extended use of the reference-dose procedure to a new drug (ethanol) and species (mouse). Of primary interest was whether the reference-dose procedure would reveal ethanol dose-effects that were not apparent in the standard procedure, especially in the range below the reference dose. Finally, we offer a theoretical analysis of CPP performance in both the standard and reference-dose procedures that combines assumptions about the behavioral performance window with a hypothetical model for the relationship between associative strength and number of conditioning trials derived from the Rescorla-Wagner model (Rescorla and Wagner 1972).

Materials and methods

Subjects

Adult male inbred DBA/2J mice ($n = 382$) were obtained from the Jackson Laboratory (Bar Harbor, ME) at 6 weeks of age and allowed to acclimate to the colony for 2–3 weeks before training. Mice were housed in groups of 4 in polycarbonate cages with cob bedding in a Thoren rack. Water and food were continuously available in the home cage. The room was maintained at a temperature of $21 \pm 1^\circ \text{C}$ with a normal 12 hr light cycle (lights on at 7:00 a.m.). All experimental procedures occurred during the light phase.

Apparatus

Experiments were conducted using 12 identical acrylic and aluminum boxes ($30 \times 15 \times 15 \text{ cm}$) enclosed in separate ventilated, light and sound-attenuating chambers (Coulbourn Model E10–20). Six sets of infrared light sources and detectors were mounted opposite each other at 5.0 cm intervals along the acrylic walls of each box, 2.2 cm above the floor. The detectors were connected to a computer that recorded activity counts and amount of time spent on each side of the box. The conditioned stimuli (CSs) were interchangeable floors within the apparatus. These floors had two distinct textures—grid or hole. Grid floors were made of 2.3 mm stainless steel rods mounted 6.4 mm apart in acrylic rails. Hole floors were made from 16-gauge stainless steel perforated with 6.4 mm round holes on 9.5 mm staggered centers. This combination of floor textures was selected on the basis of previous studies demonstrating that drug naïve DBA/2J mice spend approximately equal time on each floor type during drug-free preference tests (e.g., Cunningham et al. 2003). For a more detailed description of the conditioning apparatus and general CPP procedure used in our laboratory, see Cunningham et al. (2006).

Ethanol Doses

Ethanol solutions were prepared from 95% stock solution using saline as the vehicle. In experiments 1 and 2, ethanol doses of 0.5, 2, or 4 g/kg (10–20%, v/v) were injected intraperitoneally in volumes of 6.25, 12.5, or 25.0 ml/kg, respectively. In experiment 3, ethanol doses of 0.5, 1.5, or 4 g/kg (20%, v/v) were injected in volumes of 3.125, 9.375, or 25.0 ml/kg, respectively.

Experimental design

Three experiments were conducted using an unbiased apparatus and procedure in order to characterize both the reference-dose and standard CPP procedures. Previous CPP experiments in our laboratory using the standard procedure had shown that 0.5 g/kg is too low to produce significant place preference after four drug pairings, whereas 4 g/kg, which is the highest dose we have used, has been found to produce a strong preference that is typically the same, or marginally stronger, than that produced by 2 g/kg in our standard procedure (unpublished observations). Thus, we examined doses between 0.5 and 4 g/kg in both in the standard and reference-dose procedures. Overall, standard procedures were performed with 0.5 (Exp. 1), 1.5 (Exp. 3), 2 (Exps. 1–2) and 4 (Exp. 2) g/kg, whereas reference-dose procedures involved

the following comparisons: 2-vs.-0.5 g/kg (Exp. 1), 2-vs.-4 g/kg (Exp. 2), 1.5-vs.-0.5 g/kg, 1.5-vs.-1.5 g/kg, and 1.5-vs.-4 g/kg (Exp. 3). Table 1 provides a summary of the design for each experiment.

Due to initial uncertainty about the optimal reference dose and limitations on the number of mice that could be run concurrently, our first two experiments explored the efficacy of a 2 g/kg reference dose with a comparison dose that was either lower (2.0-vs.-0.5 g/kg, Exp. 1) or higher (2-vs.-4 g/kg, Exp. 2) than the reference dose. Each of these studies also included groups that received each dose in a standard procedure for comparison (Exp. 1: 0.5-vs.-0 g/kg and 2-vs.-0 g/kg; Exp. 2: 2-vs.-0 g/kg and 4-vs.-0 g/kg). A reference dose of 2 g/kg was selected for these initial studies because it had previously been found to reliably produce CPP in mice (e.g., Cunningham et al. 2003) and because it appeared only slightly above the threshold dose for inducing conditioned place preference in previous studies using our apparatus and conditioning parameters (Risinger et al. 1994).

Although the reference-dose procedure in Experiment 1 (Group 2-vs.-0.5 g/kg) was more sensitive to the low ethanol dose than the standard procedure (Group 0.5-vs.-0 g/kg), the reference-dose procedure in Experiment 2 (Group 2-vs.-4 g/kg) failed to detect a difference between 2 vs. 4 g/kg. Because this outcome was unexpected and because there appeared to be a trend toward a weak conditioning effect in the initial cohort of Group 2-vs.-4 g/kg mice, we increased the sample size (see Table 1). However, we were still unable to find a significant effect. Thus, Experiment 3 was designed with a lower reference dose (1.5 g/kg) to determine whether we could enhance sensitivity to dose effects both above (1.5-vs.-4 g/kg) and below (1.5-vs. 0.5-g/kg) the reference dose. This experiment also included a group that received a standard procedure with 1.5 g/kg (i.e., 1.5-vs.-0 g/kg) and a group that received a reference-dose procedure in which the two doses were identical (1.5-vs.-1.5 g/kg). The latter group, a control group that was not included in either the Barr et al. (1985) or Bevins (2005) studies, was not expected to develop a significant place preference.

Procedure

Each experiment included three phases: habituation (one session), conditioning (eight sessions) and preference testing (one session). Sessions were conducted 5 days per week, with a 2-day break between the first four and last four conditioning sessions.

The habituation session was intended to adapt mice to handling, injection and the apparatus. Mice were weighed, injected with saline (12.5 ml/kg) and immediately placed in the conditioning box for 5 min on a smooth paper floor.

During the conditioning phase, mice within each dose group were randomly assigned to one of two conditioning subgroups and exposed to an unbiased Pavlovian conditioning procedure (Cunningham et al. 2003). These conditioning subgroups differed in terms of which dose was assigned to the grid floor, i.e., dose assignment was counterbalanced. For example, in the 2-vs.-0.5-g/kg group (Exp. 1), mice in the GRID₂ subgroup received injections of 2 g/kg ethanol paired with the grid floor and 0.5 g/kg ethanol paired with the hole floor. For the GRID_{0.5} subgroup, these contingencies were reversed, i.e., 0.5 g/kg ethanol was paired with the grid floor and 2 g/kg ethanol was paired with the hole floor. All experiments used a one-compartment training procedure such that the assigned floor cue was present on both sides and mice had access to the entire apparatus on every trial (Cunningham et al. 2006). Each mouse received four 5-min conditioning sessions with each floor type. One trial was given each day and order of exposure to each floor type was counterbalanced over days within each conditioning subgroup.

The place preference test was conducted 24 hrs after the last conditioning trial. Mice were weighed, injected with an intermediate volume of saline (12.5 ml/kg for Exps. 1 and 2; 9.375 ml/kg for Exp. 3) and placed in the apparatus for 30 min. The floor of the apparatus was half grid and half hole. Floor position (i.e., left versus right) was counterbalanced within each group. The primary dependent variable was the amount of time spent on the grid floor (Cunningham et al. 2003).

In experiments 1 and 2, we controlled overall exposure to ethanol within each experiment by giving a second injection in the home cage 60 min after the first injection during the conditioning phase. Specifically, all mice in Exp. 1 received a total of 2.5 g/kg ethanol over each pair of conditioning days, whereas all mice in Exp. 2 received a total of 6 g/kg. Mice in the standard groups (0.5-vs.-0, 2-vs.-0 and 4-vs.-0 g/kg) received their matching ethanol injection (0.5, 2 or 4 g/kg) on days when they received saline in the conditioning apparatus; saline was injected in the home cage on days when they received ethanol in the apparatus. Mice in the reference-dose groups (2-vs.-0.5 and 2-vs.-4 g/kg) received only saline injections in the home cage. Home-cage injections were omitted in Experiment 3 to simplify the dosing regimen and because a comparison of CPP in mice that received standard place conditioning with 2 g/kg in Exps. 1 and 2 showed similar preferences despite the between-experiment discrepancy in overall ethanol exposure. These data are also in agreement with a previous study (Cunningham et al. 2002b) that showed no differences in CPP expression between a group that received only the paired (CS+ trial) ethanol injections and groups that received extra ethanol injections (either 65 min before or after each CS+ trial). Thus, although mice from each experiment experienced different total amounts of ethanol exposure, we feel that it is reasonable to compare groups across experiments.

Data Analysis

Preference test data are presented and analyzed in two ways. First, raw grid time scores were analyzed by two-way analysis of variance (ANOVA) using Dose Group and Conditioning Subgroup as factors. As discussed elsewhere (Cunningham et al. 2003), grid time differences between the counterbalanced conditioning subgroups (e.g., GRID₂ vs. GRID_{0.5}) provide the best index of place conditioning in our unbiased design. Second, to facilitate comparisons across experiments, we collapsed over the conditioning subgroups and used one-way ANOVAs to analyze the percentage of the 30-min test that each comparison dose group spent on the comparison dose-paired floor under all possible reference dose conditions. The data from each of the dose groups was used twice for these analyses (except for the 1.5-vs.-1.5 group in Exp. 3), once with the first dose in the group label used as the *reference* dose and the second dose used as the *comparison* dose and again with those assignments reversed. Because there were differences in the number of groups tested at each reference dose and because comparisons between reference dose conditions were not statistically independent, the comparison-dose-effect relationship for each reference dose was analyzed separately by one-way ANOVA. Test session activity data were analyzed using one-way ANOVAs to examine the dose group effect. All post-hoc pair-wise comparisons were Bonferroni-corrected to limit overall alpha level to 0.05.

Results

Data from three mice (two from Exp. 2 and one from Exp. 3) were removed due to procedural errors. Data from three other mice that died during Exp. 2 were also removed. Final group sizes are shown in Table 1.

Preference Test: Grid Times

The primary goals of the grid time analyses were to determine which dose groups yielded significant CPP and to compare dose groups within each experiment. The conditioning subgroup means (sec/min $\pm$ SEM) for each experiment are shown in Table 1, which also summarizes the outcomes of the two-way ANOVAs and post-hoc pairwise comparisons. These comparisons confirmed the development of significant CPP in the groups that received standard place conditioning with 1.5 (Exp. 3), 2 (Exps. 1 and 2) or 4 (Exp. 2) g/kg, but not in the group that received standard place conditioning with 0.5 g/kg (Exp. 1, Bonferroni-corrected $p = .13$). Moreover, these analyses showed significant place conditioning in the reference-dose groups that were conditioned with 2-vs.-0.5 g/kg (Exp. 1) and with 1.5-vs.-4 g/kg (Exp. 3), indicating that mice can distinguish between the rewarding values of the doses used in each of these combinations. However, there was no significant place conditioning in the groups that were conditioned with 2-vs.-4 g/kg (Exp. 2), 1.5- vs.-0.5 g/kg (Exp. 3) or 1.5-vs.-1.5 g/kg (Exp. 3), suggesting that the doses in each of these combinations had undetectable differences in rewarding value.

To further examine the sources of the interactions in the overall ANOVAs, separate two-way (Dose Group $\times$ Conditioning Subgroup) ANOVAs were applied to the data from all possible pair-wise combinations of dose groups within each experiment. The brackets in the middle column of Table 1 indicate the significant interactions from these analyses. In Exp. 1, these analyses showed that Group 2-vs.-0.5 g/kg developed a weaker preference than Group 2-vs.-0 g/kg [$F(1, 59) = 4.3, p < .05$], indicating that preference for the floor paired with 2 g/kg was significantly reduced when the other floor was paired with 0.5 g/kg instead of saline. Thus, although 0.5 g/kg failed to produce a significant CPP in the standard procedure (Group 0.5-vs.-0 g/kg), this comparison between the reference-dose and standard procedure provides evidence that 0.5 g/kg has a rewarding value greater than saline. In addition, these analyses showed that Group 2-vs.-0 g/kg developed a stronger preference than Group 0.5-vs.-0 g/kg [$F(1, 59) = 5.4, p < .05$], thereby providing evidence of a dose effect between the groups given the standard procedure.

In Exp. 2, the two-way follow-up ANOVAs showed that both groups exposed to the standard procedure developed stronger CPP than the 2-vs.-4 g/kg reference-dose group [Group 2-vs.-0 g/kg: $F(1, 152) = 35.1, p < .0001$; Group 4-vs.-0 g/kg: $F(1, 120) = 27.9, p < .0001$]. However, there was no significant interaction in the comparison between 2-vs.-0 and 4-vs.-0 g/kg groups [$F < 1$], indicating no dose effect between groups given the standard procedure. Thus, the conclusion of no difference between the effects of 2 and 4 g/kg ethanol was confirmed both in the standard and reference-dose procedures.

In Exp. 3, the standard procedure group (Group 1.5-vs.-0 g/kg) expressed stronger CPP than the reference-dose groups that received comparison doses of 1.5-vs.-0.5 g/kg [$F(1,44) = 13.9, p < .001$] or 1.5-vs.-1.5 g/kg [$F(1,44) = 16.6, p < .001$]. Thus, as in Exp. 1, the significant difference between the reference-dose group that received 0.5 g/kg (Group 1.5-vs.-0.5 g/kg) and the standard-procedure group (Group 1.5-vs.-0 g/kg) provides further evidence that 0.5 g/kg has a rewarding value greater than saline. However, the absence of CPP in the 1.5-vs.-0.5 g/kg group (as well as the lack of difference between the 1.5-vs.-0.5 and 1.5-vs.-1.5 g/kg groups) suggests that mice could not distinguish between the rewarding effects of 0.5 and 1.5 g/kg. The follow-up ANOVAs also showed that the reference-dose group that received 4 g/kg (i.e., 1.5-vs.-4 g/kg group) differed significantly from all other groups [Group 1.5-vs.-0 g/kg: $F(1,43) = 61.1, p < .0001$; Group 1.5-vs.-0.5: $F(1,43) = 13.4, p < .001$; Group 1.5-vs.-1.5 g/kg: $F(1,43) = 9.7, p < .004$], indicating that mice could distinguish between the rewarding effects of 4 g/kg and the rewarding effects of the lower doses (0, 0.5 and 1.5 g/kg).

Preference Test: Percent Time on Comparison Dose-Paired Floor

As noted earlier, to facilitate comparisons across experiments, data were collapsed over conditioning subgroups and depicted as the percentage of the 30-min test that each dose group spent on the comparison dose-paired floor for all possible reference-comparison dose combinations (see Figure 1). As can be seen, there was a positive comparison-dose-effect relationship for all of the reference dose conditions. Separate one-way ANOVAs at each reference dose yielded a significant comparison dose effect at all reference doses except reference dose 0 [Ref 0: $F(4, 174) = 1.8$, $p \pm .1$; Ref 0.5: $F(2, 85) = 6.8$, $p < .002$; Ref 1.5: $F(3, 91) = 16.0$, $p < .0001$; Ref 2: $F(3, 215) = 16.9$, $p < .0001$; Ref 4: $F(2, 144) = 8.0$, $p < .001$].

In general, post-hoc pair-wise tests confirmed conclusions derived from the analyses of grid time data. At the 0.5 g/kg reference dose, there was a significant difference between the 0 and 2 g/kg comparison dose groups ($p < .002$). At the 1.5 g/kg reference dose, post-hoc tests indicated significant differences between the 0 g/kg comparison dose group and the 0.5, 1.5 and 4 g/kg comparison dose groups (p 's $< .005$). Also, the 4 g/kg comparison dose group differed from the 0.5 and 1.5 g/kg comparison dose groups (p 's $< .02$). At the 2 g/kg reference dose, both 0 g/kg comparison dose groups and the 0.5 g/kg comparison dose group differed from the 4 g/kg comparison dose group (p 's $< .02$). Finally, at the 4 g/kg reference dose, there was a significant difference between the 0 and 2 g/kg comparison dose groups ($p < .001$).

The failure to see a significant dose effect at reference dose 0 is likely due to the absence of a comparison dose 0 group (i.e., saline vs. saline). Several previous studies have shown a significant ethanol dose effect in the standard CPP procedure when a saline-only group is compared to a group that receives an ethanol dose of 2 g/kg (e.g., Cunningham et al. 2003).

Test Session Activity

In Exps. 1 and 2, mean test activity rates (Table 1) were generally higher in groups that had previously received ethanol injections on both types of conditioning trials (i.e., the reference-dose groups) than in groups that had received saline on one of the conditioning trials (i.e., the standard procedure groups). In Exp. 3, however, only Group 1.5-vs.1.5 g/kg differed from Group 1.5 vs. 0 g/kg. One-way ANOVAs yielded a significant dose group effect in all three experiments (Table 1). Pair-wise follow-up comparisons indicated that mice in the reference-dose procedures tended to show more conditioned activity during the preference tests than the mice in the standard procedures (Table 1). The finding of greater test session activity in the reference-dose groups might reflect stronger conditioning of ethanol-induced activation to general apparatus cues (Cunningham and Noble 1992) as a result of receiving twice as many ethanol injections in the conditioning box compared to the standard procedure groups.

Discussion

These experiments show that by using a reference-dose procedure or a combination of reference-dose and standard procedures, one can demonstrate graded dose effects in the place-conditioning task. Moreover, in contrast to previous studies reported by Barr et al. (1985) and Bevins (2005), these studies illustrate the advantage of using the reference-dose procedure for making comparisons to doses both above and below the reference dose. If one focuses only on findings from groups that received the standard procedure (i.e., ethanol vs. saline), the conclusion from our studies would be that ethanol doses of 1.5, 2 and 4 g/kg yielded similar levels of CPP, whereas 0.5 g/kg did not produce significant CPP, suggesting that the former doses are equally rewarding while the latter dose has no appreciable rewarding effect. However, by using the reference-dose procedure, we were able to extend our understanding of the ethanol dose-effect function in two important ways. First, our finding that the reference-dose groups that received 0.5 g/kg ethanol in combination with intermediate ethanol doses (i.e., Group 2-

vs. -0.5 g/kg in Exp. 1 and Group 1.5-vs. -0.5 g/kg in Exp. 3) developed significantly weaker preference than standard-procedure groups that received only the higher dose (i.e., Groups 2-vs. -0 g/kg and 1.5-vs. -0 g/kg, respectively) implies that mice distinguished between the effects of 0.5 g/kg ethanol and saline, a finding that was not apparent from the standard procedure alone. Second, the finding of significant preference in Group 1.5-vs. -4 g/kg shows that the reference-dose procedure was able to detect a difference between an intermediate and high ethanol dose that was not detected in the standard procedure.

Our studies support the following conclusions about the relative rewarding efficacy of various ethanol doses in DBA/2J mice as indexed by the CPP procedure. Starting at the low end of the dose-effect curve, we conclude that 0.5 g/kg is more rewarding than saline. The lack of conditioning in Group 1.5-vs. -0.5 g/kg suggests that the rewarding effects of 0.5 and 1.5 g/kg ethanol cannot be distinguished. However, the significant conditioning effect in Group 2-vs. -0.5 g/kg indicates that DBA/2J mice can distinguish between the rewarding effects of 0.5 and 2 g/kg ethanol. Finally, at the high end of the dose-effect curve, the lack of significant conditioning in Group 2-vs. -4 g/kg suggests difficulty in distinguishing between the rewarding effects of 2 and 4 g/kg, whereas the significant conditioned effect in Group 1.5-vs. -4 g/kg indicates that DBA/2J mice are able to distinguish between the effects of 1.5 and 4 g/kg.

By comparing the results of multiple standard and reference-dose procedures, the current experiments were able to more fully characterize ethanol's effects across a wide range of doses. These experiments also illustrate the importance of examining more than one reference dose to characterize CPP dose effects. For example, the combined results of Exps. 1 and 2 showed that comparisons to a reference dose of 2 g/kg could detect a difference between the reference dose and a lower comparison dose (0.5 g/kg), but not a higher comparison dose (4 g/kg). In contrast, by slightly reducing the reference dose to 1.5 g/kg in Exp. 3, we were able to detect a difference between the reference dose and a higher comparison dose (4 g/kg), but not a lower comparison dose (0.5 g/kg). Nevertheless, in both cases, by combining the results from reference-dose and standard procedures, we were able to infer that 0.5 g/kg was more rewarding than saline, even though the standard procedure with 0.5 g/kg failed to yield significant CPP.

One issue that must be considered in the interpretation of these studies is whether the greater sensitivity of the reference dose procedure was due, in part, to the higher test session activity produced by the reference-dose procedure. For example, one might argue that the lower CPP in the reference-dose group in Exp. 1 (Group 2-vs. -0.5 g/kg) relative to the 2 g/kg standard procedure group (Group 2-vs. -0 g/kg) was a byproduct of the greater test session activity level in the reference-dose group, a possibility that is generally consistent with recent studies showing an inverse relationship between test session activity and CPP magnitude (Gremel and Cunningham 2007). Although a possible contribution of test activity cannot be dismissed, several findings argue against this interpretation. For instance, the ability of 0.5 g/kg to offset the rewarding effect of a 1.5-g/kg injection in the reference dose procedure (Group 1.5-vs. -0.5 g/kg compared to Group 1.5-vs. -0 g/kg in Exp. 3) was not accompanied by a significant group difference in test activity. Moreover, the reference-dose procedure that successfully detected a difference between 1.5 and 4 g/kg (Group 1.5-vs. -4 g/kg in Exp. 3) produced a test activity rate that was similar or greater than that produced in standard procedures with either dose alone, an outcome contrary to predictions based simply on group differences in test session activity.

As in previous CPP dose-effect studies with other abused drugs (e.g., cocaine: Bevins 2005; morphine: Barr et al. 1985), our study showed a monotonic dose-effect curve for ethanol-induced CPP. These findings contrast with the biphasic dose-effect relationship typically reported for self-administration of ethanol (e.g., Meisch and Thompson 1974) and other abused drugs (e.g., Yokel 1987). The decrease in rate of self-administration that occurs at higher doses has been explained in several ways, including satiation, drug-induced interference with

responding and aversive effects of high drug doses (Wise 1987; Yokel 1987). Although CPP has been shown to be sensitive to high dose aversive drug effects (e.g., methamphetamine: Cunningham and Noble 1992), it is presumably less sensitive to satiation and response interference because animals are tested in the absence of drug.

One potential advantage of the reference-dose procedure over the standard CPP procedure is that the reference-dose procedure may be able to detect small changes in the rewarding effects of ethanol produced by pharmacological manipulations. For example, if a treatment drug co-administered with an ethanol dose of 2 g/kg produced only a small decrease in ethanol reward, a standard CPP procedure might be insensitive to this manipulation, suggesting that the treatment drug had no effect. However, if the same treatment drug were co-administered with a low comparison dose of ethanol in a reference-dose procedure (e.g., 2 g/kg + vehicle vs. 0.5 g/kg + treatment drug), a small drug-induced decrease in ethanol reward might have a greater likelihood of being detected. In this case, one would actually expect the treatment drug to increase CPP (by reducing the effect of the low comparison dose) relative to a reference-dose group given vehicle treatment (e.g., 2 g/kg + vehicle vs. 0.5 g/kg + vehicle).

A variation on this strategy would be to co-administer the treatment drug with a comparison dose that is identical to the reference dose (e.g., 2 g/kg + vehicle vs. 2 g/kg + treatment drug). Because no CPP is expected in the control condition (2 g/kg + vehicle vs. 2 g/kg + vehicle), a treatment drug effect would be manifest either by development of preference for the compartment paired with the reference dose (indicating that the treatment drug had reduced the rewarding effect of the comparison ethanol dose) or aversion for that compartment (indicating that the treatment drug had enhanced the rewarding effect of the comparison ethanol dose). In fact, this approach was recently used successfully to characterize a treatment that reduced ethanol reward. Specifically, Font et al. (2006) reported that Swiss mice developed a significant preference for a tactile cue previously paired with an ethanol reference dose of 2 g/kg relative to a cue that had been paired with the same ethanol comparison dose combined with D-penicillamine, a drug that reduces the ethanol metabolite acetaldehyde.

Theoretical Analysis of Dose Effects on Performance of CPP

One theoretical approach for understanding why the standard and reference-dose procedures might yield different conclusions is illustrated in Figure 2, which depicts hypothetical values of the stimulus-ethanol association (Associative Strength, V) as a function of increasing numbers of conditioning trials for each ethanol dose used in the standard procedure (solid lines). A key assumption in this analysis is that dose-effects on performance in the standard CPP procedure may be obscured either by a response floor or response ceiling, represented by the shaded regions in Figure 2. The potential problem can be seen most clearly after a relatively large number of trials (C). Because asymptotic associative strength (V) exceeds the response ceiling, doses of 1.5 g/kg and higher are all assumed to produce the same (maximal) effect on CPP performance. In contrast, because the asymptotic V value produced by 0.5 g/kg is assumed to be near the response floor, CPP performance is expected to be marginal or non-significant.

Predictions for the reference-dose groups (dashed lines in Fig. 2) are based on the arithmetic differences in associative strength between cues that were paired with different combinations of ethanol doses in the reference dose procedure. It was assumed that the associative value for each cue was the same as that produced when the paired dose was used in a standard CPP procedure. Regardless of the amount of training, one can use this analysis to explain why the reference-dose procedure was able to detect effects of the lowest ethanol dose. More specifically, as shown in Figure 2, the dashed lines for the reference dose groups that received 2-vs.-0.5 or 1.5-vs.-0.5 g/kg both fell below the response ceiling, which would explain why CPP performance in those groups was weaker than that seen in the groups that received the standard procedure with either of the higher doses alone. The finding of significant CPP in the

2-vs.-0.5 g/kg group is consistent with the placement of their dashed line in the middle of the performance range whereas the absence of CPP in the 1.5-vs.-0.5 group is consistent with the placement of their dashed line below the response floor. Moreover, the ability to detect a difference between 1.5-vs.-4 g/kg in the reference dose procedure is explained by the positioning of that dashed line below the response ceiling in the middle of the performance range.

Although the foregoing analysis is admittedly post hoc, it yields several predictions that could be tested in future studies. For example, the ability to detect differences between high ethanol doses in the standard procedure should be greater after a relatively low number of conditioning trials, before associative strength has reached the response ceiling (e.g., point A in Fig. 2). Based on previous studies suggesting that CPP performance produced by 2 g/kg in the standard procedure is below asymptote after two CS+ conditioning trials (Cunningham et al. 1997, 2002b), our analysis would predict that a standard procedure should be more likely to reveal differences between high ethanol dose groups after only one or two trials. Nevertheless, because CPP learning is so rapid, it may be difficult to find the exact amount of training required to optimize the dose-effect curve in the standard procedure. An alternative strategy for assessing dose effects in the standard procedure might be to examine differences in rate of extinction between groups that have been trained to asymptotic levels of performance. In general, higher doses would be expected to produce greater resistance to extinction because more CS exposure would be needed to induce sufficient inhibitory learning to offset associative strength below the performance response ceiling. Because the rate of CPP extinction generally appears to be slower than the rate of CPP acquisition (e.g., Cunningham et al. 1998), the extinction strategy might be better for revealing dose effects.

The theoretical analysis outlined here is based on the simplifying assumption that all other variables known to affect CPP magnitude are held constant. It should be noted, however, that variations in many other conditioning parameters can shift the CPP dose-effect curve. One such parameter is conditioning trial duration, which is inversely related to the strength of CPP conditioned by 2g/kg ethanol in the range between 5 and 30 min (Cunningham and Prather 1992). In fact, an early ethanol dose-effect study that used 30-min trials showed significant CPP at 3 and 4 g/kg, but not at 1 or 2 g/kg (Cunningham et al. 1992), suggesting that the ethanol dose-effect curve is shifted to the right when longer trial durations are used. Route of administration is another important variable that influences the dose-effect curve. For example, in contrast to the strong CPP produced at both 2 and 4 g/kg when ethanol was administered intraperitoneally in the present studies, only 4 g/kg was able to induce CPP when ethanol was infused intragastrically in a previous study (Cunningham et al. 2002a), again suggesting a rightward shift in the dose-effect curve.

In summary, the present experiments have confirmed the utility of the reference-dose procedure for studying ethanol dose effects in the CPP procedure and have offered insight into alternative strategies for assessing the rewarding properties of ethanol, as well as other drugs of abuse, in a CPP framework. Standard CPP procedures showed that ethanol doses of 1.5, 2, and 4 g/kg elicited similar levels of preference, whereas a dose of 0.5 g/kg was unable to induce a significant preference. However, the reference-dose CPP procedure was able to distinguish between the rewarding effects of higher doses (1.5 vs. 4 g/kg) and, when used in combination with a standard procedure, revealed rewarding effects of a relatively low dose (0.5 g/kg). Thus, the reference-dose procedure provides a potentially more sensitive method for detecting small differences or changes in ethanol's rewarding effect and may serve as an important alternative or supplement to the standard CPP procedure.

Acknowledgments

This research was supported by NIH-NIAAA grants AA07468 and AA07702. Thanks are extended to Jessica Knowles for assistance in data collection and to Christina Gremel and Charlene Voorhees for comments on the manuscript.

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Figure 1.

Mean ($\pm$ SEM) percent time spent on the comparison dose-paired floor (collapsed across conditioning subgroups). Each symbol depicts a different reference (Ref) dose condition. A second-order polynomial curve was drawn for each reference dose merely to facilitate visual comparison across reference doses. No attempt was made to determine best-fit curves. Symbols without error bars represent instances where it was assumed (but not tested) that equivalent reference and comparison doses would yield no place conditioning (i.e., preference = 50%). All other symbols reflect data shown in Table 1.

Figure 2.

This figure depicts hypothesized strength (V) of the stimulus-ethanol association as a function of number of conditioning trials. Solid lines represent associative strength conditioned to the CS+ by increasing doses of ethanol (0.5, 1.5, 2.0 and 4.0 g/kg) in a standard CPP procedure. These curves were generated using the Rescorla-Wagner (1972) equation for growth in associative strength, $\Delta V = k(\lambda - V)$, where ΔV is the change in associative strength on each trial, k is a learning rate parameter, and λ is the dose-dependent asymptote for conditioning. The value of the learning rate parameter was arbitrarily set at 0.09 and it was assumed that increases in ethanol dose produced a monotonic increase in the learning asymptote (λ was set at 0.65, 1.2, 1.6 and 2.0 for ethanol doses of 0.5, 1.5, 2.0 and 4.0 g/kg, respectively). Dashed lines represent the arithmetic differences in associative strength between cues that were paired with various combinations of ethanol doses in a reference dose procedure. It was assumed that the associative value for each cue was the same as that produced when the paired dose was used in a standard CPP procedure. The shaded portions of the figure shown below the “response floor” or above the “response ceiling” depict conditions in which differences in associative strength do not produce differences in CPP performance. That is, below the response floor, no value of associative strength will produce significant CPP whereas above the response ceiling, all values of associative strength will produce a similar, maximal level of CPP. The un-shaded portion of the figure (“performance range”) indicates the conditions in which CPP performance is generally assumed to vary with changes in associative strength. Within this range, no specific relationship between associative strength and CPP performance is assumed except that CPP performance should be positively related to associative strength. Points A, B and C represent training after a low, intermediate or high number of conditioning trials. See Discussion section for further explanation.

Table 1
Experimental Designs, Ethanol Dose Conditions, Preference Test Grid Time Means and Grid Time ANOVAs

Exp	Dose Group (g/kg)	Conditioning Subgroup	n	Time (sec/min) on Grid Floor (mean ± SEM)	Post-hoc tests ¹	% Time on 1 st Dose Floor	% Time on 2 nd Dose Floor	Preference Test Activity counts/min (mean ± SEM)	Post-Hoc tests ²		
1	0.5 vs. 0	GRID _{0.5}	16	37.7 ± 3.3	}	ns	58.0 ± 4.7	42.0 ± 4.7	}		
		GRID ₀	16	28.1 ± 4.6							
	2 vs. 0	GRID ₂	16	45.4 ± 2.9			72.3 ± 3.8	27.7 ± 3.8		35.2 ± 1.6	
		GRID ₀	15	18.8 ± 3.6							
	2 vs. 0.5	GRID ₂	16	39.9 ± 3.0			61.8 ± 3.4	38.2 ± 3.4		42.7 ± 1.6	
		GRID _{0.5}	16	25.8 ± 2.6							
1				Dose Group: F(2,89) = 0.03 Conditioning Subgroup: F(1,89) = 37.0 ^{*****} Interaction: F(2,89) = 3.4 [*]				Dose Group: F(2,92) = 13.3 ^{*****}			
2	2 vs. 0	GRID ₂	32	42.5 ± 3.3	}	****	71.5 ± 3.7	28.5 ± 3.7	}		
		GRID ₀	30	16.7 ± 3.1						29.6 ± 1.2	
	4 vs. 0	GRID ₄	16	36.1 ± 4.9			71.5 ± 5.0	28.5 ± 5.0		30.3 ± 2.1	
		GRID ₀	14	9.4 ± 1.8							
	2 vs. 4	GRID ₂	47	29.5 ± 1.9			ns	46.9 ± 2.3		53.1 ± 2.3	42.6 ± 1.1
		GRID ₄	47	33.2 ± 1.9							
2				Dose Group: F(2,180) = 3.7 [*] Conditioning Subgroup: F(1,180) = 42.8 ^{*****} Interaction: F(2,180) = 22.3 ^{*****}				Dose Group: F(2,183) = 37.1 ^{****}			
3	1.5 vs. 0	GRID _{1.5}	12	43.0 ± 2.5	}	****	69.7 ± 2.8	30.3 ± 2.8	}		
		GRID ₀	12	19.3 ± 2.4						36.5 ± 2.3	
	1.5 vs. 0.5	GRID _{1.5}	12	31.4 ± 2.2			ns	53.3 ± 3.3		46.7 ± 3.3	42.8 ± 1.2
		GRID _{0.5}	12	27.4 ± 3.4							
	1.5 vs. 1.5	GRID _{1.5}	12	29.8 ± 3.0			ns	48.8 ± 3.5 ³		45.9 ± 2.6	

Exp	Dose Group (g/kg)	Conditioning Subgroup	n	Time (sec/min) on Grid Floor (mean ± SEM)	Post-hoc tests ¹		% Time on 1 st Dose Floor	% Time on 2 nd Dose Floor	Preference Test Activity counts/min (mean ± SEM)	Post-Hoc tests ²
	1.5 vs. 4	GRID _{1.5}	12	28.3 ± 3.0	****		36.8 ± 3.8	63.2 ± 3.8	38.5 ± 1.4	
		GRID _{1.5}	12	27.9 ± 2.7						
		GRID ₄	11	44.3 ± 2.8						
3	Dose Group: F(3,87) = 2.7* Conditioning Subgroup: F(1,87) = 2.7 Interaction: F(3,87) = **** Dose Group: F(3,91) = 4.6***									

¹ Brackets indicate significant differences between Dose groups based on Dose Group × Conditioning Subgroup interaction (p < .05)

² Brackets indicate significant differences between Dose groups based on post-hoc F-tests (Bonferroni-corrected p < .05).

³ Because the same dose was paired with both floors, only one mean is shown for the 1.5 vs. 1.5 g/kg dose group

* p < .05,

** p < .01,

*** p < .001,

**** p < .0001 (p-values for all conditioning subgroup comparisons are Bonferroni-corrected within each experiment)