Psychopharmacology (2002) 160:414–424
DOI 10.1007/s00213-001-0990-1

ORIGINAL INVESTIGATION

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Distal and proximal pre-exposure to ethanol in the place conditioning task: tolerance to aversive effect, sensitization to activating effect, but no change in rewarding effect

Received: 10 September 2001 / Accepted: 17 November 2001 / Published online: 31 January 2002

Abstract *Rationale:* The literature offers many examples of tolerance to ethanol's inhibitory/depressant effects and sensitization to its activating effects. There are also many examples of tolerance to ethanol's aversive effects as measured in the conditioned taste aversion and conditioned place aversion (CPA) procedures. However, there are very few demonstrations of either tolerance or sensitization to ethanol's rewarding or reinforcing effects. *Objective:* The present studies were designed to examine effects of two forms of ethanol pre-exposure (distal or proximal) on ethanol's rewarding and aversive effects as indexed by the place conditioning procedure. *Method:* Male inbred (DBA/2J) mice were exposed to ethanol (2 g/kg IP) in an unbiased place conditioning procedure that normally produces either conditioned place preference (CPP) (ethanol injection before CS exposure) or CPA (ethanol injection after CS exposure). In the distal pre-exposure studies (experiments 1 and 2), mice initially received a series of four ethanol injections (0, 2, or 4 g/kg) in the home cage at 48-h intervals during the week before place conditioning. In the proximal pre-exposure studies (experiments 3–4), mice were injected with ethanol 65 min before (experimental groups) or 65 min after (control groups) each paired ethanol injection on CS+ trials. *Results:* Distal pre-exposure produced a robust sensitization to ethanol's activating effect, whereas proximal pre-exposure generally reduced the activation normally produced by the paired ethanol injection. Both forms of pre-exposure interfered with CPA, but had no effect on CPP. *Conclusions:* These studies suggest that both forms of pre-exposure reduced ethanol's aversive effect, but had no impact on ethanol's rewarding effect. In general, the detrimental effects of pre-exposure on CPA are explained best in terms of a reduction in ethanol's efficacy as an aversive unconditioned stimulus (i.e. tolerance), although explanations based on other types of associative interference are also possible. The failure to affect CPP with pre-exposure treatments that reduced or eliminated CPA suggests that these behaviors are mediated by independent, motivationally opposite effects of ethanol. Moreover, these results indicate dissociation between sensitization to ethanol's locomotor activating effect and changes in its rewarding effect. To the extent that motivational processes measured by CPP and CPA normally contribute to ethanol drinking, the present findings suggest that increases in ethanol intake seen after chronic ethanol exposure are more likely caused by tolerance to ethanol's aversive effect rather than sensitization to its rewarding or reinforcing effect.

Keywords Ethanol · Conditioned place preference · Conditioned place aversion · Ethanol pre-exposure · Tolerance · Sensitization · Locomotor activity · Reward · Inbred mice · DBA/2J

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Introduction

Tolerance and sensitization are the two most commonly studied forms of neuroadaptation produced by repeated drug exposure. In the case of ethanol, repeated exposure has been found to produce tolerance to (i.e. a decrease in) several different inhibitory/depressant effects of ethanol, including hypothermia (e.g. Kalant and Le 1991), ataxia (e.g. LeBlanc et al. 1969; Kalant et al. 1971, 1978) and loss of the righting response (e.g. Tabakoff et al. 1980). These effects have been observed in many different rat and mouse strains (e.g. Crabbe et al. 1981, 1982; Tabakoff and Culp 1984; Phillips and Crabbe 1991). In contrast, repeated ethanol exposure has been found consistently to produce sensitization to (i.e. an increase in) only one excitatory/stimulant effect of ethanol, namely, the locomotor activating effect produced by low to moderate doses (e.g. Cunningham and Noble 1992;

Phillips et al. 1994; Broadbent et al. 1995; Lessov and Phillips 1998). Moreover, this effect is typically seen only in mice.

Both types of neuroadaptation have been implicated in theoretical discussions of the progressive increase in ethanol drinking shown by alcohol abusers and alcoholics. For example, increased drinking has sometimes been attributed to tolerance to ethanol's rewarding or reinforcing effects, which requires individuals to increase intake in order to maintain a desired drug effect (e.g. Tabakoff and Hoffman 1988; Gauvin and Holloway 1992). Increased drinking can also be explained in terms of tolerance to an inhibitory or aversive drug effect that initially limits ethanol intake, but dissipates with repeated exposure, thereby allowing greater intakes (e.g. Cappell and LeBlanc 1983; Tabakoff and Hoffman 1988; Gauvin et al. 2000). Finally, increased drinking has been explained by sensitization to ethanol's rewarding or reinforcing effects. That is, progressive increases in drinking may be attributed to an increase in the positive affective consequences of exposure to a given amount of drug (e.g. Newlin and Thomson 1991, 1999; Hunt and Lands 1992; Schmidt et al. 2000; Lessov et al. 2001).

Empirical evidence in support of these theoretical alternatives is strongest for the suggestion that ethanol intake is enhanced by development of tolerance to an aversive ethanol effect or to a motor debilitating effect (e.g. ataxia) that otherwise interferes with continued drinking (Cappell and LeBlanc 1983). For example, many studies have shown that ethanol's aversive effects in conditioned taste aversion procedures (e.g. Berman and Cannon 1974; Cannon et al. 1975; Barker and Johns 1978; Risinger and Cunningham 1995) and conditioned place aversion procedures (e.g. Gauvin and Holloway 1992; Holloway et al. 1992; Cunningham and Henderson 2000) are reduced by repeated ethanol exposure. However, relatively few studies have yielded convincing demonstrations that repeated ethanol exposure produces either tolerance or sensitization to ethanol's rewarding or reinforcing effects (Cappell and LeBlanc 1983; Lessov et al. 2001).

One difficulty in this area of research stems from the technique most often used to index effects of prior ethanol exposure on ethanol reinforcement. Specifically, oral self administration typically provides an ambiguous answer to this question due to the well-known inverted U-shaped relationship between ethanol intake and variables that presumably affect ethanol's reinforcing efficacy, such as dose or concentration (e.g. Richter and Campbell 1940; Meisch and Thompson 1974). Thus, treatments that are assumed to enhance ethanol's reinforcing efficacy (e.g. sensitization) can either increase or decrease ethanol intake, depending on where control intakes fall on the concentration-response function. A similar ambiguity occurs with treatments assumed to reduce reinforcing efficacy (e.g. tolerance), which can also produce either increases or decreases in intake. The overall implication of this analysis is that changes in intake of a fixed ethanol concentration after repeated ethanol exposure are difficult to attribute exclusively to either tolerance or sensitization. Moreover, it is difficult to know whether the behavioral change reflects alteration in a rewarding or aversive effect of ethanol.

The place conditioning procedure offers an alternative approach that may be better suited for assessing effects of prior ethanol exposure for two reasons: (1) the task allows one to distinguish between rewarding and aversive drug effects (Tzchentke 1998), and (2) the relationship between strength of place conditioning and variables thought to influence reinforcing efficacy (e.g. dose) is monotonic (e.g. Cunningham et al. 1992; Risinger et al. 1994; Risinger and Oakes 1996). Thus, this procedure has the potential to yield less ambiguous conclusions about the impact of ethanol pre-exposure on ethanol's rewarding and aversive effects. Also, because locomotor activity is easily recorded during place conditioning, this approach provides an independent measure of tolerance or sensitization to ethanol's effects on activity.

The present studies were designed to examine effects of repeated home-cage ethanol injections on subsequent development of ethanol-induced conditioned place preference (CPP) or place aversion (CPA). These studies take advantage of recent findings that a given dose of ethanol will produce either place preference or place aversion in the same mouse strain depending on whether ethanol is injected immediately before exposure to the conditioned stimulus (CS) or immediately after CS exposure. More specifically, pre-CS ethanol injection produces CPP whereas post-CS ethanol injection produces CPA (Cunningham et al. 1997, 1998; Cunningham and Henderson 2000). Although the mechanisms underlying these opposite behavioral effects of ethanol are not yet known, it has been postulated that these outcomes reflect independently mediated rewarding and aversive effects of ethanol (Cunningham and Henderson 2000). Methodologically, this strategy allows one to examine ethanol pre-exposure effects on these opposite behavioral outcomes using the exact same strain, apparatus, CS, ethanol dose and dependent variable.

Two forms of ethanol pre-exposure were examined here. In experiments 1 and 2, mice received a series of four ethanol injections (0, 2 or 4 g/kg) at 48-h intervals in the home cage during the week prior to the beginning of place conditioning (referred to here as "distal" pre-exposure). This approach, which represents the most common form of pre-exposure, assessed the impact of an historical treatment that would presumably alter the motivational effect of ethanol experienced on each conditioning trial. Experiments 3 and 4 examined a less-frequently studied form of pre-exposure in which mice received a home cage injection of ethanol or saline about 1 h before each successive ethanol place conditioning trial (referred to here as "proximal" pre-exposure). Thus, experimental subjects were still intoxicated at the time they received each CS-ethanol pairing. In order to control for the historical effect of these additional ethanol exposures over trials, a control group in each experiment received an extra home cage ethanol injection about 1 h after each ethanol conditioning trial.

Materials and methods

Subjects

Naive adult male mice (DBA/2J) were shipped from the Jackson Laboratory (Bar Harbor, Maine, USA) at 6 weeks of age (*n*=88–96/experiment). They were housed in groups of three or four in polycarbonate cages with cob bedding in a Thoren rack and allowed to adapt to the colony for 2 weeks before testing. The colony room was maintained at 21±1°C with a normal 12-h light-dark cycle (lights on at 7 a.m.). Testing occurred during the light cycle. Food and water were available at all times in the home cage. The NIH "Principles of laboratory animal care" were followed in conducting these studies.

Apparatus

Place conditioning and preference testing were conducted in 12 aluminum and acrylic chambers (30×15×15 cm) contained in separate ventilated sound- and light-attenuating enclosures (Coulbourn Instruments Model E10-20). Activity and side position were detected by six sets of infrared light sources and photodetectors mounted at equal intervals, 2.2 cm above the floor along the length of each chamber. Data were automatically recorded by microcomputer. Conditioned stimuli were provided by the tactile cues of the interchangeable floor halves placed beneath each chamber. The grid floor was constructed from 2.3 mm stainless steel rods mounted 6.4 mm apart in acrylic rails; the hole floor consisted of perforated stainless steel sheet metal (16 gauge) containing 6.4 mm round holes on 9.5 mm staggered centers. This combination of floor stimuli was selected on the basis of many previous studies indicating that saline-treated mice spend about half their time on each floor type during choice tests (e.g. Cunningham et al. 1992, 1997; Cunningham 1995). The inside of the chamber and floors were wiped with a damp sponge, and the litter paper beneath the floors was changed after each animal.

Distal pre-exposure and conditioning: experiments 1–2

Both of the distal pre-exposure experiments included three phases: ethanol pre-exposure (four injections), conditioning (eight sessions), and testing (two sessions). For pre-exposure, mice in both experiments were randomly assigned to one of three ethanol dose groups: 0, 2 or 4 g/kg. Dose was manipulated by adjusting the volume of a 20% (v/v) solution of ethanol mixed in saline (Linakis and Cunningham 1979). Four pre-exposure injections were given in the home cage at 48-h intervals.

Place conditioning began 48 h after the last pre-exposure injection. Mice within each pre-exposure group were randomly assigned to one of two conditioning subgroups (*n*=15–16 per subgroup) and exposed to a series of Pavlovian conditioning trials. Each trial lasted 5 min and mice had access to the entire chamber, which contained one floor type throughout. On alternate days, the grid floor was paired with ethanol (CS+ trials) and the hole floor was paired with saline (CS– trials) for mice in the GRID+ conditioning subgroups. These contingencies were reversed for mice in the GRID– subgroups. Order of exposure to CS+ and CS– was counterbalanced within each subgroup. Ethanol dose was 2 g/kg for all groups on CS+ trials. All injections were given immediately before placement on the floor in experiment 1, whereas all injections were given immediately after removal from the floor in experiment 2. As noted earlier, pre-CS injection was expected to produce CPP whereas post-CS injection was expected to produce CPA (Cunningham et al. 1997, 1998; Cunningham and Henderson 2000).

Proximal pre-exposure and conditioning: experiments 3–4

Both of the proximal pre-exposure experiments included three phases: habituation (one session), conditioning (eight sessions), and testing (1 session). On the habituation day, mice received three saline injections separated by 65 min. The first and third injections were given in the home cage, whereas the second injection was given immediately *before* (experiment 3) or *after* (experiment 4) 5-min exposure to a smooth paper floor in the apparatus.

Subjects were then randomly assigned to GRID+ or GRID– conditioning subgroups (*n*=13–16 per subgroup) within one of three treatment groups. On each of the 8 conditioning days, all mice received a series of three injections timed identically to the habituation day. On CS+ trials, the middle ("paired") injection was ethanol (2 g/kg) for all groups. In general, this injection was expected to produce conditioned preference when given before CS exposure (experiment 3) or conditioned aversion when given after CS exposure (experiment 4). Mice in the experimental group, group EES, received another ethanol injection (2 g/kg) in the home cage 65 min before the paired ethanol injection. Thus, mice in group EES were intoxicated at the time of the paired injection. Mice in one of the control groups, group SES, received a saline injection in the home cage 65 min before the paired ethanol injection. This manipulation controlled for the stress of the pretreatment injection using a procedure that was otherwise expected to produce a "normal" level of place conditioning. Mice in the second control group, group SEE, also received a saline pretreatment injection. However, to control for overall exposure to ethanol, this group received a second ethanol injection (2 g/kg) in the home cage, 65 min after the paired ethanol injection. Thus, group SEE received the same total exposure to ethanol as group EES on CS+ days. However, in contrast to group EES, they were not intoxicated when the paired injection was given. To match groups for total number of injections, groups EES and SES also received a saline injection in the home cage 65 min after the paired injection. On CS– trials, subjects in all groups were treated identically, receiving a saline injection at each of the three time points. As in experiments 1 and 2, each exposure to the apparatus lasted 5 min and mice had access to the entire chamber, which contained one floor type throughout. Moreover, order of exposure to CS+ and CS– was counterbalanced within each subgroup.

It should be noted that proximal ethanol pre-exposure was not expected to influence place conditioning via direct association with the CS because previous research has shown that injection-to-CS intervals of 60 min or longer are ineffective (Cunningham et al. 1997).

Preference tests

In experiments 1 and 2, two 60-min floor preference tests were given. The first test occurred 24 h after the fourth conditioning trial, whereas the second test occurred 24 h after the eighth conditioning trial. In experiments 3 and 4, only one preference test was given, 24 h after the eighth conditioning trial. On all tests, the apparatus floor was half grid and half hole, with relative position counterbalanced within each subgroup. Time spent on the grid floor was the primary dependent variable during each 60-min session. A 2-day (weekend) break occurred before the fifth conditioning trial in all experiments.

Data analysis

Data were evaluated by analysis of variance (ANOVA) with the alpha level set at 0.05. Pre-exposure group and Conditioning subgroup were treated as between-group factors, whereas Trials, CS type and Test were treated as within-group factors. For purposes of analysis, preference data were expressed as the mean seconds per minute spent on the grid floor averaged over each 60-min test session. To control overall alpha level within each experiment, *P*-values were Bonferroni-corrected for the number of post-hoc

comparisons between pairs of group means. In the unbiased, counterbalanced design used in these studies, place conditioning is defined by the difference between the GRID+ and GRID– conditioning subgroups within each of the main treatment groups (Cunningham 1993). CPP is observed when mice that have received grid-ethanol pairings (GRID+) spend more time on the grid floor during choice tests than mice that have received grid-saline pairings (GRID–). Conversely, CPA is observed when the GRID+ subgroup spends less time on the grid floor than the GRID– subgroup.

Results

Distal pre-exposure: conditioning trial activity

Experiment 1

As expected, injection of ethanol increased activity on CS+ trials in experiment 1 (Fig. 1). Moreover, home-cage ethanol pre-exposure during the week prior to conditioning produced sensitization to ethanol's activating effect as shown by higher levels of ethanol-stimulated activity in the 2 and 4 g/kg groups compared to the 0 g/kg group on trial 1. However, magnitude of sensitization to the 2 g/kg conditioning dose did not differ as a function of pre-exposure dose. Repeated exposure to ethanol during conditioning produced additional sensitization in all groups. In contrast, activity on saline (CS–) trials decreased over trials in a similar manner for all three pre-exposure groups.

The foregoing observations were supported by a three-way mixed ANOVA (Pre-exposure group×CS type×Trials) that revealed a significant three-way interaction [$F(6,279)=4.6$, $P<0.001$] in addition to significant main effects for each factor and all two-way interactions [all $P<0.0001$]. Separate two-way follow-up ANOVAs suggested the three-way interaction could be attributed to a significant Pre-exposure group×Trials interaction on CS+ trials [$F(6,279)=8.3$, $P<0.0001$], but not on CS– trials. Only the Trials effect was significant for CS– [$F(3,279)=9.8$, $P<0.0001$]. Additional follow-up analyses confirmed that all three pre-exposure groups showed a significant increase in CS+ activity between trials 1 and 4 [$Fs(3,93)>10.8$, $P<0.0001$]. CS+ activity of the 0 g/kg group was significantly lower than that of the 2 g/kg group on all four trials and lower than that of the 4 g/kg group on all but the last trial (Bonferroni-adjusted $P<0.05$). The 2 and 4 g/kg groups did not differ on any trial.

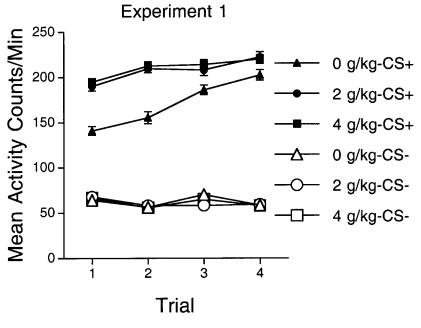

Fig. 1 Mean activity counts per minute (+SEM) during each CS+ (*solid symbols*) and CS– (*closed symbols*) conditioning trial in experiment 1. All mice received an ethanol (2 g/kg) injection before each CS+ trial and a saline injection before each CS– trial. Distal pre-exposure groups differed in the dose of ethanol (0, 2 or 4 g/kg) injected at 48-h intervals in the home cage during the week before conditioning. Data are collapsed over GRID+ and GRID– conditioning subgroups (n=32/group)

Experiment 2

Although ethanol pre-exposure clearly produced sensitization to ethanol's activating effect in experiment 1, the same pre-exposure treatment had little effect on activity recorded in the apparatus during the 5 min just before paired ethanol injection on conditioning trials in experiment 2 (data not shown). Activity rates generally declined over trials and were slightly higher on CS+ trials (38.4±1.1) than on CS– trials (35.5±0.9). These conclusions were supported by a three-way mixed ANOVA (Pre-exposure group×CS type×Trials) that yielded significant main effects of CS type [$F(1,92)=10.0$, $P<0.01$] and Trials [$F(3,276)=69.0$, $P<0.0001$], but no other effects or interactions.

Distal pre-exposure: preference tests

Experiment 1

As noted earlier, place conditioning was assessed by comparing the magnitude of the difference between the counterbalanced GRID+ and GRID– subgroups within each of the main treatment groups. This comparison indicated that all groups in experiment 1 developed a CPP, regardless of ethanol pre-exposure dose (Fig. 2). This

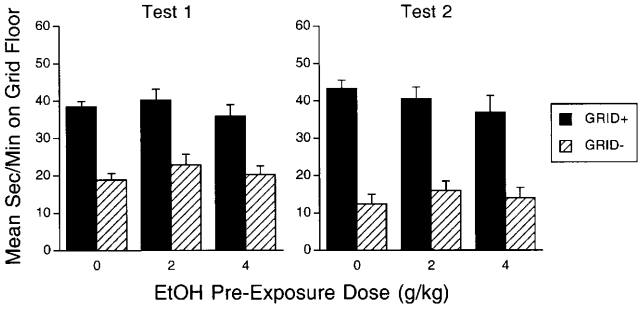

Fig. 2 Mean seconds per minute (+SEM) spent on the grid floor during two 60-min test sessions in experiment 1. Test 1 was given after the first four conditioning trials (two CS+ and two CS– trials), whereas test 2 was given after eight conditioning trials (four CS+ and four CS– trials). Distal pre-exposure groups differed in the dose of ethanol (0, 2 or 4 g/kg) injected at 48-h intervals in the home cage during the week before conditioning. During the conditioning phase, mice in the GRID+ conditioning subgroups received ethanol (2 g/kg) immediately before 5-min exposure to the grid floor on CS+ trials; saline was injected before exposure to the hole floor on CS– trials. These contingencies were reversed for mice in the GRID– conditioning subgroups. Each conditioning subgroup contained 16 mice

Table 1 Mean activity counts per minute (±SEM) during preference tests

Group	Experiment 1		Experiment 2	
	Test 1	Test 2	Test 1	Test 2
0	33.8±0.9	29.0±1.6	25.6±1.0	19.3±1.3
2	34.9±1.3	31.8±1.7	26.9±1.5	23.1±1.8
4	37.2±1.5	29.7±1.6	27.6±0.8	24.0±1.4
	Experiment 3		Experiment 4	
	Test 1		Test 1	
SES	28.1±0.9		15.5±1.3	
EES	27.4±1.0		22.4±1.2	
SEE	25.2±1.4		13.0±1.1	

preference was apparent on the first test (left panel) and increased on the second test (right panel) as a result of two additional conditioning trials. However, there were no differences among ethanol pre-exposure groups on either test. Three-way ANOVA (Pre-exposure group×Conditioning subgroup×Test) supported these observations, yielding significant main effects of Conditioning subgroup [$F(1,90)=123.7$, $P<0.0001$] and Test [$F(1,90)=4.2$, $P<0.05$], but no main effect or interactions involving Pre-exposure group. There was also a significant Conditioning subgroup×Test interaction [$F(1,90)=14.8$, $P<0.001$], reflecting the increase in magnitude of conditioned preference across tests. Separate follow-up ANOVAs indicated the Conditioning subgroup effect was significant on both tests [$Fs(1,90)>77.8$, $P<0.0001$]. Test session activity rates (Table 1) decreased between tests 1 and 2 [$F(1,93)=44.8$, $P<0.0001$], but did not differ among pre-exposure groups.

Experiment 2

Post-CS injection of 2 g/kg ethanol on conditioning trials produced a conditioned aversion for the ethanol-paired floor on both tests, but only in the 0 g/kg pre-exposure group (Fig. 3). Neither of the ethanol pre-exposed groups displayed appreciable conditioning. These conclusions were supported by a three way ANOVA (Pre-exposure group×Conditioning subgroup×Test) that produced a significant Conditioning subgroup effect [$F(1,89)=10.4$, $P<0.002$] and a significant Pre-exposure group×Conditioning subgroup interaction [$F(2,89)=3.9$, $P<0.05$]. Separate comparisons between GRID+ and GRID– conditioning subgroups (collapsed across tests) were conducted to determine whether place conditioning had occurred within each pre-exposure group. These tests indicated significant place aversion in the 0 g/kg group (Bonferroni-adjusted $P<0.0005$), but not in the 2 or 4 g/kg groups. Test session activity rates (Table 1) decreased across tests [$F(1,92)=31.3$, $P<0.0001$], but did not differ among pre-exposure groups.

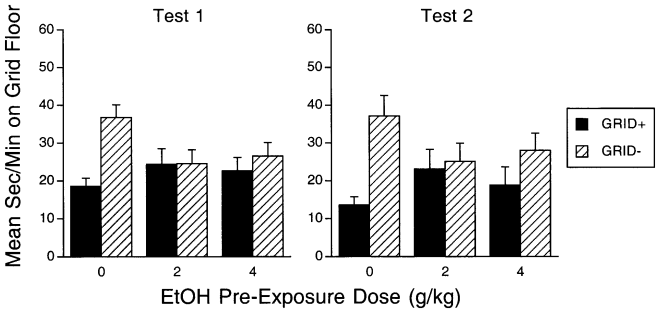

Fig. 3 Mean seconds per minute (+SEM) spent on the grid floor during two 60-min test sessions in experiment 2. Test 1 was given after the first four conditioning trials (two CS+ and two CS– trials), whereas test 2 was given after eight conditioning trials (fours CS+ and four CS– trials). Distal pre-exposure groups differed in the dose of ethanol (0, 2 or 4 g/kg) injected at 48-h intervals in the home cage during the week before conditioning. During the conditioning phase, mice in the GRID+ conditioning subgroups received ethanol (2 g/kg) immediately after 5-min exposure to the grid floor on CS+ trials; saline was injected after exposure to the hole floor on CS– trials. These contingencies were reversed for mice in the GRID– conditioning subgroups. Each conditioning subgroup contained 15–16 mice

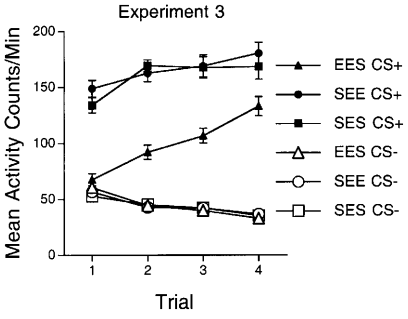

Fig. 4 Mean activity counts per minute (+SEM) during each CS+ (*solid symbols*) and CS– (*closed symbols*) conditioning trial in experiment 3. All mice received an ethanol (2 g/kg) injection immediately before each CS+ trial and a saline injection immediately before each CS– trial. The proximal pre-exposure groups were injected with 2 g/kg ethanol (*group EES*) or saline (*groups SEE and SES*) in the home cage 65 min before each CS+ trial. These groups also received a 2 g/kg ethanol (SEE) or saline (EES and SES) injection in the home cage 65 min after each CS+ trial. To equate for handling and injection experience, all groups were injected with saline in the home cage 65 min before and 65 min after each CS– trial. Data are collapsed over GRID+ and GRID– conditioning subgroups (n=28–32/group)

Proximal pre-exposure: conditioning trial activity

Experiment 3

As in experiment 1, conditioning trial injections of ethanol increased locomotor activity and repeated exposure over trials produced sensitization (Fig. 4). Administration of a second ethanol injection 65 min after each CS+ trial (group SEE) had no effect on ethanol-induced activation or sensitization compared to a procedure in which mice received only a single paired ethanol injection (group SES). In contrast, injection of ethanol 65 min be-

fore CS+ trials (group EES) appeared to reduce the typical activating effect of the first ethanol conditioning trial injection, although it did not prevent development of sensitization across trials. Examination of activity on a minute-by-minute basis during the first ethanol trial (data not shown) actually revealed a higher activity rate in group EES (166.7±7.0) compared to groups SES (93.9±8.6) and SEE (106.6±8.4) during the first minute after injection, but a lower activity rate during the fifth minute (EES=25.9±6.9; SES=141.5±6.5; SEE=147.9±8.4). Treatments given on CS+ days generally had little effect on CS– trial activity levels, which decreased over trials in all groups.

Three-way mixed ANOVA (Pre-exposure group×CS type×Trials) applied to data shown in Fig. 4 yielded a significant three-way interaction [$F(6,255)=3.6$, $P<0.005$] in addition to significant main effects for each factor and all two-way interactions (all $P<0.05$). Separate two-way follow-up ANOVAs indicated significant main effects of Trials [$Fs(3,255)>28.2$, $P<0.001$] and significant Pre-exposure group×Trials interactions [$Fs(6,255)>2.3$, $P<0.05$] for both types of conditioning trials. However, the main effect of Pre-exposure group was significant only for CS+ trials [$F(2,85)=37.9$, $P<0.001$], confirming the general suppressive effect of proximal ethanol pre-exposure. Additional analyses showed that Trials effects were significant for both types of CS in all three Pre-exposure groups ($P<0.005$).

Experiment 4

Activity levels were generally low in most groups during the 5 min before conditioning trial injections in experiment 4 (Fig. 5). The exception was group EES, which showed a much higher level of activity compared to other groups on CS+ trials, reflecting the residual activating effects of the ethanol injection given 65 min earlier in the home cage. Group EES also showed higher activity levels than other groups on CS– trials. Although both of the other groups showed a trend toward higher activity on CS+ trials compared to CS– trials, overall activity was relatively low. All groups showed a decrease in activity across both types of trials.

ANOVA (Pre-exposure group×CS type×Trials) indicated that all of the main effects were significant [group: $F(2,91)=123.2$; CS: $F(1,91)=154.8$; Trials: $F(3,273)=135.8$; all $P<0.0001$] as were the Pre-exposure group×CS type [$F(2,91)=93.9$, $P<0.0001$] and Pre-exposure group×Trials [$F(6,273)=6.4$, $P<0.0001$] interactions. Follow-up analyses indicated that the group×CS interaction was due primarily to differences between group EES and each of the other groups (Bonferroni-adjusted $P<0.001$), which did not differ on either type of trial.

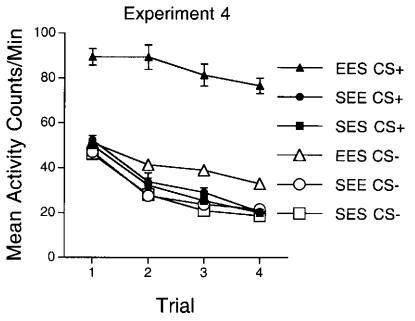

Fig. 5 Mean activity counts per minute (+SEM) during each CS+ (*solid symbols*) and CS– (*closed symbols*) conditioning trial in experiment 4. All mice received an ethanol (2 g/kg) injection immediately after each CS+ trial and a saline injection after each CS– trial. The proximal pre-exposure groups were injected with 2 g/kg ethanol (*group EES*) or saline (*groups SEE and SES*) in the home cage 65 min before each CS+ trial. These groups also received a 2 g/kg ethanol (SEE) or saline (EES and SES) injection in the home cage 65 min after each CS+ trial. To equate for handling and injection experience, all groups were injected with saline in the home cage 65 min before and 65 min after each CS– trial. Data are collapsed over GRID+ and GRID– conditioning subgroups (n=31–32/group)

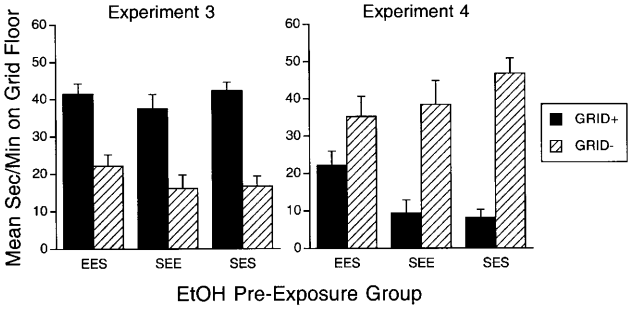

Fig. 6 Mean seconds per minute (+SEM) spent on the grid floor during 60-min test sessions in experiments 3 (*left panel*) and 4 (*right panel*). experimental contingencies during proximal pre-exposure and conditioning trials are described in the captions for Figs. 4 and 5. Each conditioning subgroup contained 13–16 mice

Proximal pre-exposure: preference test

Experiment 3

Proximal ethanol pre-exposure had no effect on strength of CPP (Fig. 6, left panel). Two-way ANOVA (Pre-exposure group×Conditioning subgroup) confirmed this conclusion by yielding a significant main effect of Conditioning subgroup [$F(1,82)=78.0$, $P<0.0001$], but no other effects. Moreover, there was no group difference in test session activity rates [$F(2,85)=1.8$; see Table 1].

Experiment 4

In contrast to the null effect on CPP (experiment 3), proximal ethanol pre-exposure reduced CPA in group EES relative to both control groups, which did not differ (Fig. 6, right panel). This conclusion was supported by a

two-way ANOVA (Pre-exposure group×Conditioning subgroup) that yielded a significant interaction [$F(2,88)=4.2$, $P<0.02$] in addition to a significant main effect of Conditioning subgroup [$F(1,88)=55.7$, $P<0.0001$]. Separate comparisons between GRID+ and GRID– conditioning subgroups within each pre-exposure group indicated significant place aversion in groups SEE and SES (Bonferroni-adjusted $P<0.0001$), but not in group EES (Bonferroni-adjusted $P>0.1$). Analysis of test session activity rates revealed a significant Pre-exposure group effect [$F(2,91)=33.7$, $P<0.0001$; see Table 1]. Follow-up comparisons indicated that group EES was significantly more active than either of the other groups (Bonferroni-adjusted $P<0.001$), which did not differ.

Discussion

These experiments showed that ethanol pre-exposure, given either distally or proximally, reduced the strength of ethanol-induced CPA but had no impact on ethanol-induced CPP. Despite strong evidence that distal pre-exposure produced sensitization to ethanol's locomotor activating effect at both doses (Fig. 1), there was no change in ethanol's rewarding effect as indexed by the CPP procedure (Fig. 2). In contrast, the same pre-exposure treatment completely eliminated the CPA normally produced by post-CS ethanol injections (Fig. 3), suggesting that pre-exposure reduced ethanol's aversive effects. Although the proximal pre-exposure studies did not address whether groups differed in locomotor sensitization produced by ethanol, the overall pattern of place conditioning results was similar in suggesting a reduction in ethanol's aversive effect but no change in its rewarding effects (Fig. 6).

Distal pre-exposure

Place aversion

The finding of reduced CPA after distal pre-exposure is consistent with previous studies involving many different drugs, including ethanol. For example, distal pre-exposure has been reported to attenuate the CPA normally produced by phencyclidine (Iwamoto 1985; Kitaichi et al. 1996) and the kappa agonist U-69593 (Shippenberg et al. 1988). Distal pre-exposure has also been found to reduce or eliminate the CPA normally produced by ethanol in rats (Gauvin and Holloway 1992; Holloway et al. 1992) and mice (Cunningham and Henderson 2000). The suggestion that such effects reflect a reduction in ethanol's aversive properties is further supported by studies showing that distal pre-exposure weakens ethanol-induced conditioned taste aversion (Berman and Cannon 1974; Cannon et al. 1975; Barker and Johns 1978; Risinger and Cunningham 1995; Gauvin et al. 2000). At a more general level, the distal pre-exposure effect on ethanol CPA is consistent with previous findings that pre-exposure to the unconditioned stimulus (US) before CS-US pairings interferes with subsequent development of conditioned responses in a variety of Pavlovian conditioning preparations (see review by Randich and LoLordo 1979).

Theoretical analyses of such US pre-exposure effects have suggested both non-associative and associative accounts of this phenomenon (Randich and LoLordo 1979), either of which might be applied here. According to a prominent non-associative account, US pre-exposure might interfere with subsequent conditioning due to habituation or tolerance to unconditioned responses (URs) elicited by the US. Although no independent evidence of tolerance was provided in the present studies, this possibility is supported by a previous study in which rats that developed tolerance to ethanol's rate decreasing effects in an operant task later showed a reduced CPA (Holloway et al. 1992).

Alternatively, the most plausible associative account of US pre-exposure provides an explanation based on the phenomenon of context blocking (Kamin 1969). According to this theory, cues that became associated with the ethanol US during pre-exposure presumably blocked the later development of an association between the CS+ and ethanol. However, to apply this theory to studies involving home cage pre-exposure injections, one must argue that handling and injection stimuli served as the blocking stimuli (Randich and LoLordo 1979). That is, one must assume that the paired relationship between handling/injection cues and ethanol during the pre-exposure phase established an association that interfered with learning about the CS-ethanol relationship during subsequent place conditioning. Although there is no independent evidence for this association in the present study, the treatment contingencies would certainly have allowed it.

Place preference

The absence of a distal pre-exposure effect on ethanol CPP suggests that pre-exposure produced neither tolerance nor sensitization to ethanol's rewarding effect. This null outcome is consistent with a previous study in which C57BL/6 mice self-administered ethanol orally during pre-exposure (Nocjar et al. 1999). However, that study did not show that the pre-exposure regimen produced tolerance or sensitization to any ethanol effect. In contrast, the same pre-exposure treatment that failed to affect CPP in our study produced both a significant reduction in ethanol CPA (Fig. 3) and a robust sensitization of ethanol's locomotor activating effect (Fig. 1). Although the latter effect is often hypothesized to predict enhancement of ethanol's rewarding or reinforcing effects (Hunt and Lands 1992; Lessov et al. 2001), there was no effect on ethanol CPP. One might be concerned that ethanol pre-exposure (sensitization) had no effect because CPP had reached a performance ceiling. However, because the first preference test was conducted after only two trials, before conditioning had reached asymp-

tote, it is difficult to argue that our test parameters were insensitive to a treatment that might have enhanced ethanol reward. The significant increase in CPP magnitude between the first and second tests (left versus right panels of Fig. 2) indicates that the conditions of test 1 provided a good opportunity to detect pre-exposure effects that were not limited by a performance ceiling. Despite these considerations, one could still argue that additional pre-exposures might have affected ethanol CPP. It is also possible that conditioning with a lower ethanol dose would have enhanced sensitivity to pre-exposure. Nevertheless, one must conclude that ethanol's rewarding effect as measured by CPP is more resistant to distal pre-exposure than ethanol's aversive effect as measured by CPA.

At first glance, the null effect of distal pre-exposure on ethanol CPP in mice does not appear consistent with several previous studies in rats in which distal pre-exposure has been shown or was alleged to promote the development of ethanol CPP (Reid et al. 1985; Gauvin and Holloway 1992; Holloway et al. 1992; Bienkowski et al. 1995, 1996; Biala and Kotlinska 1999; Ciccocioppo et al. 1999; Gauvin et al. 2000). However, interpretation of this apparent species difference is complicated by the fact that most studies using the pre-CS procedure have not found a reliable ethanol-induced CPP in drug-naive rats (Sherman et al. 1988; Tzchentke 1998), whereas this outcome is quite common in drug-naive mice (e.g. Cunningham et al. 1991, 1992, 2000; Risinger et al. 1994; Cunningham 1995; Risinger and Oakes 1996; Chester et al. 1998; Nocjar et al. 1999; Itzhak and Martin 2000; Grahame et al. 2001).

While some authors have suggested that distal pre-exposure effects obtained in these rat studies reflect sensitization to ethanol's rewarding effect (Bienkowski et al. 1995, 1996), most have offered a more parsimonious interpretation based on tolerance to ethanol's aversive effect (Gauvin and Holloway 1992; Holloway et al. 1992; Biala and Kotlinska 1999; Ciccocioppo et al. 1999). According to this analysis, pre-CS administration of ethanol is assumed to have both rewarding and aversive effects in rats, but tolerance to its aversive effect is usually required before its rewarding effect can be revealed. Thus, the general conclusions from rat pre-exposure studies appear consistent with those from our mouse studies, i.e. distal pre-exposure produces tolerance to ethanol's aversive effect but no change in its rewarding effect. In addition to extending this conclusion to a different species, the present studies offer more compelling evidence that pre-exposure does not alter ethanol reward by showing that ethanol CPP is unaffected even when the pre-exposure treatment produces a reduction in ethanol's aversive effects and sensitization to its locomotor activating effects. Our findings might also be interpreted as supporting the idea that locomotor sensitization actually reflects tolerance to an activity suppressing effect of ethanol (Tritto and Dudek 1997) that is aversive.

Although the foregoing analysis allows reconciliation of the ethanol place conditioning literature, the null effect of pre-exposure on ethanol CPP is not congruent with studies showing significant pre-exposure effects on CPP induced by other abused drugs such as morphine (Shippenberg et al. 1988, 1989, 1996, 1998; Lett 1989), cocaine (Lett 1989; Shippenberg and Heidbreder 1995a, 1995b; Shippenberg et al. 1996, 1998), amphetamine (Lett 1989) and nicotine (Shoaib et al. 1994). Our analysis also fails to explain why the well-known US pre-exposure effect (Randich and LoLordo 1979) occurs with ethanol CPA, but not with ethanol CPP, even though both behaviors are viewed as instances of Pavlovian conditioning (Cunningham and Henderson 2000). One possibility is that ethanol's rewarding effect is simply much more resistant to change than either its aversive effect or the rewarding effects of opiate and psychostimulant drugs.

Proximal pre-exposure

Experiments 3 and 4 are the first to examine effects of proximal US pre-exposure on drug-induced CPP and CPA. Although the effects of proximal pre-exposure appear to parallel those of distal pre-exposure in showing a reduction in CPA but no change in CPP, the interpretation of these findings is quite different. The distal pre-exposure studies examined the historical impact of a series of ethanol exposures well after the direct pharmacological effects of pre-exposure had dissipated. In contrast, the proximal pre-exposure studies examined the effects of a relatively recent drug exposure at a time when blood ethanol concentrations were still elevated.

The finding that proximal pre-exposure interfered with development of CPA is generally consistent with an older literature showing that proximal US pre-exposure impedes rabbit eyelid conditioning and lithium chloride-induced conditioned taste aversion (see review by Domjan 1980). That literature suggested two possible mechanisms for proximal US pre-exposure effects: (a) reduced effectiveness of the conditioning US presentation, and (b) interference with (overshadowing of) the target CS (Best and Domjan 1979). According to the first interpretation, proximal exposure to ethanol might have reduced the UR to the second (paired) ethanol injection through either an opponent-process mechanism (Solomon 1977) or a memory "priming" mechanism (Wagner 1975, 1981). In pharmacological terms, this might represent an instance of "acute tolerance" in which the ability of a given drug concentration to produce its normal effect is reduced after a prolonged continuous exposure to ethanol (Le and Mayer 1996). According to the second interpretation, strong sensory experiences produced by the initial ethanol injection might have reduced learning about the target (floor) CS due to attentional or associative competition (Best and Domjan 1979, p. 439). The associative version of this second interpretation is related to another potential explanation of proximal pre-exposure based on state-dependent learning. That is, one might explain the weaker CPA produced by proximal

ethanol pre-exposure as resulting from the removal of an important component of the overall stimulus context present at the time of original conditioning (generalization decrement). Given ethanol's postulated amnesic properties (Ryabinin 1998), the reduction in CPA produced by proximal pre-exposure might also be explained directly in terms of memory interference.

Although all of these interpretations seem to offer reasonable accounts of the proximal pre-exposure effect on CPA, interpretations that appeal to competition with the target CS, generalization decrement or memory interference are much less compelling when one considers that the same ethanol pre-exposure treatment had little effect on the ability of the target CS to control CPP. Thus, interpretations based on a change in US efficacy (e.g. UR magnitude) seem more plausible. For example, if one assumes that CPA is caused by an aversive effect of ethanol injection that is independent from the rewarding effect that produces CPP (Cunningham et al. 1997; Cunningham and Henderson 2000), the different outcomes of experiments 3 and 4 can be explained by arguing that the aversive UR is simply more sensitive to proximal pre-exposure than the rewarding UR. However, this interpretation does not work if one assumes that ethanol-induced CPA and CPP are produced by a common rewarding effect (see Cunningham and Henderson 2000, for further discussion of this issue).

Previous discussions of ethanol's bivalent effects on mice in the place conditioning task have suggested that IP injection of ethanol produces an initial short-duration aversive effect that is followed by a longer lasting positive motivational effect (Cunningham et al. 1997; Cunningham and Henderson 2000). Presumably, post-CS injection favors conditioning of the aversive component whereas pre-CS injection favors conditioning of the rewarding component. Although the exact nature of the initial aversive component is unknown, one possibility is that it reflects a relatively nonspecific effect of the rapid transition from the sober state to the intoxicated state (Cunningham et al. 1997). This interpretation is easily extended to explain why post-CS injection of psychostimulant drugs like amphetamine and nicotine also produce CPA (Fudala and Iwamoto 1987, 1990). Furthermore, because the aversiveness of the drug-state transition produced by the conditioning US injection would presumably be reduced in an animal that was already intoxicated, this interpretation also explains why proximal pre-exposure weakens CPA.

The null effect of proximal pre-exposure on ethanol CPP suggests that intoxication did not change the rewarding effect of the paired ethanol injection or the animal's ability to encode or to subsequently recall the association between ethanol and CS+. Based solely on pharmacological considerations, one might have predicted either a reduction or an enhancement in ethanol's rewarding effect. For example, the proximal injection could have reduced the rewarding effect of the US injection through an acute tolerance mechanism. Alternatively, because total brain ethanol concentration during the 5-min conditioning trials was greater in proximally pre-exposed mice, one might have expected greater CPP based on dose-response considerations (Cunningham et al. 1992; Risinger and Oakes 1996). One obvious shortcoming of the present studies is the examination of only one combination of pre-exposure and conditioning doses at a single time interval. It is quite possible that proximal pre-exposure would influence CPP under a different set of parameters. Nevertheless, the present studies reveal a clear difference in the sensitivity of CPP and CPA to effects of proximal ethanol exposure.

The proximal pre-exposure studies may have an interesting broader implication for studies that examine effects of other pharmacological pretreatments on CPP or CPA, especially treatments thought to mimic ethanol's "agonist" effects. Given that ethanol is its own best agonist, the present findings suggest that ethanol CPP may be relatively resistant to agonist pretreatment and more likely to yield "false negatives." At present, the relevant literature is simply too sparse to draw any firm conclusions. Nevertheless, it is interesting to note that the pattern of findings in experiment 3 is quite similar to that reported in a recent study of the $GABA_B$ agonist baclofen on ethanol CPP. That is, baclofen reduced ethanol stimulated activation, but did not alter CPP (Chester and Cunningham 1999). Given that pretreatment with ethanol itself has little impact on CPP, those authors may have been premature in dismissing a role for $GABA_B$ receptors in ethanol reward.

General conclusions

To the extent that motivational processes measured by CPP and CPA normally contribute to ethanol drinking, the present findings suggest that increases in ethanol intake seen after chronic ethanol exposure are more likely caused by tolerance to ethanol's aversive effect rather than by sensitization to its rewarding or reinforcing effect. These findings also suggest that ethanol's rewarding effect, as indexed by CPP, is generally much more resistant to effects of either distal or proximal ethanol pre-exposure than ethanol's aversive effect as indexed by CPA. Although pre-exposure had no effect on ethanol reward in the present studies, it is still possible that tolerance or sensitization to that effect might be observed in a different mouse strain or under different (e.g. longer) schedules of ethanol pre-exposure.

Distal ethanol pre-exposure may be more effective in producing sensitization to ethanol reward when ethanol is voluntarily self-administered during pre-exposure rather than passively administered as in the present studies (see Hunt and Lands 1992). Indeed, many (though not all) of the studies that have been successful in demonstrating ethanol CPP in rats after distal pre-exposure allowed animals to self-administer ethanol during pre-exposure (e.g. Reid et al. 1985; Gauvin and Holloway 1992; Ciccocioppo et al. 1999). However, in the one published study of distal pre-exposure on ethanol CPP in

mice, voluntary consumption of ethanol during the pre-exposure phase had no impact on subsequent development of CPP (Nocjar et al. 1999). Although the present and many previous studies make it clear that locomotor sensitization is rapidly acquired with passive ethanol exposure, future studies must determine whether neuroadaptation to ethanol reward requires or is facilitated by self-administration.

Finally, the present studies do not support the hypothesis that ethanol-induced CPP and CPA are caused by a monovalent rewarding effect of ethanol (see Cunningham and Henderson 2000). If CPP and CPA were both induced by the same motivational effect of ethanol, one would expect ethanol pre-exposure to have a similar influence on both types of behavior. However, the finding that pre-exposure (distal or proximal) has different effects on CPP and CPA lends support to the conclusion that CPP and CPA are mediated by independent, motivationally opposed effects of ethanol.

Acknowledgements This research was supported by NIAAA grants AA07702 and AA07468. We thank Anita Bechtholt, Tara Fidler and Katherine Hill for comments on the manuscript. Portions of these data were presented at the annual Research Society on Alcoholism meeting in Denver, Col., USA, June, 2000.

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