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Repeated microinjections into the medial prefrontal cortex (mPFC) impair extinction of conditioned place preference in mice

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Abstract

The medial prefrontal cortex (mPFC) is important for extinction of many behaviors including conditioned place preference (CPP). We examined the effects of intra-mPFC inactivation (with bupivacaine) on extinction of ethanol-induced CPP in mice. Injections of both bupivacaine and vehicle impaired extinction whereas No-Surgery control mice extinguished normally. Consistent with recently reported effects of mPFC lesions, these data suggest that extinction was impaired by excessive mPFC damage induced by repeated intracranial infusions.

Keywords

Extinction; mPFC; CPP; ethanol; mice; intra-cranial

Recent evidence from our laboratory has shown that the medial prefrontal cortex (mPFC) plays an integral role in extinction of ethanol (EtOH)-induced associative learning in mice [1]. Specifically, Groblewski et al. showed that the prelimbic (PL) and infralimbic (IL) subregions of the mPFC exhibited significant activation of cAMP response element-binding (CREB) protein following a brief exposure to a cue that had previously been paired with ethanol. However, this increase in the number of pCREB-positive cells in the mPFC was not evident in animals that had had the EtOH-cue association extinguished. Additionally, Groblewski et al. then showed that electrolytic lesions of the mPFC following conditioning significantly impaired extinction of EtOH-induced conditioned place preference (CPP) in mice—a finding that is in agreement with previous reports implicating the mPFC in extinction of CPP [2, 3].

In order to expand upon the lesion results we recently attempted to determine the effects of reversible mPFC inactivation on extinction of EtOH-CPP in mice via microinjections of bupivacaine (similar to a previous procedure used in rats [2]). Similar to lidocaine, bupivacaine is a sodium channel blocker that, when injected, temporarily inactivates neuronal signaling by preventing membrane depolarization and subsequent action potentials [4]. We hypothesized that intra-mPFC injection of bupivacaine prior to each of the

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extinction trials would prevent mPFC function thereby blocking extinction of CPP. These experiments were intended to identify the parameters required for future explorations of the receptor systems and signaling pathways in the mPFC that are required for extinction of EtOH-induced associative learning in mice.

Adult, male DBA/2J mice (n=108) were obtained from Jackson Laboratory (Sacramento, CA) at 6–7 weeks old and allowed to acclimate to the animal colony for 2 or 3 weeks before experiments commenced. Mice were housed, four to a cage, in cob bedding in a Thoren rack with water and food available *ad libitum* throughout each experiment. All experiments were conducted during the light phase (7:00–19:00). The Oregon Health & Science University IACUC approved all experimental procedures.

All behavioral procedures were performed in custom made, acrylic and aluminum conditioning boxes previously described in detail [5]. The conditioned stimuli (CSs) consisted of two distinct tactile cues—grid and hole floors. Grid floors (2.3 mm stainless steel rods, 6.4 mm apart) and hole floors (16-gauge stainless steel perforated with 6.4-mm round holes) were interchangeable allowing for either full- or split-cue configurations during conditioning/extinction and testing, respectively. These cues are unbiased in that naïve DBA/2J mice show equal preference for the two floors during drug-free preference tests [6].

In Experiment 1, mice first received EtOH-CPP conditioning as described previously [5]. Briefly, on the first day, mice were given a saline injection (12.5 ml/kg) and habituated to the conditioning apparatus, equipped with white paper flooring, for 5 min. Twenty-four hrs later animals received daily CPP conditioning trials during which EtOH (20% v/v in isotonic saline administered intraperitoneally (IP) at a dose of 2 g/kg) was paired with one of the tactile cues while saline was paired with the other cue on alternating days. Conditioning subgroup (the floor with which EtOH was paired) and trial-type order (S-E-S-E or E-S-E-S) were fully counterbalanced. One day after the last conditioning trial, all animals received a drug-free, 30-min preference test (Test 1) during which both tactile cues were presented and place preference was assessed.

Three days following Test 1, all animals underwent surgical implantation of a single, midline microinjection guide-cannula targeting the mPFC. Mice received an injection of meloxicam (0.2 mg/kg, SC) prior to being placed and maintained under deep anesthesia with isoflurane. Mice were then placed in a stereotaxic frame (Model 1900, KOPF Instruments, Tujunga, CA) with the skull horizontal and an anchor screw was affixed. A single, midline burr hole was drilled 1.8 mm rostral to bregma. An indwelling, stainless steel guide cannula (10 mm, 28g) was implanted, aimed 1 mm dorsal to the target area in the mPFC (AP: +1.8 mm, ML: 0.0 mm, DV: –2.5 mm) using a mouse brain atlas [7]. Guide cannulae were secured to the skull with carboxylate dental cement (Durelon, 3M, St. Paul, MN) and a 32 g stainless steel stylet was inserted.

After recovery from surgery (5–7 days), mice received 3 days of extinction, each of which consisted of a morning (AM) and afternoon (PM) session [8]. During AM sessions, all animals received a sham microinjection followed by an IP saline injection (12.5 ml/kg) and immediate placement onto the CS- floor for 30 min. In the PM sessions, animals received a microinjection of vehicle (saline) or bupivacaine (bupivacaine hydrochloride (Sigma Aldrich, St. Louis, MO) in isotonic saline at 2% w/v or 20 mg/ml) followed by an IP saline injection and immediate placement onto the CS+ floor for 30 min.

Microinjections were performed by gently restraining the mouse, removing the 10mm stylet and replacing it with the injector (11 mm, 32 g stainless steel tubing affixed inside of a 25 g tube), which was attached to a Hamilton syringe (10 µL) via polyethylene tubing (PE20). A syringe pump (Model A-74900-10, Cole Parmer, Vernon Hills, IL) delivered 0.2 µL of

vehicle or bupivacaine over 2 min (0.1 μ L/min) and injectors remained in place for 30 sec after the injection was complete in order to prevent diffusion up the cannula. Based on previous parametric studies of effective diffusion, this injection volume of bupivacaine was expected to prevent >90% of neuronal activation within the PL and IL subregions of the mPFC [9] for up 90 min [10]. Injectors were then removed and a clean stylet was inserted. Sham microinjections were performed identically except that no solution was pumped through the lowered injector. Twenty-four hrs after the last extinction trial all animals received a second, drug-free 30-min preference test (Test 2). Upon completion of each experiment, animals received an overdose of pentobarbital (150 mg/kg), brains were removed and postfixed with paraformaldehyde (2% w/v in phosphate buffered solution, PBS). Brains were cryoprotected with 20% then 30% sucrose in PBS and 0.1% NaN_3 . Frozen slices (40 μ m) were obtained on a cryostat (Leica CM1900) and thionin-stained for verification of cannula location and lesion location/size.

As described previously [1, 8], place preference was assessed using analysis of variance (ANOVA) of the percentage of each test spent on the EtOH-paired (CS+) floor (% Time on EtOH-paired floor). Extinction was defined *a priori* as a significant reduction in place preference that was determined by Bonferroni-adjusted within-subjects comparisons of preferences on Test 1 and 2 (paired t-tests) for each group following an initial 2-way ANOVA (Group $\times$ Test) and were performed whether or not the interaction was significant. This method of analysis, although not statistically justified, was implemented because of the narrow detection window and increased variability that are inherent to our extinction procedure (e.g., [1, 8]). Overall α was set at .05 for all statistical tests.

Because the goal of these experiments was to specifically manipulate extinction of an acquired EtOH-CPP, we decided, *a priori*, to remove animals that failed to express a place preference of greater than 50% on Test 1. These experiments revealed that less than 25% of all subjects failed to express significant preference following the 2-trial conditioning procedure—a finding that is consistent with previous reports that have also removed non-learners from extinction analyses [1, 8].

In Experiment 1, a total of 18 animals were removed from analysis (9 showed less than 50% preference on Test 1 and an additional 9 were removed for not meeting histological criteria). Histological analysis revealed that the injector tracks terminated at a point within the target region between the PL and IL subregions of the mPFC and there was no difference in the location of injector tips for the two groups (Figure 1).

The place preference results from Tests 1 and 2 revealed that neither the Vehicle nor Bupivacaine group showed a significant reduction in preference following extinction (Figure 2A). Specifically, the 2-way ANOVA of the Percent Time Spent on the EtOH-paired floor revealed a main effect of Test [$F(1,33) = 4.3, p < .05$] but no main effect of Group or a Group $\times$ Test interaction (p 's $> .05$). The separate paired t-tests of preferences on Test 1 and 2 revealed no significant reduction for either group (p 's $> .05$). Thus, neither the control (Vehicle) nor the experimental group (Bupivacaine) showed significant extinction of EtOH-CPP.

Because of the incomplete extinction in the Vehicle group, it remained unknown if inactivation of the mPFC with bupivacaine was able to impair extinction. In order to further investigate these findings, Experiment 2 involved three procedural changes intended to increase the extinction exhibited by the Vehicle group. First, cannula implantation surgery was performed prior to conditioning. This manipulation was intended to eliminate any potential effects of the time interval between Test 1 and extinction (9 days). Previous reports have suggested that inserting a time gap between conditioning and extinction can alter

extinction (e.g., [11]). Second, extinction was extended by an extra trial for a total of four days of extinction to increase the amount of nonreinforced exposure to the cues in hopes of further reducing the preference in the Vehicle group. Finally, an additional control group that did not receive surgery was included (No Sx group) in order to assess the effects of the handling portion of the microinjection procedure on extinction learning. Although it is unclear how stressful handling (i.e., scruffing for 2–3 min) could influence extinction, previous reports have shown that this type of handling did effect expression of EtOH-induced conditioned place aversion [12].

In Experiment 2, a total of 16 animals were removed from the analysis (8 showed less than 50% preference on Test 1 and an additional 8 were removed for not meeting histological criteria). Similar to Experiment 1, histological analysis revealed that the injector tracks were aimed between the PL and IL subregions of the mPFC and there was no difference in the location of injector tips for the two groups (Figure 1).

Analysis of the place preference from Tests 1 and 2 of Experiment 2 revealed that only the No Sx group exhibited significant extinction (Figure 2B). Specifically, the initial 2-way ANOVA revealed a main effect of Test [$F(1,40) = 9.3, p < .005$] but not Group, and no significant Group $\times$ Test interaction (p 's $> .05$). The results of the separate paired t-tests showed a significant decrease in preference for only the No Sx group [$t(11) = 2.5, p < .05$]. These data suggest that the No Sx, but not Vehicle or Bupivacaine, group significantly extinguished. Therefore, despite increasing the number of extinction trials and changing the timing of surgery, the results of Experiment 2 are similar to those in Experiment 1. Together, these experiments suggest some component of the microinjection procedure (but not merely the handling required to make the injections) interfered with extinction of EtOH-CPP.

The most parsimonious explanation for these findings is that the microinjection procedure, regardless of the compound being injected, caused tissue damage to an area of the brain shown to be necessary for extinction of EtOH-CPP [1]. The tissue damage that resulted from the multiple microinjections in Experiments 1 and 2 may have been sufficient to effectively result in a partial or complete lesion of portions of the mPFC. Post-mortem histological examination suggested that the tissue damage (i.e., cell proliferation) caused by implantation of the cannula, repeated insertion of the injector, and/or injection of solution (vehicle or bupivacaine) is substantial and limited primarily to Layers I–III of the mPFC (Figure 1B). Importantly, because these cannulae were located on the sagittal midline, tissue damage to these layers of the cortex affected both hemispheres. Retrograde tracing experiments have shown that Layers II/III of the mPFC contain dense populations of projection neurons that terminate in both the nucleus accumbens (NAc) and basolateral amygdala complex (BLC) [13, 14]. Interestingly, activity in these mPFC projection neurons is altered by exposure to drug-paired cues that are capable of eliciting approach behavior [14, 15]. Therefore, it is conceivable that the damage caused by the microinjection procedure resulted in altered mPFC regulation of the NAc and BLC, thereby impairing extinction of EtOH-CPP. Of course, further examination of the effects of the microinjection procedure on these specific projections within the mPFC, as well as their projection targets, is required to confirm this suggestion.

Most studies examining the effects of intra-mPFC pharmacological manipulations on extinction have involved conditioned fear procedures that require only a single injection prior to, or following, a single extinction session (e.g., [16]). Unlike extinction of conditioned fear, however, extinction of EtOH-CPP requires multiple extinction trials over the course of several days. Specifically, previous studies from our laboratory have shown that a minimum of 3 days of 30-min exposures to both the CS– and CS+ cues is required for

complete extinction of EtOH-CPP [8]. In order to minimize the number of microinjections administered in the current study, Experiment 1 consisted of this 3-day extinction procedure. However, because the Vehicle group exhibited incomplete extinction, Experiment 2 included an additional day of extinction. Regardless of whether the animals received 3 or 4 days of extinction, however, the Vehicle groups in both experiments failed to show significant extinction. In fact, in one of the replicates of Experiment 1 we extended extinction by an additional 3 days (for a total of 6 days). Despite doubling the number of extinction trials, we found that both mPFC-injected groups continued to exhibit impaired extinction—that is, both groups showed persistent CPP (data not shown). These findings suggest that the damage caused by intra-mPFC injections even when the number of injections was minimized, was sufficient enough to impair extinction—an effect that persisted despite extended extinction training.

It is unlikely that the observed effect was a direct result of the surgery/anesthesia *per se* as we have previously shown that a one-time insertion of an electrode into the same area of the mPFC while under general anesthesia did not impair subsequent extinction [1]. Importantly, the current surgeries and those described by Groblewski et al. (2011) used the same strain of mice (male, DBA/2J mice), stereotaxic frame and coordinates, anesthesia (type, concentration, and duration of exposure), and were all performed by the same surgeon. Additionally, the behavioral procedures (CPP acquisition and extinction) and apparatuses used in the two studies were identical. Therefore, it is unlikely that the surgery/anesthesia component of the procedure was responsible for the impaired extinction observed here. It is important to note that irrespective of how each component of the microinjection procedure contributes to the observed effect, as currently performed, this procedure is not adequately suited to examine the effects of intra-mPFC pharmacological manipulations on extinction of EtOH-CPP in mice. Further parametric studies that aim to reduce the damage caused by the microinjection procedure and/or reduce the number of required microinjections are necessary before future explorations of the different receptor systems and signaling pathways within the mPFC that are involved in extinction of CPP in mice are possible.

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RESEARCH HIGHLIGHTS

- Intra-mPFC bupivacaine and vehicle impaired extinction of ethanol-CPP.
- No-Surgery control mice extinguished normally.
- Extinction was likely impaired by excessive mPFC damage caused by the infusions
- These results support a role for the mPFC in extinction of EtOH-CPP.
- These findings caution the use of multiple intra-mPFC microinjections in mice.

Figure 1.

A) Injector-tip location for each subject in Experiments 1 & 2 across four stereotaxic levels (adapted from Paxinos & Franklin, 2001).

B) Representative photomicrograph of the cannula and injector track from a subject in Experiment 1. The injector tip was aimed at the mPFC (AP: +1.8mm, ML: 0.0mm, DV: -2.5mm).

A)

B)

Figure 2.

Preference Test data (expressed as Percent Time on EtOH-Paired Floor) before (Test 1) and after (Test 2) extinction for (A) Experiment 1 (B) Experiment 2. Significant reduction in place preference (*) as determined by Bonferroni-corrected comparisons of Tests 1 and 2 for each group ($p < .05$). Data are expressed as mean ($\pm$ SEM).