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Pentylenetetrazol produces a state-dependent conditioned place aversion to alcohol withdrawal in mice

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ABSTRACT

The purpose of this study was to determine if aversive effects of alcohol withdrawal could be detected in mice using the place conditioning procedure and whether the $GABA_A$ receptor antagonist, pentylenetetrazol (PTZ), would increase the aversive effects of alcohol withdrawal and increase the probability of detecting conditioned place aversion. Subjects were alcohol-naïve mice from a specific line selectively bred for low alcohol preference (LAP1; $n=91$) and were assigned to three groups: alcohol withdrawal, PTZ alone, and PTZ + alcohol withdrawal. On four trials, mice received either a 4.0 g/kg intraperitoneal (i.p.) injection of alcohol (alcohol withdrawal, PTZ + alcohol withdrawal groups) or saline (PTZ group) 8 h prior to being placed on a distinctive floor texture for a 30-min conditioning session. Five minutes before these sessions, mice in the PTZ and PTZ + alcohol withdrawal groups received PTZ (5.0 mg/kg; i.p.) and the alcohol withdrawal group received saline. On intervening days mice received two saline injections at the same time points prior to being placed on a different floor texture. Post-conditioning floor preference was assessed in two 60-min tests; the first test was drug-free and the second test was state-dependent. Neither alcohol withdrawal nor PTZ produced significant place conditioning. The PTZ + alcohol withdrawal group showed a significant place aversion during the state-dependent test. These data suggest that the combined stimulus properties of PTZ and alcohol withdrawal facilitated the expression of conditioned place aversion to alcohol withdrawal.

1. Introduction

The alcohol withdrawal syndrome consists of overt, physical signs and subjective, motivational symptoms that occur when blood alcohol levels are falling and after blood alcohol levels have reached zero mg%. Signs of alcohol withdrawal can range from mild to severe and are similar in both humans and rodents (Kalant, 1977). These signs include tremors, convulsions, increased heat rate, and increased body temperature (Holloway et al., 1993; Majchrowicz, 1975). Symptoms of alcohol withdrawal, which are reportedly aversive based on subjective human descriptions, include irritability, nausea, headache, anxiety, and craving (Swift and Davidson, 1998; Tiffany, 1990). These aversive symptoms of alcohol withdrawal are thought to largely contribute to an individual's propensity to consume alcohol and to their risk for alcoholism (Chester et al., 2002; 2003; Koob, 2003; Wall and Ehlers, 1995). However, discrepancies in the human literature indicate that aversive alcohol withdrawal symptoms may be associated with either increased (McCaul et al., 1991; Newlin and Pretorius, 1990; Span and Earleywine, 1999) or decreased (Wall et al., 2000) subsequent alcohol consumption. Thus, the relationship between alcohol withdrawal and alcohol drinking behavior is not well understood in humans. It is likely that both the type and the severity of the alcohol withdrawal symptom may influence subsequent alcohol drinking behavior. For instance, craving has been associated with a propensity to consume excessive amounts of alcohol (Koob, 2003), whereas headache and nausea have been associated with alcohol avoidance (Wall et al., 2000).

Animal models of alcohol withdrawal are advantageous because potentially confounding variables can be better controlled, such as amount of alcohol exposure and individual (e.g., genetic) and environmental factors that are known to influence the expression of the alcohol withdrawal syndrome in humans. Symptoms of alcohol withdrawal in rodents have been difficult to measure because of their subjective nature (see Emmett-Oglesby et al., 1990 for review). However, several models have been used to index withdrawal symptoms such as anxiety and craving in rodents, including pentylenetetrazole (PTZ) drug discrimination (Gauvin et al., 1989), acoustic startle responding (e.g., Rassnick et al., 1992), the social interaction test (e.g., Overstreet et al., 2002), the elevated plus maze (e.g., Valdez et al., 2002), and operant self-administration (e.g., Roberts et al., 2000). Although anxiety and self-administration models have high face validity, it is important to remember that alcohol withdrawal-induced

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changes in anxiety or self-administration behavior do not necessarily reflect the motivational (rewarding or aversive) effects of the alcohol withdrawal state.

The place conditioning procedure has been used as a sensitive measure of both rewarding and aversive motivational effects of drug intoxication and withdrawal (discussed in Bozarth, 1987; Chester and Cunningham, 2002; Cunningham et al., 2000). This procedure involves establishing an association between a neutral environmental conditioning stimulus (CS) and the unconditioned motivational effects of drug intoxication or withdrawal. The motivational effect of this association is determined by examining approach to or avoidance of the drug-paired CS. Greater approach toward and contact with the CS is interpreted as evidence of the rewarding effect of the drug, whereas stronger avoidance of the CS is seen as indicative of the aversive effect of the drug. Advantages and disadvantages of this procedure as a model to study the motivational effects of drugs have recently been reviewed (Cunningham et al., 2006). One valuable aspect of this procedure is that it specifically assesses the learned relationship between environmental stimuli and motivational effects of the drug. Thus, this procedure may serve as a particularly useful model for understanding certain learning and memory processes and the role of environmental cues in influencing craving, relapse, and alcohol-seeking behavior in humans (Cunningham et al., 2000). Another potential advantage, particularly when applied to the study of alcohol withdrawal, is that it may detect motivational effects of alcohol withdrawal after relatively small amounts of alcohol exposure compared to other withdrawal models that often require extended exposure to alcohol before alcohol withdrawal signs are observable. This feature of the place conditioning procedure is useful because initial sensitivity to the aversive motivational effects of alcohol withdrawal following a single or several discrete alcohol exposures may be regulated by mechanisms that are different from those that regulate motivational effects of alcohol withdrawal following continuous or chronic alcohol exposure.

Place conditioning has often been used in rats as a sensitive measure of aversive motivational effects of withdrawal from both acute (e.g., Azar et al., 2003; Parker et al., 2002) and chronic (e.g. Mucha, 1987; Stinus et al., 1990) opiate exposure, largely because withdrawal from opiates can easily be precipitated using opiate antagonists. There are only two studies in rats that have used the place conditioning procedure to study the motivational effects of alcohol withdrawal. In one study, a place preference was observed (Gauvin et al., 1997) and in another study, a place aversion was observed (Morse et al., 2000). There are no studies in mice that have demonstrated place conditioning to the motivational effects of alcohol withdrawal. The lack of studies showing place conditioning to alcohol withdrawal could be partly due to the lack of an established procedure for precipitating withdrawal from alcohol. Similar to that employed with opiates, such a procedure would produce a discrete period of aversive effects during alcohol withdrawal that could be more easily paired with a neutral CS to produce a conditioned response.

The goal of the present study was to determine whether aversive motivational effects of alcohol withdrawal could be detected in mice using the place conditioning procedure. It was hypothesized that the time course of physical and motivational effects of alcohol withdrawal may overlap. Thus, we chose to condition mice at 8 h in withdrawal, a time point at which peak physical signs of acute withdrawal, assessed via handling-induced convulsions (HICs), are evident in mice (e.g., Crabbe et al., 1991; Crabbe, 1998; Metten and Crabbe, 1994). Further, we assessed the effect of a $GABA_A$ receptor antagonist (PTZ) on alcohol withdrawal induced-place conditioning. PTZ has been shown to enhance the physical signs of alcohol withdrawal (e.g., Chesher and Jackson, 1974; Crabbe et al., 1991; Finn and Crabbe, 1999). Thus, we hypothesized that administration of a subconvulsant dose of PTZ would enhance the aversive motivational effects of alcohol withdrawal and thereby increase the strength of conditioned place aversion.

2. Method

2.1. Subjects

Subjects were alcohol-naïve, adult male mice selectively bred for low alcohol preference (LAP1 line). Mice used in the place conditioning procedures were 82–124 days old and mice used in the HIC procedure were 52–64 days old at the start of each experiment. LAP1 mice were used in the current studies because they are readily available in our laboratory and they exhibit greater HICs during withdrawal from both acute and chronic (vapor chamber) alcohol exposure compared to their counterparts bred for high alcohol preference (HAP) (unpublished data from our laboratory and from the laboratory of Dr. John C. Crabbe at Oregon Health & Science University: P. Metten, N.J. Grahame, J.C. Crabbe, personal communication). This finding is consistent with the well-documented negative correlation between magnitude of alcohol withdrawal signs and alcohol drinking propensity in rodents (Chester et al., 2002; 2003; Metten et al., 1998). Thus, LAP1 mice were used as subjects (as opposed to HAP mice) in an effort to maximize our ability to detect aversive motivational effects of alcohol withdrawal in the place conditioning procedure.

The LAP1 mouse line was derived from a foundation stock of outbred HS/Ibg mice (Boulder, Colorado, USA) at the Indiana Alcohol Research Center (IARC) (Grahame et al., 1999). HS/Ibg mice were originally created by an intercross of eight different inbred mouse strains (A, AKR, BALB/c, C3H/2, C57BL, DBA/2, Is/Bi, and RIII) (McClearn et al., 1970). Subjects were generated at Purdue University from 8 original LAP1 breeder pairs (generation 27) obtained from the IARC. The mice used in the present study were 3rd, 4th, and 5th generation offspring from these original breeders maintained with relaxed selection. There were on average 22 breeder pairs used to generate experimental subjects for the current studies and care was taken to ensure heterogeneity in subjects' genetic background by avoiding genetic conflicts between breeder pairs at the parental and grandparental levels.

Mice were weaned at 20–23 days old and housed in polycarbonate cages (11.5×7.5×5.0 in.) in groups of 2–4 per cage with aspen wood shavings. Ambient temperature in the colony room was maintained at 21±1 °C and mice had free-access to food (Rodent Lab Diet 5001, Purina Mills Inc., St. Louis, MO) and water in the home cage. Experimental procedures were conducted during the light phase of a 12:12 light:dark cycle (lights on at 0700). Experiments were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and the experimental procedures were approved by the Purdue Animal Care and Use Committee.

2.2. Apparatus

The apparatus consisted of 8 identical open-top boxes made of Plexiglas (43.2×21.6×25.4 cm) enclosed in separate ventilated sound- and light-attenuated chambers (76.2×50.8×20.3 cm). Each box was surrounded by an open field activity frame (SmartFrame Low Density, Lafayette Instrument Co, Lafayette, IN, USA) that contained eight infrared photobeams along the length and four along the width of each frame (internal frame dimensions: 24.1×45.7 cm). The floor of each box consisted of interchangeable halves with distinct floor textures. One floor texture (the grid floor) consisted of 4 mm steel rods mounted 3.5 mm apart and the other floor texture (the hole floor) was made up of perforated 16 gauge stainless steel with 6.4 mm holes on 9.5 mm staggered centers. The photobeams were approximately 2 cm above the floor of each box. Locomotor activity and side position (left or right) for each mouse was continuously monitored by a computer program (Hamilton-Kinder MotorMonitor, Model HMM100, Hamilton-Kinder Motor Monitor, San Diego, California, USA). General activity and location of the animal (left or right) within

the box are continuously measured by occlusion of the infrared photobeams. This place conditioning apparatus was constructed with slight modifications based on that used in prior work with mice (e.g., Chester and Cunningham, 1998; 1999a,b; Chester et al., 1998; Grahame et al., 2001).

2.3. Drugs

Alcohol was diluted from a 95% (v/v) solution to a concentration of 20% (v/v) with 0.9% saline and administered in a dose of 4.0 g/kg (25.0 ml/kg injection volume). PTZ was dissolved in 0.9% saline and administered in a dose of 5.0 mg/kg (10.0 ml/kg injection volume).

2.4. Procedures

2.4.1. Handling-induced convulsions

As previously mentioned, we chose to condition mice at 8 h in withdrawal because this is the time point at which peak HICs have been demonstrated in mice exposed to an acute dose of 4.0 g/kg alcohol (e.g., Crabbe et al., 1991; Crabbe, 1998; Metten and Crabbe, 1994). Similar findings have previously been found in LAP1 mice (Metten et al., personal communication). Furthermore, we have previously demonstrated that blood alcohol levels in LAP1 mice exposed to an acute dose of 4.0 g/kg alcohol are negligible at 8 h in withdrawal (Chester and Barrenha, 2007). However, to confirm the time course of physical withdrawal in LAP1 mice and provide additional support for our choice of withdrawal conditioning time point, we assessed HICs in a group of male LAP1 mice following a single injection of 4.0 g/kg alcohol.

HICs were assessed in LAP1 mice ($n=10$) using a 7-point rating scale modified from Goldstein (1972) and previously described (e.g., Metten and Crabbe, 1994). Each mouse was lifted by the tail and observed for signs of convulsive behavior. If no signs were observed within 2 s the mouse was gently rotated 180° and observed again. Two baseline HICs scores were taken 20 min apart, the second of which occurred immediately prior to injection of 4.0 g/kg alcohol. Mice were then scored for HICs every hour for 12 h and again at 24 h post-alcohol injection.

2.4.2. Place conditioning

The place conditioning procedure involved one 60-min pre-test, eight 30-min conditioning sessions, and two 60-min post-tests. Conditioning sessions were conducted on consecutive days except that a 48-h break separated the first four and the second four sessions. The alcohol withdrawal study was conducted in four replications due to limitations associated with breeding, apparatus number, and conditioning time parameters.

An independent control experiment was conducted to verify that evidence for alcohol withdrawal-induced place conditioning was not influenced by potential changes in unconditioned floor preference due to repeated exposure to the apparatus, handling, injections, and floor stimulus cues over the course of the experiment (Cunningham et al., 2003). In this study, mice were exposed to the same experimental procedures but received saline throughout the experiment.

Pre-test. Initial preference for the two floor textures used as conditioning stimuli was assessed 24 h prior to the start of the first conditioning trial. All mice were placed in the apparatus on a half grid/half hole floor for 60 min. Floor position (left versus right side of the box) was counterbalanced within each conditioning subgroup.

Conditioning. Mice were subjected to a differential place conditioning procedure where they were randomly assigned to one of two separate conditioning subgroups within each experimental group. Each conditioning subgroup received exposure to either a grid or hole floor paired with drug treatments (CS+ conditioning sessions) and the other floor type paired with saline (CS− conditioning sessions) for a total of 4 four CS+ and CS− conditioning sessions. All subjects in each group received equal exposure to drug/saline treatments and to both floor textures. The advantage of this differential conditioning procedure is that it provides control over the subjects' exposure to the floor types and to drug exposure so that the conditioning subgroups differ only in the specific floor that is paired with drug effects (Cunningham, 1993). Assignment of mice to experimental groups and conditioning subgroups was counterbalanced by litter of origin (genetic background), order of exposure to the CS and drug exposure, and apparatus enclosure.

For the alcohol withdrawal study, mice were assigned to one of three experimental groups: alcohol withdrawal ($n=30$), PTZ ($n=30$), and PTZ + alcohol withdrawal ($n=31$). On alternating CS+ conditioning days, mice in the alcohol withdrawal and PTZ + alcohol withdrawal groups received an intraperitoneal (i.p) injection of 4.0 g/kg alcohol and the PTZ group received an equal volume of saline 8 h prior to being placed on a homogeneous grid or hole floor for 30 min. Five minutes before the CS+ conditioning session, mice in the PTZ and PTZ + alcohol withdrawal groups received an i.p. injection of 5.0 mg/kg PTZ (10.0 ml/kg) and the alcohol withdrawal group received an equal volume of saline. The 5-min pretreatment time was chosen based on the fact that PTZ produces effects on behavior within 5 min following i.p. injection (Crabbe et al., 1991; Freund et al., 1987). On intervening CS− conditioning days, all groups received two saline injections, one at 8 h and one at 5 min, prior to being placed on the alternate floor type. The floors and inside of the box were wiped with a damp sponge between each subject. All mice were handled by the scruff of the neck rather than the tail in order to avoid eliciting HICs during conditioning. No evidence of convulsive behavior was seen during conditioning.

For the saline control study, mice ($n=16$) were randomly assigned to conditioning subgroups but they received saline injections at 8 h and at 5 min before each conditioning session.

Post-tests. The post-tests were conducted in the same manner as the pre-test. The first post-test was conducted in a drug-free state 24 h after the last conditioning session. The second post-test was "state-dependent," conducted 72 h after the first post-test. In the alcohol withdrawal study, a small portion of the mice received their state-dependent test at 96 h after the first post-test because one of the replications occurred over a holiday. Prior to the state-dependent test, all groups received their same respective drug treatments as that given on CS+ conditioning sessions at 8 h and 5 min before the test session. For the control study, two post-tests were given in the same manner as the alcohol withdrawal study but only saline injections were administered.

2.5. Statistical analyses

Data were analyzed using analysis of variance (ANOVA) with the significance level set at $p<0.05$. Between-group factors included Group (alcohol withdrawal, PTZ, PTZ + alcohol withdrawal, saline) or Pretreatment (saline or PTZ) and Conditioning Treatment (saline or alcohol withdrawal). Within-group factors included Floor Type (grid or hole), CS Session Type (CS+ or CS−), Conditioning Session (1–4), Test Phase (pre- versus post-conditioning), and Minute or Hour. Because experimental procedures were identical, place conditioning data from the alcohol withdrawal and the saline control study were analyzed together. For the HICs study, the two baseline scores were averaged and compared to the peak withdrawal magnitude score which was calculated by averaging the highest HIC score and the HIC score taken before and after the highest HIC score (Metten and Crabbe, 1994). Significant main effects and interactions were followed using lower-order one-way ANOVAs and paired samples *t*-tests, where appropriate (Keppel, 1991). Pearson product moment correlations were conducted to determine the relationship between locomotor activity during conditioning and testing and the magnitude of conditioned place aversion.

3. Results

3.1. Handling-induced convulsions

Fig. 1 shows the time course of HIC scores in LAP1 mice following a single injection of 4.0 g/kg alcohol. As can be seen in the figure, the peak magnitude of convulsions occurred on average at 8 h in withdrawal. One mouse was removed from the analysis because it showed the highest HIC score during the last (24) h of measurement and thus a peak withdrawal score could not be calculated. Paired samples *t*-test revealed a significant difference between the average baseline scores and the peak withdrawal magnitude scores ($t=-3.3$, $df=8$, $p=0.01$).

3.2. Alcohol withdrawal place conditioning study

Two mice died during the course of the study and were removed from all analyses.

3.2.1. Pre-test

Overall analysis of the 60-min pretest data (Group × Floor Type × Minute ANOVA) yielded main effects of Floor Type [$F(1,103)=12.8$, $p=0.001$] and Minute [$F(59,6077)=1.4$, $p<0.05$] and a Floor Type × Minute interaction [$F(59,6077)=1.6$, $p<0.01$]. Follow-up one-way ANOVAs indicated main effects of Floor Type during both the first 30 and last 30 min of the session [$Fs>8.0$, $ps<0.01$], due to mice spending on average more time on the grid floor (32.1 ± 0.6 s/min) versus the hole floor (27.8 ± 0.6 s/min) during the 60-min pre-test. However, the average amount of time mice spent on their assigned CS+ floor versus CS− floor during the 60-min pre-test was not statistically different (CS+ floor: 29.8 ± 0.6 s/min; CS− floor 30.2 ± 0.6 s/min).

Mean (± sem) activity counts during the pre-test were 82.6 ± 1.9, 81.1 ± 2.3, 83.6 ± 2.1, and 82.7 ± 2.6 for the alcohol withdrawal, PTZ, PTZ + alcohol withdrawal, and saline group, respectively. One-way ANOVA showed no group differences in activity levels.

3.3. Conditioning trial activity

Fig. 2 depicts mean (± sem) activity levels during CS+ and CS− conditioning sessions collapsed across conditioning sessions 1–4 in each group. Activity levels in the alcohol withdrawal and PTZ + alcohol withdrawal groups were reduced during CS+ conditioning sessions compared to CS− session activity levels and compared to the PTZ group. Overall analysis of the data (Group × Conditioning Session × CS Session Type) yielded main effects of Group [$F(3,103)=2.7$, $p<0.05$], Conditioning Session [$F(3,309)=6.5$, $p<0.001$], CS Session Type [$F(1,103)=36.5$, $p<0.001$], and Group × Conditioning Session [$F(9,309)=3.9$, $p<0.001$] and Group × CS Session Type [$F(3,103)=11.7$, $p<0.001$]. To explore the Group × CS Session Type interaction, follow-up one-way ANOVAs of activity levels on CS+ versus CS− conditioning sessions were conducted within each group (collapsed across the four conditioning sessions). These analyses indicated significantly lower activity levels in the alcohol withdrawal and PTZ + alcohol withdrawal groups on CS+ conditioning sessions compared to CS− conditioning sessions ($Fs>58.2$, $ps<0.001$). Activity levels on both trial types were comparable in the saline and PTZ groups. In addition, activity levels on CS+ sessions did not differ between the alcohol withdrawal and PTZ + alcohol withdrawal groups. A three-way ANOVA (Pretreatment × Conditioning Treatment × CS Session Type) indicated main effects of Conditioning Treatment [$F(1,103)=6.8$, $p=0.01$; alcohol withdrawal < saline] and CS Session Type [$F(1,103)=36.5$, $p<0.05$; CS+ < CS−] and a Conditioning Treatment × CS Session Type interaction [$F(1,103)=33.3$, $p<0.001$] but no interaction with Pretreatment. Reduced locomotor activity during alcohol withdrawal is a finding consistent with prior reports in rodents (e.g., File et al., 1989; Jung et al., 2000; Kliethermes et al., 2004; Knapp et al., 2005; Onaivi et al., 1989; Rasmussen et al., 2001).

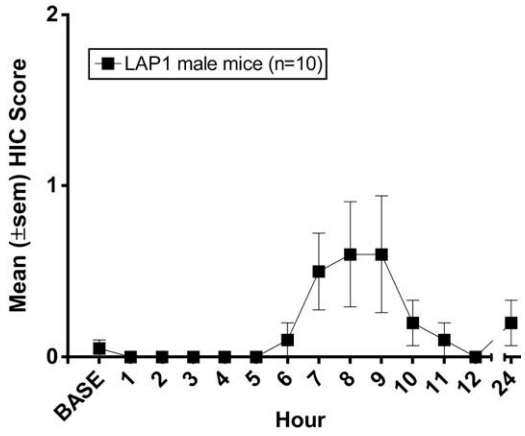

Fig. 1. Mean (± sem) HIC scores in male LAP1 mice following a single i.p. injection of 4.0 g/kg alcohol. BASE indicates the average of two baseline scores taken 20 min apart just prior to the alcohol injection.

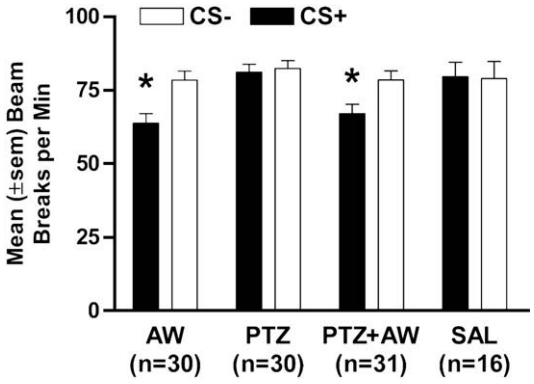

Fig. 2. Mean (± sem) activity levels during CS+ and CS− conditioning sessions in the alcohol withdrawal (AW), PTZ, PTZ + alcohol withdrawal (PTZ + AW), and saline (SAL) groups collapsed across conditioning sessions 1–4; *$p<0.05$ CS+ versus CS− sessions.

3.4. Post-test 1: drug-free

Evidence for place conditioning was assessed by conducting within-subject comparisons of the amount of time spent on the CS+ floor during the pre-test to the amount of time spent on the CS+ floor during the post-tests. All pre- versus post-test comparisons were conducted on data averaged across the 60-min test session because initial analyses indicated that time spent on the CS+ floor was relatively constant across the 60-min test in all groups. Fig. 3 shows mean (± sem) difference scores for each group calculated by subtracting the time spent on the CS+ floor during the pre-test from time spent on the CS+ floor during post-test 1 (drug-free).

The repeated-measures analysis of time on the CS+ floor [Group × Test Phase ANOVA] indicated a Group × Test Phase interaction [$F(2,88)=3.3$, $p<0.05$]. However, follow-up comparisons of Test Phase within each Group indicated no significant effects. Pre- versus post-test difference scores were also analyzed with a two-way ANOVA (Pretreatment × Conditioning Treatment) to determine whether PTZ pretreatment affected the development of conditioned place aversion to alcohol withdrawal as evidenced during the drug-free test. This analysis showed a main effect of Pretreatment [$F(1,103)=4.7$, $p<0.05$; PTZ-treated < saline-treated] but no other significant effects were found.

Mean (± sem) activity counts during post-test 1 were 72.7 ± 3.6, 84.1 ± 3.6, 73.6 ± 3.6, and 83.3 ± 4.0 for the alcohol withdrawal, PTZ, PTZ + alcohol withdrawal, and saline group, respectively. Two-way ANOVA (Pretreatment × Conditioning Treatment) indicated a main

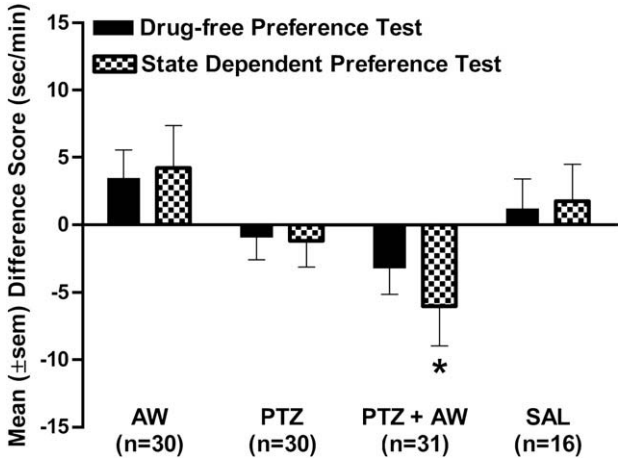

Fig. 3. Mean (±sem) difference scores for each group calculated by subtracting the time spent on the CS+ floor during the pre-test from time spent on the CS+ floor during the drug-free and state-dependent preference tests; *$p<0.05$ post-test CS+ time versus pre-test CS+ time.

effect of Conditioning Treatment [$F(1,103)=7.5$, $p<0.01$; alcohol withdrawal-treated<saline-treated] but no other effects were found.

Pearson correlations between post-test 1 difference scores and average activity levels during CS+ conditioning trials ($r=-0.037$, $p=0.71$, $n=107$) and during post-test 1 ($r=-0.119$, $p=0.22$, $n=107$) were not significant.

3.5. Post-test 2: state-dependent

Fig. 3 shows mean (±sem) difference scores for each group calculated by subtracting the time spent on the CS+ floor during the pre-test from time spent on the CS+ floor during post-test 2 (state-dependent).

The repeated-measures analysis of time on the CS+ floor [Group×Test Phase ANOVA] indicated a Group×Test Phase interaction [$F(3,103)=2.7$, $p=0.05$]. Follow-up comparisons of Test Phase within each group indicated a significant place aversion in the PTZ+alcohol withdrawal group [$F(1,30)=4.2$, $p<0.05$; post-test CS+ time<pre-test CS+ time]. As was done with post-test 1 data, pre- versus post-test difference scores were analyzed with a two-way ANOVA (Pretreatment×Conditioning Treatment) which showed a main effect of Pretreatment [$F(1,103)=5.1$, $p<0.05$; PTZ-treated<saline-treated]. This effect is clearly due to the significant aversion in the PTZ+alcohol withdrawal group (see Fig. 3) but there was not enough statistical power to detect a Pretreatment×Conditioning Treatment interaction.

Mean (±sem) activity counts during post-test 2 were 44.2±4.2, 73.7±4.2, 50.0±4.1, and 82.3±5.0 for the alcohol withdrawal, PTZ, PTZ+alcohol withdrawal, and saline group, respectively. Two-way ANOVA (Pretreatment×Conditioning Treatment) indicated a main effect of Conditioning Treatment [$F(1,103)=46.5$, $p<0.001$; alcohol withdrawal-treated<saline-treated] but no other effects were found.

Pearson correlations between post-test 2 difference scores and average activity levels during CS+ conditioning trials ($r=0.067$, $p=0.49$, $n=107$) and during post-test 2 ($r=0.002$, $p=0.98$, $n=107$) were not significant.

4. Discussion

Alcohol withdrawal produces aversive motivational effects which have been hypothesized to play a primary role in the development of alcoholism (Chester et al., 2002; 2003; Koob, 2003; Wall and Ehlers, 1995). Relatively few animal models have been developed that assess the motivational effects of alcohol withdrawal. The goal of the present study was two-fold: 1) to determine whether a conditioned place aversion to alcohol withdrawal could be detected in mice and 2) whether PTZ administration during alcohol withdrawal would increase the strength of the conditioned place aversion.

With regard to the first study goal, conditioning during alcohol withdrawal did not produce a significant conditioned place aversion during either the drug-free or state-dependent preference test. Only two prior studies in rats have reported place conditioning to alcohol withdrawal. Gauvin et al. (1997) reported a place preference and Morse et al. (2000) reported a place aversion to alcohol withdrawal. Comparison between these discrepant studies and with the present data is particularly difficult because there is no consistency among the experimental procedures used. For example, Gauvin et al. found a place preference after 8 pairings of the non-preferred environmental stimuli (biased procedure) with alcohol withdrawal whereas Morse et al. found a place aversion after a single pairing of neutral environmental stimuli (unbiased procedure) with alcohol withdrawal. Another major difference between studies was that Gauvin et al. conditioned rats at 18 h in withdrawal whereas Morse et al. conditioned rats at 10 h in withdrawal. We chose to condition mice at 8 h in withdrawal because it is the time point at which peak physical signs of alcohol withdrawal, assessed via HICs, were evident in LAP1 mice following a single injection of 4.0 g/kg alcohol. However, it is possible that we missed the ideal conditioning time point that would have produced a conditioned place aversion. Indeed, there is evidence in rodents that affective or motivational effects of alcohol withdrawal extend past the time that physical signs of withdrawal are evident (e.g., Prediger et al., 2006; Roberts et al., 2000). For example, Prediger et al. (2006) showed that alcohol withdrawal-induced anxiety in mice was greatest between 12 and 18 h in withdrawal from a single injection of 4.0 g/kg alcohol. Finally, it should be noted that species differences could account for the discrepant results between the present and prior studies. Alcohol intoxication has been shown to produce place aversion in rats but place preference in mice exposed to identical conditioning parameters (Cunningham et al., 1993). Thus, there may also be relevant species differences in sensitivity to the rewarding or aversive effects of alcohol withdrawal. The overall lack of studies showing place conditioning to alcohol withdrawal, unlike with opiate withdrawal, could partly be due to the fact that alcohol withdrawal is not a discrete stimulus and there is no established procedure for precipitating withdrawal from alcohol. This situation, and the cumbersome nature of the place conditioning procedure, makes it challenging to identify effective conditioning parameters that will capture the aversive effects of alcohol withdrawal and maximize the salience of alcohol withdrawal as an unconditioned stimulus.

This is the first study to report a place aversion to alcohol withdrawal in mice using a drug (PTZ) to "precipitate" or increase the aversive effects of alcohol withdrawal. The PTZ+alcohol withdrawal group showed a significant place aversion during the state-dependent test. These data suggest that the combined stimulus properties of PTZ and alcohol withdrawal facilitated the expression of conditioned place aversion to alcohol withdrawal. Further, this finding suggests that PTZ can reveal sub-threshold aversive motivational effects of alcohol withdrawal that are present at 8 h in withdrawal, a time point when peak physical signs of alcohol withdrawal are evident in mice (See Fig. 1; Crabbe et al., 1991; Crabbe, 1998; Metten and Crabbe, 1994). However, additional studies are needed to determine whether this effect is dependent on the presence of PTZ during conditioning trials or whether acute administration of PTZ during the expression test is sufficient to reveal a conditioned place aversion to alcohol withdrawal. Interestingly, we have previously shown that pretreatment with the $GABA_A$ receptor antagonists, picrotoxin and bicuculline, prior to conditioning trials with alcohol enhanced alcohol-induced conditioned place preference; however, these drugs were not given prior to the preference test (Chester and Cunningham, 1998). It should also be noted that the magnitude of conditioned place aversion seen in the PTZ+alcohol withdrawal group was small and more work is needed to explore whether other experimental parameters, such as a different

PTZ dose or conditioning time point, may produce a greater effect. The lack of preference or aversion behavior in the PTZ alone and saline-treated groups indicates that the observed effect in the PTZ + alcohol withdrawal group is not due to rewarding or aversive effects of PTZ itself or to changes in unconditioned preference for the conditioning stimuli during repeated conditioning and testing procedures.

PTZ is a $GABA_A$ receptor antagonist and is frequently used as a chemoconvulsant to precipitate the physical signs of alcohol withdrawal and assess alcohol dependence-related phenomena (e.g., Cagetti et al., 2004; Chesher and Jackson, 1974; Crabbe et al., 1991; Finn and Crabbe, 1999; Ripley et al., 2002). However, little is known about the effects of PTZ on the motivational or affective symptoms of alcohol withdrawal. Prior studies indicate that the discriminative stimulus effects of PTZ are similar to alcohol withdrawal (Gauvin et al., 1989, 1993; Lal et al., 1988), particularly in male rats (Jung et al., 1999). Thus, the current findings could be explained via an additive effect where combined sub-threshold aversive effects of PTZ and alcohol withdrawal produce a salient enough unconditioned stimulus to support the development of a conditioned place aversion. One potential mechanism for this effect is that PTZ increases anxiety during alcohol withdrawal. Both PTZ (see Jung et al., 2002 for review) and withdrawal from high doses of alcohol produces anxiety-related behavior in both rats and mice (e.g., Overstreet et al., 2002; Valdez et al., 2002; Verleye et al., 2009; Zhang et al., 2007) and drugs that enhance activity at the $GABA_A$ receptor can reverse the anxiogenic effects of alcohol withdrawal (e.g., Knapp et al., 2005; Roberto et al., 2008; Verleye et al., 2009; Watson et al., 1997). However, Gauvin et al. (1991) showed that PTZ can also produce a conditioned place preference in rats. This result led to the suggestion that PTZ may have some rewarding motivational effects in addition to its anxiogenic effects and that anxiety induced with low to moderate doses of PTZ may serve to enhance learning in the place conditioning task, similar to that reported in other classical conditioning procedures (e.g., Taylor, 1951). It should also be noted that an alternative interpretation of the current results is that PTZ facilitates learning about stimulus effects of the alcohol withdrawal state independent of its influence on the motivational aspects alcohol withdrawal. $GABA_A$ receptor antagonists are known to enhance memory-related processes in different types of learning tasks (see reviews by Chapouthier and Venault, 2002; Izquierdo and Medina, 1991).

The significant conditioned place aversion to alcohol withdrawal in the PTZ + alcohol withdrawal group during the second post-conditioning preference test suggests that the expression of the aversion is influenced by a state-dependent memory retrieval process. State-dependent retrieval occurs when the recall of a previously learned response is facilitated by, or entirely dependent upon, the physiological or affective "state" under which the organism originally learned the response (see review by Overton, 1991). In the drug place conditioning literature, a wide range of findings have been reported in rodents, with many studies indicating that state-dependent processes do not influence the expression of conditioned drug responses (e.g., substance P: Elliott, 1988; alcohol: Gremel and Cunningham, 2007; morphine: Mucha and Iversen, 1984; amphetamine: Reicher and Holman, 1977; diazepam: Spyraki et al., 1985) while others have shown that administration of the training drug prior to testing enhances (heroin: Bozarth, 1987; alcohol: Cunningham, 1979; cholecystokinin: Swerdlow et al., 1983) or reveals (morphine: Bespalov et al., 1999; WAY161503: Mosher et al., 2006; lithium chloride, naloxone, FG1742: Oberling et al., 1993) a conditioned place preference or aversion. To our knowledge, this is the first report indicating state-dependent effects on the expression of place aversion to withdrawal from a drug, in this case, alcohol. In the two prior reports of place conditioning to alcohol withdrawal in rats (Gauvin et al., 1997; Morse et al., 2000), the authors did not address the question of whether conditioned behavior was influenced by a state-dependent mechanism.

A point worth mentioning is that reduced locomotor activity during alcohol withdrawal may have somehow influenced the acquisition of conditioned place aversion or the expression of conditioned place aversion during the state-dependent test. However, this possibility seems unlikely because both alcohol withdrawal groups showed reduced locomotor activity during conditioning trials and during the state-dependent test but only the PTZ + alcohol withdrawal group displayed a conditioned place aversion. Consistent with prior studies of conditioned place preference (e.g., Chester and Cunningham, 1999a,b; Cunningham, 1995; Gremel and Cunningham, 2007), we found no correlation between activity levels on CS+ conditioning trials and place aversion magnitude during the state-dependent test. In addition, although some previous studies have indicated a positive correlation between magnitude of place preference and test activity levels (e.g., Gremel and Cunningham, 2007; Neisewander et al., 1990), we found no correlation between place aversion magnitude and activity levels during the state-dependent test.

In summary, PTZ administration during alcohol withdrawal conditioning trials produced a state-dependent conditioned place aversion. This finding perhaps reflects a phenomenon whereby PTZ increases or "precipitates" the aversive effects of alcohol withdrawal and provides further support for a role of the $GABA_A$ receptor in modulating the neurochemical and behavioral effects of alcohol withdrawal (e.g., Cagetti et al., 2003; Devaud et al., 2003; Follesa et al., 2006; Knapp et al., 2005; Morrow et al., 1991). $GABA_A$ receptor antagonists such as PTZ may be useful to increase the sensitivity of the place conditioning procedure as a measure of the aversive effects of alcohol withdrawal in rodents. A model such as this is a valuable tool to identify and characterize genetic and neurochemical mechanisms that mediate sensitivity to aversive motivational effects of alcohol withdrawal that, in turn, may influence alcohol drinking behavior. Future studies using this technique could eventually lead to the discovery of novel behavioral and pharmacological treatments for alcoholism.

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