Interstimulus interval determines whether ethanol produces conditioned place preference or aversion in mice

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DBA/2J mice were exposed to a distinctive floor stimulus (CS+) and ethanol (2 g/kg) in a place conditioning paradigm. A different floor stimulus (CS-) was presented with saline. Mice injected just before or 30 min before CS exposure (Groups 0, -30) showed conditioned place preference, whereas mice injected right after exposure to the CS (Group 5) displayed place aversion (Experiment 1). None of the other groups (-120, -60, 15, 60) showed place conditioning. Handling and saline injection given just before or after CS exposure were unable to produce place conditioning (Experiment 2). However, there was a positive relationship between ethanol concentration (10% vs. 20%) and test performance, suggesting that peritoneal irritation influences place conditioning (Experiment 3). Overall, these findings support the suggestion that intraperitoneal injection of ethanol produces an initial short-duration aversive effect that is followed by a longer lasting positive motivational effect.

The place conditioning task is widely used to study the motivational effects of drugs in rats and mice (see reviews by Bozarth, 1987; Carr, Fibiger, & Phillips, 1989; Hoffman, 1989; Schechter & Calcagnetti, 1993; Swerdlow, Gilbert, & Koob, 1989; van der Kooy, 1987). Operationally, the training phase for this task involves a Pavlovian conditioning procedure in which a distinctive set of environmental stimuli serving as the conditioned stimulus (CS) is paired with administration of a drug that serves as the unconditioned stimulus (US) (Cunningham, 1993). The effect of this conditioning is typically assessed by measuring the amount of time that animals spend in contact with the drug-paired CS when offered a choice between spatial locations that contain the CS or an alternative set of environmental stimuli. Because the drug is not presented, the test can be viewed as providing a measure of the conditioned reinforcing or punishing value of the drug-paired CS. Approach toward or withdrawal from the target CS during testing presumably results from the instrumental contingency between spatial choice and exposure to the CS, or it might emerge as a result of sign-tracking.

A Pavlovian analysis of the place conditioning task has been supported by studies showing that test performance is positively related to the number of conditioning trials

(e.g., Mucha & Iversen, 1984) and to US magnitude (i.e., drug dose) (e.g., Cunningham, Niehus, Malott, & Prather, 1992; Mucha & Iversen, 1984; Risinger, Malott, Prather, Niehus, & Cunningham, 1994; White, Messier, & Carr, 1987). A Pavlovian analysis is also consistent with studies showing an influence of the temporal relationship between exposure to the environmental CS and drug US. The most commonly used temporal arrangement has involved "simultaneous" exposure to both events (i.e., the drug is injected immediately before placement of the subject in the CS environment). When exposure to the CS is delayed following drug administration (i.e., backward conditioning), conditioned place preference has sometimes been reported at intervals of up to 2 h (but not 4 h) with an amphetamine US (Reicher & Holman, 1977; Sherman, Roberts, Roskam, & Holman, 1980; but see Wall, Hinson, Schmidt, Johnston, & Streather, 1990). With nicotine, however, backward intervals as short as 20 min have yielded no place conditioning (Fudala & Iwamoto, 1986).

Introducing a forward delay between the CS and US by administering drug when the animal is removed from the CS environment has been found to eliminate the conditioned place preference normally produced by diazepam (Spyraki, Kazandjian, & Varonos, 1985) or morphine (Bardo, Miller, & Neisewander, 1984; Kumar, 1972; Sherman, Pickman, Rice, Liebeskind, & Holman, 1980). Such findings are easily explained in terms of an unfavorable interstimulus interval (ISI) or by the lack of overlap between the CS and the drug's effects (Broadbent & Cunningham, 1996). However, a few recent studies involving forward delays have yielded unexpected outcomes. In these cases, two drugs known to produce conditioned place preference in the standard "simultaneous" procedure (amphetamine or nicotine) were found to produce a condi-

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tioned place *aversion* when given after termination of the CS (Fudala & Iwamoto, 1986, 1987, 1990; Wall et al., 1990). Although a Pavlovian analysis clearly predicts a decrement in conditioning as the ISI is increased (Mackintosh, 1974), it has difficulty explaining a reversal in direction of the conditioned response unless one assumes that the US has bivalent effects.

The present set of experiments was initiated to extend the study of CS-US interval effects in place conditioning to an ethanol US. Several recent studies have shown that administration of an ethanol US immediately before CS exposure produces a robust conditioned place preference in many different mouse strains (e.g., Cunningham, 1995; Cunningham et al., 1992; Risinger et al., 1994), but no study has systematically examined the temporal relationship between CS and ethanol. In Experiment 1, we assessed a wide range of forward and backward CS-US intervals and established that post-CS administration of ethanol, like nicotine and amphetamine, produced a conditioned place aversion. In Experiments 2 and 3, we examined two possible explanations of this effect.

EXPERIMENT 1 Effect of Interstimulus Interval

Experiment 1 summarizes the results of three separate place conditioning studies (Experiments 1A–1C) in which the time interval between ethanol injection and exposure to the CS+ was varied (see Table 1). All three studies were conducted by the same experimenter within a consecutive 2-month period of time. In Experiment 1A, ethanol was administered 30 min before (Group –30), immediately before (Group 0), or 5 min after (Group 5)

the onset of 5-min exposure to the CS+. In Experiment 1B, the Group 5 treatment was replicated, and two additional forward delay intervals, 15 and 60 min (Groups 15 and 60), were examined. In Experiment 1C, the Group –30 treatment was replicated, and two additional backward delay intervals, –60 and –120 min (Groups –60 and –120), were examined. Preliminary statistical analyses indicated that the replicated groups (i.e., Groups 5 and –30) showed equivalent test performance across studies. In order to simplify presentation and to control overall alpha level, the data from Experiments 1A–1C have been combined.

Method

Subjects

Adult male inbred mice (DBA/2J) were shipped from the Jackson Laboratory (Bar Harbor, ME) at 6 weeks of age and allowed to acclimate to the animal colony for 2 weeks before training. They were housed in groups of 4 at an ambient temperature of $21^{\circ}\pm 1^{\circ}\text{C}$. Water and lab chow were freely available in the home cage. Experimental procedures were conducted during the light phase of a 12:12-h light:dark cycle (lights on at 0700).

Apparatus

The apparatus consisted of 12 identical Plexiglas and aluminum boxes ($30 \times 15 \times 15$ cm) enclosed in separate ventilated light- and sound-attenuating enclosures (Coulbourn Instruments Model E10-20). General activity and position were recorded automatically by computer using infrared detectors. The floor of each box consisted of interchangeable halves made of one of two textures. The "grid" floor was composed of 2.3-mm stainless steel rods mounted 6.4 mm apart in Plexiglas rails. The "hole" floor was made from perforated stainless steel (16 ga) 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 showing that control groups given saline injections paired with both sides spend about half their time on each floor type during preference tests (Cunningham, 1995; Cunningham et al., 1992). The floors and inside of the box were wiped with a damp sponge and the litter paper beneath the floors was changed after each animal.

Procedure

These experiments involved three phases: habituation (one session), conditioning (eight sessions), and testing (one session). Sessions were conducted 5 days a week with a 2-day break between the first four and second four conditioning sessions.

Habituation. The habituation session was intended to reduce the novelty and stress associated with handling and exposure to the apparatus. Each mouse was weighed and placed in the conditioning box on a smooth floor covered with paper for 5 min. Subjects were not exposed to the distinctive floor textures, in order to avoid latent inhibition.

Conditioning. During the conditioning phase, mice were randomly assigned to one of seven ISI groups: –120, –60, –30, 0, 5, 15, or 60 min (see Table 1). Within each of these groups, mice were randomly assigned to one of two conditioning subgroups ($n = 15$ –16/group) and exposed to a Pavlovian discrimination conditioning procedure. On alternate days, mice in the GRID+ subgroups received an intraperitoneal (i.p.) injection of ethanol (2 g/kg, 20% v/v in saline vehicle) before or after placement on the grid floor (CS+ trial), and an injection of saline before or after placement on the hole floor (CS– trial). These contingencies were reversed for mice in the GRID– subgroups. The subjects were weighed immediately before each injection. On all conditioning trials, the subjects had access to both sides of the apparatus and the floor texture was homogeneous. Four 5-min conditioning trials of each type were given over an 8-day

Table 1
Design for Experiment 1

Experiment	CS-US Delay	Conditioning Group	<i>n</i>
1A	–30	GRID+	16
	–30	GRID–	16
	0	GRID+	16
	0	GRID–	16
	5	GRID+	16
	5	GRID–	16
1B	5	GRID+	15
	5	GRID–	15
	15	GRID+	16
	15	GRID–	16
	60	GRID+	16
	60	GRID–	16
1C	–120	GRID+	16
	–120	GRID–	16
	–60	GRID+	16
	–60	GRID–	16
	–30	GRID+	16
	–30	GRID–	16

Note.—The delay represents the time interval (in minutes) between the beginning of the 5-min conditioning trial (CS) and injection of ethanol (US). Group 0 received ethanol immediately before placement in the chamber, whereas Group 5 received ethanol immediately after removal from the chamber. Subjects in the longer forward or backward delay groups spent the interval between CS and US exposure in the home cage.

period; order of exposure to the CS+ and CS- was counterbalanced within each subgroup. Because the two conditioning subgroups within each ISI group were matched for overall exposure to each floor type, ethanol and saline, and differed only in the floor-drug contingency, differences between the subgroups during preference testing should be attributed to learning (Cunningham, 1993).

Place preference test. A 60-min floor preference test was given 24 h after the last conditioning trial. The subjects were not weighed or injected before testing. The apparatus was configured with half grid floor and half hole floor. The relative position of the floors (i.e., left vs. right) was counterbalanced within each subgroup. The primary dependent variable was the amount of time spent on the grid floor during the test session.

Results

Activity and preference test data were initially evaluated by analysis of variance (ANOVA; $\alpha < .05$). Probability values reported for post hoc pairwise comparisons are Bonferroni corrected.

Conditioning Trials

Because of a computer error, activity data from part of the first conditioning trial were not available for 4 subjects from each of the three groups in Experiment 1B. Those subjects were omitted from analyses that involved the missing data, but were included in all other analyses.

CS+ (ethanol). Mean activity counts per minute on the first and last CS+ (ethanol) trials are shown for each of the ISI groups in Figure 1 (data are collapsed across GRID+ and GRID- subgroups). In accord with previous studies (e.g., Cunningham & Prather, 1992), ethanol-stimulated activity was greatest during the 5-min period

immediately after injection (Group 0) and decreased over time in the groups whose activity was measured for 5 min at various time points after ethanol injection (Groups -30, -60, and -120). In contrast, groups that were exposed to ethanol after their 5-min trial (i.e., Groups 5, 15, and 60) showed relatively low activity levels in the range seen on CS- trials (see next section). Repeated exposure to the conditioning procedure produced changes in activity on CS+ trials in most groups. However, direction of the change between Trials 1 and 4 varied as a function of ISI. Groups 0 and -30 showed an increase in ethanol-stimulated activity, suggesting development of sensitization to ethanol's activating effect (Cunningham & Noble, 1992). With the exception of Group -60, all of the remaining groups showed a decrease in activity over trials, consistent with the development of either ethanol tolerance or habituation to the conditioning apparatus.

A two-way mixed ANOVA (7 ISI groups $\times$ 2 trials) yielded significant main effects of ISI [$F(6,273) = 369.9$, $p < .001$] and trial [$F(1,273) = 11.2$, $p < .001$], and a significant ISI $\times$ trial interaction [$F(6,273) = 41.2$, $p < .001$]. Separate analyses indicated that the ISI effect was significant on both trials (both F s > 167 , $p < .001$). Pairwise group comparisons on each trial indicated that the differences between all group pairs were significant (all p s $< .005$), except within the subset comprising Groups 5, 15, and 60. Within-group comparisons showed a significant difference between Trial 1 and Trial 4 in all but Group -60 (all p s $< .001$).

CS- (saline). Figure 2 depicts mean activity on the first and last CS- (saline) conditioning trials. Activity

Figure 1. Mean activity counts per minute ($\pm$ SEM) during the first and last CS+ (ethanol) conditioning trials for each ISI group in Experiment 1. Group labels refer to the time interval between onset of the 5-min CS exposure and injection of ethanol (2 g/kg). Data are collapsed across GRID+ and GRID- subgroups.

levels were generally similar across groups on the first CS- trial, regardless of whether saline was administered before or after the 5-min trial. Activity levels decreased over trials in all but Group 0. Furthermore, the general pattern of ISI group differences on Trial 4 resembled that seen on CS+ trials, with Group 0 showing the highest level of activity (see Figure 1).

A two-way ANOVA (7 ISI groups $\times$ 2 trials) produced significant main effects of ISI [$F(6,273) = 11.5, p < .001$] and trial [$F(1,273) = 194.6, p < .001$], and a significant ISI $\times$ trial interaction [$F(6,273) = 8.8, p < .001$]. Separate analyses revealed a significant ISI effect on both trials (both $F_s > 3.2, p < .005$). Pairwise group comparisons on Trial 1 indicated significant differences only between Group -120 and Groups -60 and -30 (both $p_s < .05$). Pairwise comparisons on Trial 4 showed a significant difference between Group 0 and Groups -120, -60, 5, 15, and 60 (all $p_s < .002$). Also, Group -30 differed from Groups 5, 15, and 60 (all $p_s < .01$) and Group -60 differed from Group 5 ($p < .03$). Within-group comparisons showed a significant decrease between Trial 1 and Trial 4 in all but Group 0 (all $p_s < .001$).

Place Preference Test

Figure 3 shows the average time spent on the grid floor during preference testing by each of the conditioning subgroups. To facilitate comparisons across ISI groups, the figure inset depicts mean percent time spent on the ethanol-paired floor, averaged across conditioning subgroups. In this experimental design, evidence of

place conditioning is provided by comparing time spent on one particular floor by the GRID+ and GRID- subgroups within each ISI group. As expected, Group 0 showed a conditioned place preference in that mice exposed to the GRID+ treatment spent *more* time on the grid floor than did mice exposed to the GRID- treatment. Conditioned place preference was also seen in Group -30. However, Group 5 showed conditioned place aversion (i.e., GRID+ mice spent *less* time on the grid floor than did GRID- mice). None of the other ISI groups showed place conditioning. These observations were supported by ANOVA (7 ISI groups $\times$ 2 conditioning groups), which yielded a significant ISI $\times$ conditioning group interaction [$F(6,272) = 10.4, p < .001$], but no main effects. To determine the source of ISI effects on place conditioning, follow-up tests compared GRID+ and GRID- groups within each ISI group. These tests showed significant place conditioning only in Groups -30, 0, and 5 (all $p_s < .001$).

The general pattern of group differences in activity during the preference test resembled that seen on the last CS- trial. Mean activity rates (counts per minute $\pm$ SEM) were 23.8 ± 1.0 , 27.1 ± 1.1 , 26.7 ± 0.8 , 28.9 ± 0.9 , 17.8 ± 1.1 , 21.0 ± 1.2 , and 21.5 ± 1.5 for Groups -120, -60, -30, 0, 5, 15, and 60, respectively. A two-way ANOVA (ISI $\times$ conditioning group) showed a significant ISI effect [$F(6,272) = 14.9, p < .001$], but no effects of conditioning group. Pairwise comparisons indicated that activity was significantly higher in Group 0 than in Groups 5, 15, and 60 (all $p_s < .001$). Further, Group -30 differed from Groups 5, 15, and 60 (all $p_s < .02$), Group -60 differed

Figure 2. Mean activity counts per minute ($\pm$ SEM) during the first and last CS- (saline) conditioning trials for each ISI group in Experiment 1. Group labels refer to the time interval between onset of the 5-min CS exposure and injection of saline. Data are collapsed across GRID+ and GRID- subgroups.

Figure 3. Mean seconds per minute (+SEM) spent on the grid floor by each group during the 60-min preference test in Experiment 1. Group labels refer to ISI on conditioning trials. GRID+ and GRID- refer to the subgroups within each ISI group that had previously received either the grid floor (GRID+) or hole floor (GRID-) and ethanol on CS+ conditioning trials. These subgroups were exposed to the opposite floor type and saline on CS- conditioning trials. The inset depicts the same data converted to percent time spent on the floor paired with ethanol (EtOH) collapsed across GRID+ and GRID- subgroups within each ISI group.

from Groups 5, 15, and 60 (all $ps < .03$), while Group -120 differed only from Group 5 ($p < .01$).

Discussion

This study is the first to show that the direction of the conditioned motivational response produced by ethanol depends on the ISI. When ethanol was administered immediately or 30 min before CS exposure (Groups 0 and -30), place preference was conditioned. However, administration of ethanol immediately after the 5-min CS exposure (Group 5) induced place aversion. Thus, the ISI appears to have the same effect on place conditioning with ethanol as it does on place conditioning with nicotine and amphetamine (Fudala & Iwamoto, 1986, 1987, 1990).

The finding of place preference with a 30-min backward interval (Group -30) is not consistent with a preliminary finding reported by Risinger and Cunningham (1992a). In an experiment designed to test the hypothesis that ethanol's immediate effects are rewarding but that its delayed effects are aversive (Cunningham & Prather, 1992), Risinger and Cunningham found a trend toward place aversion in mice that received a 30-min CS exposure 30 min after injection of a 3-g/kg dose of ethanol. The discrepancy between that previous observation and the present findings may be related to differences in

ethanol dose or trial duration, or the previous result might have been due to sampling error. In general, the present finding of conditioned preference at the 30-min backward interval (replicated independently in Experiments 1A and 1C) and no conditioning at longer backward delays (-60, -120) argues against the notion of a biphasic motivational effect that is initially rewarding but aversive after a delay (Cunningham & Prather, 1992).

Two aspects of the activity data recorded on conditioning trials are of potential interest. First, the two groups that showed sensitization to ethanol's activating effect over conditioning trials (Groups 0 and -30) were the only groups to show conditioned place preference during testing. Given previous findings indicating an associative component to sensitization in this task (Cunningham & Noble, 1992), the present data offer further support for the suggestion that a common learning process mediates ethanol-induced conditioned place preference and behavioral sensitization to ethanol (Cunningham & Noble, 1992; Cunningham & Prather, 1992). These data also lend general support to the incentive sensitization theory of addiction, which postulates a link between sensitization and drug craving (Robinson & Berridge, 1993; but see Cunningham, 1995). However, the foregoing conclusions must be tempered by the fact that the present experiment was not specifically designed to provide a test for sensitization under comparable conditions in all groups.

The other interesting aspect of the activity data is the development over CS- trials of a between-groups pattern of activity similar to that observed on CS+ trials. Although all groups showed similar levels of activity on the first CS- trial, the groups showing the highest levels of activity on CS+ trials also showed the highest activity levels on the final CS- trial. It is possible that the between-groups pattern of responding on the final CS- trial was due to the acquisition of conditioned activity responses evoked by general contextual cues associated with ethanol on CS+ trials (Cunningham & Noble, 1992). As suggested by Cunningham and Noble (1992), these conditioned activity responses may have contributed to the sensitization of ethanol-induced activation measured on CS+ trials. Alternatively, because the activity response to the CS- on later trials never exceeded that recorded on the first trial, one might also argue that the final pattern of group differences in CS- activity was due to differential effects of ethanol on habituation to contextual cues rather than to differences in conditioning.

EXPERIMENT 2

Effect of Handling and Injection

The finding that the same dose of a drug will establish either a conditioned preference or a conditioned aversion depending on its temporal relationship with the CS poses a serious challenge for interpretations of place conditioning that assume a unitary motivational effect of the drug US. One solution to this problem is to argue that administration of ethanol produces an initial short-lived aversive effect that is followed by a longer lasting rewarding effect. Thus, conditioning of preference or aversion could be explained in terms of the temporal relationship between the CS and these opposite motivational effects. There appear to be at least three different potential sources of the hypothesized initial aversive effect of ethanol injection: (1) the pain/distress caused by handling and i.p. injection, (2) peritoneal irritation by a concentrated ethanol solution, and (3) pharmacological effects of ethanol at specific target tissues (e.g., central nervous system [CNS]).

Experiment 2 was designed to shed light on the first of these possibilities by examining the motivational effects of handling and injection of an inert substance (saline) in the place conditioning paradigm. Because mice were injected on both CS+ and CS- trials in Experiment 1, that experiment does not provide information on effects of injection per se. It is possible, for example, that floor choice during preference testing was affected, at least in part, by approach to or withdrawal from the CS- based on its association with saline injection. One previous study in rats has shown that repeated pre-CS injections of saline produced a conditioned preference for associated place cues, an effect that was attributed to positive motivational consequences of stress-induced release of endogenous opioid peptides (Bozarth, 1987). However, there have been no similar studies in mice and no studies examining whether post-CS exposure to handling and injection

induces conditioned aversion. Thus, in Experiment 2 we assessed the effects of pre-CS (*before* groups) or post-CS (*after* groups) injections of saline on place conditioning using procedures similar to those used in Experiment 1. All mice were exposed to the alternative cue (CS-) without injection. Evidence of a conditioned motivational effect produced by saline injection or of a difference in the effects produced by pre-CS and post-CS injections could have important implications for the interpretation of ethanol's effects in Experiment 1.

Method

Subjects and Apparatus

The subjects were naive adult male DBA/2J mice maintained in the same manner as were the mice in Experiment 1. The apparatus was that used for Experiment 1.

Procedure

Sixty subjects were randomly assigned to two large groups ($n = 30/\text{group}$). After one habituation session, the mice in both groups were exposed to each floor type four times on alternating days as in Experiment 1. Mice in the *before* group consistently received an i.p. injection of saline (12.5 ml/kg) immediately before placement on one of the two floor types (CS+ trial); no injection was given on trials involving the other floor type (CS- trial). Mice in the *after* group received similar treatment, but saline injections were always given just after exposure to the CS+ floor. Floor assignment was counterbalanced within each group such that the grid floor served as the CS+ for the GRID+ subgroups and CS- for the GRID- subgroups ($n = 15/\text{subgroup}$). Ethanol was not administered at any time. The experiment concluded with a 60-min preference test identical to that described for Experiment 1. Presumably, if handling and vehicle injection produced positive or negative motivational effects, one would expect the GRID+ and GRID- subgroups within each group to differ.

The experiment also included two additional groups that received saline injections on both types of conditioning trials. The mice in the *before* control group ($n = 12$) received a saline injection before each trial, whereas the mice in the *after* control group ($n = 16$) received a saline injection after each trial. These treatments were not expected to produce place conditioning, and the mice were expected to spend approximately half of the test session on each floor type (Cunningham, 1995; Cunningham et al., 1992).

Results

Test data for one mouse (*after* group) were excluded from analysis because of an injection injury.

Conditioning Trials

Activity in the experimental groups generally decreased between the first conditioning trial (mean activity counts per minute $\pm SEM = 47.9 \pm 1.2$) and last conditioning trial (32.9 ± 1.5). However, there were no differences between groups treated before or after conditioning trials and no difference between activity recorded on saline injection trials and activity on trials without injection. Mean activity rates ($\pm SEM$) for the *before* group were 38.1 ± 2.0 and 37.5 ± 2.0 on the saline and no-injection trials, respectively. For the *after* group, these rates were 37.2 ± 1.9 and 38.0 ± 1.5 , respectively. An overall three-way ANOVA (*before/after* $\times$ trial type $\times$ trials) yielded only a significant main effect of trials [$F(3,174) = 51.1$,

$p < .001$]. Analysis of conditioning trial data from the two control groups (before/after $\times$ trials) also showed a significant trials effect [$F(3,78) = 27.3, p < .001$] and no difference between groups injected before or after each trial. Mean activity rates were 37.9 ± 2.9 and 36.1 ± 2.9 for the before and after control groups, respectively.

Place Preference Test

The outcome of the preference test is shown in Figure 4. Subgroups that received differential conditioning within each of the main experimental groups performed identically, indicating that handling and vehicle injection had not produced place conditioning. This conclusion was further supported by the lack of difference between the conditioning subgroups and control groups that had been handled and injected on both types of conditioning trials. An overall 2×3 ANOVA (before/after $\times$ conditioning groups) of time spent on the grid floor confirmed these observations by showing no main effect or interaction with the conditioning groups factor (both F s < 1). Although the after groups generally tended to spend slightly less time on the grid floor than the before groups, the main effect for that comparison fell short of the criterion for significance [$F(1,81) = 2.9, .05 < p < .10$].

Mean ($\pm$ SEM) activity rates during preference testing were 30.9 ± 1.9 , 31.6 ± 1.2 , and 34.4 ± 2.7 , for GRID+, GRID-, and control mice, respectively, in the before groups. In the after groups, these rates were 30.8 ± 1.6 , 28.3 ± 1.4 , and 28.9 ± 0.8 , respectively. A 2×3 ANOVA

indicated that the before groups were slightly more active than the after groups [$F(1,81) = 5.3, p < .03$], but there were no significant differences among conditioning groups.

Discussion

This experiment indicates that handling and vehicle injection just before or just after CS exposure are not sufficient to induce place conditioning in mice. Thus, one cannot argue that approach to or withdrawal from the CS+ in Experiment 1 was due to saline-induced conditioned aversion or preference for the CS-. This study does not eliminate the possibility that conditioning to the CS+ in Experiment 1 was influenced by an interaction between ethanol's motivational effects and the unconditioned effects of handling and injection. For example, handling and injection may enable or exacerbate ethanol's motivational effects, even though handling and injection alone are not sufficient to produce place conditioning. However, in order to explain the difference between pre-CS and post-CS exposure to ethanol, one would also need to argue that this hypothesized interaction varied as a function of ISI.

This experiment failed to support Bozarth's (1987) finding of place preference induced by pre-CS handling and vehicle injection in rats. Aside from the use of a different species, a potential explanation of this discrepancy is that Bozarth's handling procedure was probably much more stressful than that normally used in this lab-

Figure 4. Mean seconds per minute ($\pm$ SEM) spent on the grid floor by each group during the 60-min preference test in Experiment 2. Experimental groups received a saline injection before (before groups) or after (after groups) exposure to CS+; no injection was given on CS- trials. The grid floor served as CS+ for GRID+ subgroups, whereas the hole floor served as CS+ for GRID- subgroups. The control groups received saline injections on both types of trial. The inset depicts the same data converted to percent time spent on the floor paired with saline collapsed across GRID+ and GRID- subgroups within each of the two experimental groups.

oratory (Bozarth's rats were swung back and forth five times by the experimenter before each injection).

EXPERIMENT 3 Effect of Ethanol Concentration

Experiment 3 was designed to address a possible source of aversive motivation that may be unique to ethanol injection—peritoneal irritation. It has been suggested that i.p. injection of relatively high ethanol concentrations (i.e., > 10% w/v) produces potentially irritating inflammatory effects (Wallgren & Barry, 1970, p. 41). Thus, the development of conditioned aversion to a CS presented just before injection of a high ethanol concentration may reflect an association with the immediate peritoneal irritation produced by ethanol rather than an association with its subsequent CNS effects. If peritoneal irritation is relatively short lived, the finding of conditioned preference in the typical “simultaneous” procedure (i.e., Group 0) might be explained by a more favorable temporal relationship between the CS and the later rewarding pharmacological effects of ethanol.

In Experiment 3, we examined the potential role played by peritoneal irritation by varying the concentration of ethanol used for place conditioning trials in which ethanol was administered either just before (Group 0) or just after (Group 5) exposure to the CS+. All animals received the same dose of ethanol as that in Experiment 1 (2 g/kg). However, half the animals received the same concentration drug solution used in Experiment 1 (20% v/v), whereas the other half received a lower concentration solution (10% v/v). If peritoneal irritation produced by 20% ethanol is a significant source of motivation in this task, one might predict a positive relationship between ethanol concentration and strength of conditioned place aversion in groups given post-CS drug exposure. In contrast, one might expect to find a negative relationship between ethanol concentration and strength of conditioned place preference in groups given pre-CS drug exposure.

Method

Subjects and Apparatus

The subjects were 96 naive male DBA/2J mice maintained in the same manner as mice in Experiment 1. The apparatus was that used for Experiment 1.

Procedure

The subjects were randomly assigned to four groups ($n = 24$ /group) within a 2×2 design that factorially combined ISI (0 vs. 5 min) and ethanol concentration (10% vs. 20% v/v). Because the ethanol dose was held constant at 2 g/kg, mice assigned to the 10% groups received a larger injection volume (25 ml/kg) on CS+ and CS- trials than did mice assigned to the 20% groups (12.5 ml/kg). Within each of the ISI $\times$ concentration groups, mice were randomly assigned to the same two counterbalanced conditioning subgroups described in Experiment 1: GRID+ and GRID- ($n = 12$ /subgroup).

The general procedure was identical to that described for Groups 0 and 5 in Experiment 1. In order to assess the effects of additional training on conditioned place preference and aversion, an additional four conditioning trials of each type were given beginning 3 days

after the initial floor preference test. The experiment concluded with a second preference test identical to the first.

Results

Conditioning Trials

Figure 5 shows mean activity rates on all conditioning trials for each of the ISI $\times$ concentration groups (collapsed across GRID+ and GRID- subgroups). As in Experiment 1, ethanol increased activity level in Group 0 mice, and repeated exposure to ethanol produced sensitization (left panel). Moreover, Groups 0 and 5 showed similar activity levels on the first CS- trial but then diverged over trials, with Group 0 maintaining a higher activity level than Group 5. Ethanol concentration, however, had no effect on activity on either CS+ or CS- trials. Separate analyses applied to each trial type (ISI $\times$ concentration $\times$ trial) yielded significant main effects of ISI [$F_s(1,92) > 102.1, p < .001$] and trial [$F_s(7,644) > 15.9, p < .001$], and significant ISI $\times$ trial interactions [$F_s(7,644) > 5.04, p < .001$]. No effect involving the concentration factor was significant.

Place Preference Test

As in Experiment 1, the first test showed significant conditioned place preference in Group 0 mice and conditioned place aversion in Group 5 mice. However, there were no significant effects involving ethanol concentration (data not shown). Overall magnitude of place conditioning was greater on the second test (see Figure 6), suggesting that four additional conditioning trials produced further increments in learning in each group. Of greater interest, however, was the trend toward stronger place conditioning at the higher ethanol concentration.

A three-way ANOVA (ISI $\times$ concentration $\times$ conditioning group) of time spent on the grid floor yielded significant main effects of ISI [$F(1,88) = 10.5, p < .002$], concentration [$F(1,88) = 4.5, p < .04$], and conditioning group [$F(1,88) = 4.9, p < .03$]. In addition, there were two significant interactions: ISI $\times$ conditioning group [$F(1,88) = 103.2, p < .001$] and ISI $\times$ concentration $\times$ conditioning group [$F(1,88) = 4.7, p < .04$]. In order to interpret the three-way interaction, the concentration $\times$ conditioning group interaction was evaluated separately for each ISI group. These analyses showed a significant interaction in Group 0 [$F(1,44) = 5.1, p < .03$], but not in Group 5 [$F(1,44) = 2.0, p > .15$]. Thus, these follow-up analyses suggest that conditioned place preference (Group 0) but not conditioned place aversion (Group 5) was enhanced by use of a 20% ethanol concentration. Comparisons of the GRID+ and GRID- subgroups within each ISI $\times$ concentration group on Test 2 were significant, indicating development of place conditioning in all groups (Bonferroni-corrected $p_s < .04$).

Analysis of activity during each test session (ISI $\times$ concentration $\times$ conditioning group) yielded significant main effects of ISI on both tests [$F_s(1,88) > 43.0, p < .001$], but no other effects. As in Experiment 1, mean test session activity rate (counts/minute) was higher in

Figure 5. Mean activity counts per minute ($\pm$ SEM) during each CS+ trial (left panel) and each CS- trial (right panel) in Experiment 3. The number before the slash in each group label refers to the ISI (0 vs. 5 min), whereas the percentage after the slash refers to ethanol concentration (v/v). Ethanol dose was 2 g/kg for all groups, and trial duration was 5 min. Data are collapsed across GRID+ and GRID- subgroups.

Group 0 (26.8) than in Group 5 (16.1). Activity rate was also slightly higher in Test 1 (22.5) than in Test 2 (20.4).

Discussion

Experiment 3 replicated the major finding of Experiment 1: post-CS exposure to ethanol produced conditioned place aversion, whereas pre-CS exposure produced conditioned place preference. Of greater interest, ethanol concentration was found to have a significant effect on place conditioning. However, the pattern of results does not provide unequivocal support for the peritoneal irritation hypothesis. Although the data showed a trend toward the predicted enhancement of conditioned aversion at the higher concentration, this effect was not supported by statistical analysis. Inspection of the data suggests that the ability to detect enhanced aversion may have been compromised by a general bias against the grid floor and greater variability in the 5-min ISI groups (see Figure 6). Unexpectedly, the higher ethanol concentration produced a stronger conditioned place preference in the 0-min ISI groups. This outcome seems inconsistent with the suggestion that the peritoneal irritating effects of the higher concentration ethanol should have counteracted the otherwise positive effects of ethanol intoxication, thereby reducing the magnitude of conditioned preference.

However, if one is willing to accept the trend toward enhanced place aversion in the 5/20% group as support for the hypothesized aversive effects of peritoneal irritation, one might argue that the enhanced preference in the 0/20% group was the result of conditioning a motivational response whose valence was opposite to the aversive state induced by exposure to 20% ethanol. Of course, this

interpretation assumes that the direction of the conditioned response can be reversed by manipulating ISI, an outcome that does not appear to have independent support in the drug conditioning literature. An alternative possibility is that peritoneal irritation was not a critical factor in this range of ethanol concentrations, but that the more highly concentrated ethanol solution produced a dose-like increase in the CNS effects of ethanol (Linakis & Cunningham, 1979), regardless of whether they were rewarding or aversive. However, this interpretation is not consistent with the lack of an ethanol concentration effect on activity during conditioning trials.

GENERAL DISCUSSION

Experiments 1 and 3 provide strong evidence that the direction of the conditioned motivational effect produced by ethanol in the place conditioning task depends on ISI. Conditioned place preference was consistently observed when the ethanol US was given immediately or 30 min before CS exposure, whereas conditioned place aversion was observed when ethanol was injected immediately after CS exposure. Experiment 2 showed that handling and injection alone were not sufficient to induce place conditioning, suggesting that interpretation of test performance in Experiment 1 was probably not complicated by the development of a conditioned motivational response to the CS-. Experiment 3 showed a positive relationship between ethanol concentration and place conditioning in both directions, an outcome that might be explained by an aversive effect of peritoneal irritation at the higher ethanol concentration.

Figure 6. Mean seconds per minute (+SEM) spent on the grid floor by each group during the second preference test in Experiment 3. The number before the slash in each group label refers to the ISI (0 vs. 5 min), whereas the percentage after the slash refers to ethanol concentration (v/v). GRID+ and GRID- refer to the subgroups within each group that had previously received either the grid floor (GRID+) or hole floor (GRID-) and ethanol on CS+ conditioning trials. These subgroups were exposed to the opposite floor type and saline on CS- conditioning trials. The inset depicts the same data converted to percent time spent on the floor paired with ethanol (EtOH) collapsed across GRID+ and GRID- subgroups within each treatment group.

The overall pattern of findings appears consistent with the earlier suggestion that i.p. administration of ethanol produces an initial short-duration aversive effect that is followed by a longer lasting positive motivational effect. Presumably, the temporal relationship between injection and CS exposure favors conditioning of the aversive effect in Group 5 and conditioning of the positive effect in Group 0. The exact source of the hypothesized initial aversive effect is unknown. It could be entirely peripheral (e.g., peritoneal irritation), or it might reflect an early CNS effect of ethanol (e.g., the rapid transition from the sober state to the intoxicated state could be aversive even though the intoxicated state itself is rewarding). It is also possible that these effects interact with each other or are exacerbated by the stress of handling and injection, even though handling and injection alone do not produce place conditioning.

The present data do not address the issue of whether these hypothesized motivationally opposite effects of i.p. ethanol injection are linked mechanistically or are independent. For example, whether the source of the initial aversive effect is peripheral or central, the delayed development of a positive motivational state might be predicted from opponent-process models in which activation of one affective process is thought to automatically elicit an opposing affective process (Solomon, 1977). Alternatively, the initial aversiveness of ethanol injection

might be caused by peripheral effects that are completely unrelated to the subsequent CNS-mediated rewarding effects of ethanol intoxication.

It is not clear whether the foregoing interpretation of ISI effects on ethanol place conditioning can be readily applied to studies showing similar outcomes with nicotine and amphetamine (Fudala & Iwamoto, 1986, 1987, 1990). The literature does not reveal as much concern over possible peritoneal irritating effects of these drugs as in the case of ethanol. Thus, in order to apply the present interpretation, one must appeal more to short-duration aversive CNS effects or to an interaction between such effects and the stress of handling and injection. As in the case of ethanol, however, the literature does not currently provide independent evidence of a short-lived aversive CNS effect following injection of these drugs.

Fudala and Iwamoto (1987) proposed that place aversion may be produced by post-CS drug administration because the drug interferes with habituation of neophobia to the CS+. Thus, avoidance of the CS+ during choice testing is attributed to its relative novelty rather than to an association with a primary aversive drug effect. A major difficulty with this hypothesis, however, is in explaining why pre-CS administration of the drug does not produce the same effect. In fact, given that the preference test is the first time that Group 0 animals were exposed to CS+ without the drug, one might expect substantial neopho-

bia (and therefore place aversion) based on the removal of a salient component of the original stimulus complex. Interestingly, drug-induced interference with habituation has also been used to explain conditioned place preference following pre-CS injection of the US drug (Scoles & Siegel, 1986). In this case, however, it was assumed that the relatively greater novelty of the CS+ would produce approach rather than avoidance. Whichever view is adopted, the interference-with-habituation hypothesis has difficulty explaining why the direction of the effect should change as a function of ISI.

It is possible to derive explanations of the ISI effect without assuming that the drug US has multivalent effects. For example, the CS+ might become a Pavlovian conditioned inhibitor in groups receiving immediate post-CS drug exposure. As a conditioned inhibitor, the CS would be expected to elicit a conditioned motivational state opposite in valence to that controlled by a conditioned excitatory CS associated with the same US (Rescorla, 1969). According to this account, the primary motivational effect of the ethanol US is assumed to be positive and capable of establishing the CS as a conditioned excitator when ethanol is given immediately before CS exposure (Group 0). Moreover, it must be assumed that the nonoverlapping (unpaired) relationship between the CS and US when the US is given immediately after CS exposure (Group 5) was sufficient to establish the CS as a conditioned inhibitor after a relatively small number of trials. One difficulty with this hypothesis, however, is in explaining why other groups that received nonoverlapping CS and US exposures (Groups 15 and 60) failed to show evidence of this alleged inhibitory conditioning (i.e., place aversion).

Another explanation based on the assumption of a monovalent drug effect can be derived from Siegel's compensatory conditioning model of drug tolerance (e.g., Hinson & Siegel, 1980; Siegel, 1989). Many studies, including studies with ethanol, have shown that repeated CS-drug pairings will establish a conditioned response whose direction is opposite to the unconditioned response to the drug (e.g., Krank, 1987; Mansfield & Cunningham, 1980; Siegel, 1975). Furthermore, it has been argued that evocation of these learned drug-compensatory responses in the absence of drug produces a dysphoric state ("craving") with features similar to drug withdrawal (Hinson & Siegel, 1980). In order to apply this explanation to the present studies, one must argue that immediate post-CS drug exposure produced compensatory conditioned responses, whereas pre-CS drug exposure did not. Aside from the present experiments, however, the drug conditioning literature currently offers little support for concluding that the direction of drug conditioned responses varies as a function of ISI.

The paradox posed by these opposite effects of the same drug is reminiscent of another one commonly found in the literature on conditioning produced by drugs that are self-administered. Specifically, many reports show that drugs that produce conditioned place preference will also induce conditioned taste aversion (e.g., Cunningham et al.,

1991; Reicher & Holman, 1977; Smith & Holman, 1987). Indeed, several studies from this laboratory have shown that the same dose of ethanol as that used here establishes a robust conditioned taste aversion in DBA/2 mice (Risinger & Cunningham, 1992b, 1995). This apparent paradox has been interpreted in several different ways. One approach, derived from the principle of selective association, is to propose that the drug is simultaneously rewarding and aversive and that exteroceptive and gustatory cues are differentially associated with these opposite motivational effects (Reicher & Holman, 1977). A second approach argues that performance in both tasks actually reflects learning about the same underlying positive motivational effect (Hunt & Amit, 1987). According to this explanation, the reduced intake observed in a taste conditioning task is a "bait shyness" phenomenon reflecting the gustatory system's sensitivity to the novelty of the drug experience rather than any intrinsic aversive effects of the drug.

Neither of the foregoing interpretations of the place preference-taste aversion paradox provides a satisfactory explanation of the ISI effect on place conditioning. Because the same exteroceptive CS elicited either place preference or place aversion, it is difficult to use arguments based on selective association of the CS with different components of a bivalent US. Moreover, because the gustatory system played no role in these studies, explanations based on the unique sensitivities of that system are precluded. Since place conditioning typically involves pre-CS drug administration, whereas taste conditioning usually involves post-CS drug administration, it is tempting to consider the possibility that the place preference-taste aversion paradox might actually be due to ISI differences. In support of this suggestion, one study with morphine has shown that simultaneous conditioning produces a conditioned taste preference, whereas standard trace delay conditioning produces conditioned taste aversion (Lett & Grant, 1989). However, in other studies involving either simultaneous or short backward intervals with amphetamine, taste aversion was produced even though the same drug injection conditioned preference to exteroceptive stimuli presented together with the taste CS (Reicher & Holman, 1977). Thus, it does not appear that the place preference-taste aversion paradox can be explained entirely in terms of ISI differences. Nevertheless, the same processes that induce conditioned place aversion after post-CS drug exposure may contribute to development of conditioned taste aversion in the standard trace delay procedure.

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