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Naloxone effects on extinction of ethanol- and cocaine-induced conditioned place preference in mice

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Abstract

Rationale—Previous studies found that naloxone (NLX) facilitated choice extinction of ethanol (EtOH) conditioned place preference (CPP) using long (60-min) test sessions, but there is little information on the variables determining this effect.

Objectives—These studies examined repeated exposure to NLX during extinction of ethanol- or cocaine-induced CPP using both short and long tests.

Methods—DBA/2J mice were injected with NLX (0 or 10 mg/kg) before three 10- or 60-min choice extinction tests (Exp. 1). All mice received a final 60-min test without NLX. Post-test NLX was given in Exp. 2. Exp. 3 tested whether NLX would affect a forced extinction procedure. Exp. 4 tested its effect on extinction of cocaine-induced CPP.

Results—Pre-test (but not post-test) injections of NLX facilitated choice extinction of EtOH CPP at both test durations. Pre-test NLX also facilitated forced extinction. However, pre-test NLX had no effect on choice extinction of cocaine CPP.

Conclusions—Extinction test duration is not critical for engaging the opioid system during EtOH CPP extinction (Exp. 1). Moreover, NLX's effect does not depend on CPP expression during extinction, just exposure to previously conditioned cues (Exp. 3). The null effect of post-test NLX eliminates a memory consolidation interpretation (Exp. 2) and the failure to alter cocaine CPP extinction argues against alteration of general learning or memory processes (Exp. 4). Overall, these data suggest that the endogenous opioid system mediates a conditioned motivational effect that normally maintains alcohol-induced seeking behavior, which may underlie the efficacy of opiate antagonists in the treatment of alcoholism.

Keywords

alcohol; reward; conditioning; extinction; inbred mice (DBA/2J)

Drug-associated environmental stimuli can elicit compulsive drug seeking behavior and trigger relapse in pre-clinical models and humans (Childress et al. 1999; Crombag et al.

2008; Buffalari and See 2010; Bossert et al. 2013). Because addiction is maintained, in part, by the incentive motivational properties of drug-associated cues, identifying compounds that facilitate extinction of these appetitive memories offers a promising approach for developing clinical treatments (Taylor et al. 2009; Duka et al. 2011). Extinction of conditioned appetitive memories involves new inhibitory learning in which the conditioned stimulus progressively loses its ability to evoke the conditioned response (Pavlov 1927; Bouton 2004). Pharmacological strategies that reduce the behavioral impact of drug-paired cues by enhancing and maintaining extinction of these cues could potentially reduce the likelihood of future relapse (Quirk and Mueller 2008; Torregrossa and Taylor 2013).

Extinction of cues associated with alcohol-seeking behavior can be assessed using drug self-administration and conditioned place preference (CPP) models (Myers and Carlezon 2010; Millan et al. 2011). In the self-administration model, an acquired instrumental response is extinguished over the course of one or more sessions by withholding the previously response-contingent alcohol reinforcer. In the CPP model, preference is extinguished by repeated exposure to cues previously paired with drug in the absence of alcohol (Shaham et al. 2003; Cunningham et al. 2011; Bossert et al. 2013). Although the mechanisms underlying CPP and self-administration are not identical (Bardo and Bevins 2000), there is now consistent evidence showing overlap between these procedures in the role played by the opioid system in regulating the incentive properties of ethanol associated conditioned stimuli.

This evidence suggests that the endogenous opioid system influences ethanol's primary rewarding effects (Herz 1997; Mendez and Morales-Mulia 2008; Gianoulakis 2009) as well as its conditioned rewarding effects (Cunningham et al. 1995, 1998; Pastor et al. 2011). Administration of opioid receptor antagonists such as naloxone (NLX) or naltrexone (NTX) attenuate context- (Burattini et al. 2006) and cue-induced reinstatement of previously extinguished responding for ethanol self-administration (Lê et al. 1999; Ciccocioppo et al. 2002, 2003; Liu and Weiss 2002; Burattini et al. 2006; Dayas et al. 2007; Marinelli et al. 2009), suggesting that the endogenous opioid system modulates the incentive motivational properties of cues associated with ethanol. Treatment with the opioid receptor antagonists NLX (Cunningham et al. 1995, 1998; Kuzmin et al. 2003) or NTX (Middaugh and Bandy 2000; Pastor et al. 2011) similarly affects the expression or extinction of ethanol-induced CPP. Furthermore, β -endorphin and μ -opioid receptors appear to be critically involved in the mechanisms underlying the conditioned rewarding effects of ethanol since it has been shown that neurotoxic lesions of β -endorphin neurons in the arcuate nucleus also facilitate extinction of ethanol-induced CPP in mice (Pastor et al. 2011).

Here we explore further the hypothesis that the endogenous opioid system influences the conditioned motivation that normally maintains alcohol-induced seeking behavior as indexed by CPP. Our previous studies showed that NLX given before testing had little or no effect on the initial expression of CPP during the first 10-min but gradually abolished preference over the course of a 60-min session (Cunningham et al. 1995, 1998). This temporal pattern was not explained by pharmacokinetics since the same outcome was observed whether NLX was injected 15 or 45 min before testing. Additional data showed that repeated testing with NLX produced a shift in cue preference, progressively converting

the preference for the CS+ compartment into aversion over the course of three 60-min NLX extinction tests (Cunningham et al. 1998). Importantly, this reduced preference was maintained when animals were later tested in absence of NLX, suggesting a relatively permanent change in preference that was not due to acute effects of NLX exposure. The finding that NLX had little impact on CPP during the first 10 min but gradually suppressed CPP thereafter raises the possibility that this effect may depend on a relatively long CS re-exposure during opioid receptor blockade. We tested this hypothesis by examining whether NLX's effect on extinction depended on the duration of CS re-exposure.

In Experiment 1, NLX was administered before each extinction trial (10 vs. 60 min) as in our previous studies (Cunningham et al. 1995, 1998). However, when drug is administered before each trial, it can affect performance in several different ways. For example, it could alter either affective or memory consolidation processes as well as have a direct effect on motor behavior (Cunningham et al. 2011). To test whether NLX might interfere with the maintenance of preference by enhancing the consolidation of extinction rather than by reducing the affective response to ethanol associated cues, we injected NLX immediately after each extinction trial (10 vs. 60 min) in Experiment 2. If the effect produced by pre-test NLX injection was due to enhanced consolidation of extinction, post-trial NLX injections would be expected to produce the same outcome as Experiment 1. However, if this outcome resulted from a direct effect of NLX on memory retrieval or the ethanol-conditioned motivational response, post-trial NLX would presumably have no impact on behavior during extinction.

Experiments 3 and 4 examined the generality of the NLX effect on CPP extinction in two ways. First, since our previous studies had only examined NLX's effects using a choice extinction procedure, Experiment 3 tested whether NLX would have the same impact when given during a forced extinction procedure in which the cues were presented for a fixed amount of time in a response-independent manner (see Cunningham et al. 2011 for a further discussion of choice vs. forced extinction procedures). Second, to determine whether NLX's effect on extinction was specific to ethanol, we injected NLX before choice extinction (10 vs. 60 min) of cocaine-induced CPP in mice in Experiment 4. The rationale for this study was that if NLX is interfering more generally with the formation of extinction memories rather than with ethanol-specific conditioned motivational effects, NLX should also affect the extinction of cocaine-induced CPP. Moreover, if NLX accelerates the loss of ethanol-induced CPP simply by producing a non-specific aversive reaction, it would be expected to have a similar effect on the extinction of cocaine-induced CPP.

Method

Subjects

DBA/2J male mice, 6 to 7 weeks old upon arrival to the laboratory, were obtained from Jackson Laboratory (Bar Harbor, Maine, USA) and acclimated for 2 weeks before experiments started. Male mice were used here to facilitate comparison to previous studies of extinction (Groblewski et al. 2011) and NLX (Cunningham et al. 1995, 1998), which involved male mice only. Mice were housed four per cage in a Thoren rack with tap water and standard rodent chow available ad libitum. The colony room was maintained at a

temperature of $21\pm1^{\circ}\text{C}$ on a 12 h light/dark cycle (lights on at 7:00 a.m.). All procedures were approved by the OHSU Institutional Animal Care and Use Committee and were carried out in accordance with National Institutes of Health *Principles of Laboratory Animal Care*.

Apparatus

The apparatus consisted of 12 identical acrylic and aluminum boxes ($30 \times 15 \times 15$ cm) placed inside individual ventilated, light- and sound-attenuated enclosures (Model E10-20; Coulbourn Instruments, Allentown, PA). Each conditioning box was equipped with six equally spaced infrared emitter/detector pairs running the length of the box, 2.2 cm above the floor and 5 cm apart. These detectors were attached to a computer that recorded activity counts (expressed as beam breaks per min) and side preference (expressed as time spent on the left and right sides) during all conditioning, extinction and test sessions. No divider was used so animals had access to the entire box during all sessions (one-compartment procedure). For a detailed description of the apparatus, see Cunningham et al. (2006). The boxes were placed on top of two interchangeable floor halves that served as the tactile conditioned stimuli (CSs). The grid floors consisted of 2.3 mm stainless steel cylindrical rods mounted 6.4 mm apart on acrylic rails. The hole floors were stainless steel (16 gauge) perforated with 6.4 mm holes on 9.5 mm staggered centers, mounted on an acrylic base. This combination of floor textures was selected based on previous studies showing that drug-naïve control groups show no preference for either floor type (Cunningham et al. 2003). The conditioning box and floors were wiped with a damp sponge and the litter paper was replaced after each animal.

Drugs

Ethanol (20% v/v) was diluted in isotonic saline and administered intraperitoneally (IP) at a dose of 2 g/kg (12.5 mL/kg). NLX (Sigma Chemical Co., St. Louis, Missouri, USA) was dissolved in isotonic saline to a concentration of 1 mg/mL and administered at a dose of 10 mg/kg via IP injection. NLX dose and injection time (–15 min) were based on previous studies demonstrating facilitated extinction of ethanol CPP in DBA/2J mice (Cunningham et al. 1995, 1998). As noted earlier, previous research showed no difference in NLX's facilitation of extinction whether given 15 or 45 min before testing, suggesting that its time dependent effect on CPP is not due to a delay in drug absorption or distribution (Cunningham et al. 1995). Cocaine hydrochloride (Sigma Chemical Co., St. Louis, Missouri, USA) was dissolved at concentrations of 0.2, 0.4, 0.8, 1.6 mg/ml and injected IP at doses of 2, 4, 8 and 16 mg/kg. Fresh solutions of NLX and cocaine were prepared daily.

Experiment 1: Effect of NLX treatment and test duration on choice extinction of ethanol-induced CPP

The purpose of this experiment was twofold: (a) to replicate results from previous studies in which animals received repeated NLX treatment before a series of long choice extinction sessions (60 min), and (b) to investigate the impact of repeated injections of NLX before a series of short choice extinction sessions (10 min). If the effect of repeated NLX on extinction depends on expressing a reduction in CPP in the presence of NLX during the first test, NLX might not be expected to alter CPP extinction when given before a short duration test session.

This experiment consisted of the following phases: habituation, conditioning and choice extinction tests (T1-T4). Mice ($n = 96$) were randomly divided into four groups: NLX 10-min (0 or 10 mg/kg) or NLX 60-min (0 or 10 mg/kg). During habituation, mice received an IP injection of saline and were immediately placed inside the conditioning box on a smooth paper floor (without the tactile CSs) for 5-min. The conditioning phase (8 days) started the next day. An unbiased CPP procedure was used in which mice within each of the four treatment groups were randomly assigned to one of two conditioning subgroups ($n = 12$ /subgroup) that counterbalanced the floor cues paired with ethanol or saline (Cunningham et al. 2003). Mice in the Grid+ (G+) subgroups received an injection of ethanol (2 g/kg) paired with the grid floor (CS+) and an injection of saline paired with the hole floor (CS-). Alternatively, animals in the Grid- (G-) subgroups received saline paired with the grid floor (CS-) and ethanol paired with the hole floor (CS+). Mice had access to the entire apparatus for 5-min, but only one floor type was available on each trial (i.e., a one-compartment procedure) and mice received only one trial on each day. A 5-min conditioning trial duration was used on the basis of previous research showing that longer trial durations have detrimental effects on the strength of ethanol-induced CPP in mice (Cunningham and Prather 1992). Exposure to each floor CS, as well as type of injection (i.e., ethanol vs. saline), alternated across conditioning sessions in a counterbalanced manner. There was a 2-day (weekend) break between the first and second four conditioning sessions.

Testing began the day after the last conditioning session. Because preference tests involved omission of the ethanol unconditioned stimulus, they also served as extinction trials. Both floor cues (half grid and half hole) were present during tests, with positions counterbalanced within each subgroup. All mice were weighed and injected with either saline or 10 mg/kg NLX 15-min before the test and all received a saline injection immediately before being placed in the apparatus (T1). Three additional choice extinction tests were conducted at 48-h intervals (T2-T4). For T1-T3, half of the mice treated with saline or NLX were placed in the apparatus for a 10-min test while the other half received a 60-min test. To determine whether effects of NLX on CPP would be retained later in the absence of NLX, all mice received an injection of saline immediately before a final 60-min test on T4. If NLX facilitates ethanol CPP extinction, CPP should remain reduced even when NLX is no longer present. However, if NLX simply suppresses expression of CPP, removal of NLX might be expected to reinstate CPP.

Experiment 2: Effect of post-test NLX treatment on choice extinction of ethanol-induced CPP

Experiment 2 was designed to test whether the extinction facilitating effect of NLX was due to an alteration in post-extinction consolidation by injecting NLX immediately after rather than just before each extinction test session. If the previously observed enhancing effect of pre-test NLX injections on extinction was due to enhanced consolidation of a new inhibitory memory, the same result should be observed with post-test NLX injections (McGaugh 2015).

Mice ($n = 96$) were exposed to habituation, conditioning trials and choice extinction tests identical to those used in Experiment 1 except that NLX (0 or 10 mg/kg) was given

immediately after the mouse was removed from the apparatus. For the first three tests (T1-T3), half of the mice were placed in the apparatus for 10 min and the other half for 60 min, depending on their assigned group. T4 was 60-min long for all mice.

Experiment 3: Effect of NLX treatment on forced extinction of ethanol-induced CPP

This study was designed to address the possibility that NLX's effect might actually depend on the animal's ability to express CPP in the presence of NLX. Thus, we exposed mice to the conditioned floor cues individually in the absence of ethanol (i.e., forced extinction) to produce extinction of the conditioned response, but we did not allow mice to express CPP until later choice tests without NLX treatment. Thus, in contrast to Experiment 1, mice never had the opportunity to express CPP under the influence of NLX. If facilitation of extinction depends on expressing CPP in the presence of NLX, no effect of NLX would be expected using the forced extinction procedure.

The experiment consisted of 6 phases: habituation, conditioning, forced extinction, a choice extinction test, additional forced extinction, and a second choice extinction test. Mice ($n = 96$) were exposed to habituation and conditioning sessions identical to those used for Experiment 1. Extinction began 72 h after the last conditioning session. Mice were randomly assigned to three equal groups ($n = 32/\text{group}$; 16/conditioning subgroup). Two groups received extinction trials while one did not receive any extinction (NO-EXT). On each day of extinction, mice in the two extinction groups received a pretreatment injection of saline (SAL) or NLX (10 mg/kg) 15-min before an injection of saline and placement into the chambers for 10-min. We purposely used relatively short (10-min) exposures so that extinction would proceed slowly in SAL-treated control mice (see Groblewski et al. 2011), providing a better opportunity to detect facilitation of extinction in NLX-treated mice. Mice received two extinction trials per day 4 h apart, one on the CS+ floor and the other on the CS- floor (counterbalanced order), on each of 3 consecutive days (i.e., six trials total). The assigned pretreatment drug was given before both types of trial to match the drug manipulation used in the choice extinction studies, which involved exposing mice to both cues after the assigned pretreatment injection. Mice in the NO-EXT group were briefly handled for weighing, but received no other treatment. Twenty-four h later, all mice were injected with saline and placed in the apparatus for a 30-min drug-free preference test (T1). After a 3-day break, animals received six additional extinction trials followed by a second preference test (T2).

Experiment 4: Effect of NLX on extinction of cocaine-induced CPP

This study was performed to address the pharmacological specificity of NLX's effect by determining whether NLX pretreatment also facilitated extinction of cocaine-induced CPP. A facilitating effect of NLX on extinction of cocaine CPP would suggest that NLX has a more general impact on the extinction of drug-induced memories. The overall design and procedure of this experiment were similar to that of Experiment 1.

The study consisted of three phases: habituation, conditioning, and choice extinction tests (T1-T3). The 8-day conditioning phase began 24 h after habituation. Immediately before placement on the assigned floor, all mice received a dose of cocaine that increased over the

four 30-min CS+ trials (2, 4, 8 and 16 mg/kg). A saline vehicle injection was given immediately before each of the four 30-min CS-conditioning trials. The ascending dose schedule was used based on previous research suggesting enhanced conditioning compared to use of fixed cocaine dose on each trial (Itzhak and Anderson 2012; Liddie and Itzhak 2016). A 30-min conditioning trial duration was used based on previous research in DBA/2J mice showing that cocaine-induced CPP is stronger when longer trial durations are used (Cunningham et al. 1999). As in previous studies, the floor paired with cocaine and the trial order were fully counterbalanced.

Preference testing began the day after the last conditioning session. Mice ($n = 96$) were randomly assigned to one of four groups: SAL 10-min, NLX 10-min, SAL-60 min, or NLX 60-min. As in Experiment 1, mice were then exposed to two consecutive choice extinction tests at their assigned duration (10 vs. 60 min) after pretreatment with either saline or NLX (10 mg/kg). In contrast to Experiment 1, however, all mice were pretreated with saline only before the third (and final) test, which was 60 min long. These tests occurred at 48 h intervals. We gave fewer tests with NLX before the final test in this study because cocaine-induced CPP was generally weaker and extinguished more rapidly than ethanol-induced CPP.

Data analyses

The primary dependent variable in these studies was the amount of time spent on the grid floor (grid time) by each conditioning subgroup (Cunningham et al. 2003). In order to compare groups that received different extinction trial durations, our analyses focused primarily on the first 10 min of testing. To simplify presentation of the data across extinction trials, we also analyzed the percent time spent on the drug-paired floor (collapsed across conditioning subgroups). In Experiments 1, 2 and 4, percent time data were analyzed using three-way ANOVAs with repeated measures (Treatment $\times$ Test Duration $\times$ Test Day). Treatment (0 or 10 mg/kg NLX) and Test Duration (10 or 60 min) were treated as between group factors, whereas Test Day was treated as a within-group factor. Separate analyses of grid times on each test included Conditioning Subgroup (G+ and G-) as a third between-group factor (Treatment $\times$ Test Duration $\times$ Conditioning Subgroup). Grid time data are reported in Table 1 for Experiments 1, 2 and 4. Grid time data from each test were analyzed separately with two-way ANOVA in Experiment 3, using Group (SAL, NLX or NO-EXT) and Conditioning Subgroup (G+ vs. G-) as factors. Planned comparisons (Bonferroni-corrected p values) between the G+ and G- conditioning subgroups within each treatment group were used to determine whether place conditioning was significant (Cunningham et al. 2003). Alpha-level was set at 0.05 for all analyses.

Results

Data from six mice were completely deleted from all analyses either because they died ($n = 2$) or because they showed an unexpected large drop in body weight ($> 20\%$) and were removed during the study ($n = 4$). Final group sizes are indicated in Table 1 and the figure captions.

Experiment 1: Effect of NLX treatment and test duration on choice extinction of ethanol-induced CPP

Experiment 1 examined the effect of repeated pre-injections of NLX (or SAL) on the choice extinction of ethanol CPP using short (10-min) or long (60-min) extinction trials (Fig. 1A). All groups showed significant preference for the EtOH-paired floor during the first 10 min of testing on T1, but NLX pretreatment facilitated extinction of EtOH-induced CPP across T2 and T3 (Fig. 1B). As might be expected, the rate of extinction was somewhat greater in groups given 60-min tests than in groups given 10-min tests. A three-way repeated measures ANOVA applied to the percent time data yielded significant main effects of Treatment [$F(1, 91) = 29.6, p < 0.001$] and Test Day [$F(2, 182) = 22.9, p < 0.001$], as well as significant Treatment $\times$ Test Day [$F(2, 182) = 13.4, p < 0.001$] and Duration $\times$ Test Day [$F(2, 182) = 6.0, p < 0.005$] interactions. However, analysis showed no interactions involving Treatment $\times$ Duration, indicating that CS exposure duration was not critical for determining the opioid antagonist's effect on extinction of EtOH-induced CPP. As Table 1 shows, test-by-test analyses of grid times during the extinction phase generally confirmed these findings.

Fig. 1C depicts grid times during the first 10-min of final test in which all groups were pretreated with saline. Mice previously treated with saline before 10-min extinction sessions (SAL-10) continued to show a strong place preference as indicated by the greater time spent on the grid floor in the subgroup given grid-ethanol pairings (G+) than in the group given grid-saline pairings (G-). However, preference was substantially reduced in all of the other groups, with an apparent conditioned place aversion (CPA) in the NLX-60 group. Three-way ANOVA yielded significant Treatment $\times$ Conditioning Subgroup [$F(1, 87) = 20.9, p < 0.001$] and Duration $\times$ Conditioning Subgroup [$F(1, 87) = 5.3, p < 0.05$] interactions, supporting the conclusion of stronger CPP in SAL pretreated mice and in mice given 10-min extinction tests. Bonferroni-corrected comparisons of the G+ and G- groups within each treatment $\times$ duration group showed significant place preference only in the SAL-10 group ($p < 0.01$). A separate two-way ANOVA applied only to data from SAL pretreated mice showed a significant Duration $\times$ Conditioning Subgroup interaction [$F(1, 43) = 7.1, p = 0.01$], confirming that the longer extinction duration had produced a greater weakening of CPP in SAL pretreated mice. Analysis of data from all 60 min of T4 also yielded a significant Treatment $\times$ Conditioning Subgroup interaction [$F(1, 87) = 11.3, p < 0.001$], but the Duration $\times$ Conditioning Subgroup interaction ($p = 0.10$) fell short of the criterion for significance (data not shown).

Activity rates (counts/min $\pm$ SEM) during the first 10 min of each extinction session were generally lower in NLX groups than in saline groups and the group difference increased over sessions [Test 1: SAL = 49.3 ± 1.7 , NLX = 45.9 ± 1.3 ; Test 3: SAL = 49.1 ± 2.5 , NLX = 37.4 ± 1.6]. Three-way ANOVA (Treatment $\times$ Duration $\times$ Test Day) indicated significant main effects of Treatment [$F(1,91) = 10.9, p < 0.001$] and Test Day [$F(2,182) = 5.8, p < 0.005$] as well as the Treatment $\times$ Test Day interaction [$F(2,182) = 4.9, p < 0.01$]. Mice previously pretreated with NLX (44.8 ± 1.6) also showed lower activity rates than mice previously pretreated with SAL (54.9 ± 2.3) during the first 10 min of the final test, even though both groups received only saline before that test [$F(1, 91) = 13.4, p < 0.001$].

Thus, the results of Study 1 confirm previous studies showing that repeated NLX treatment during CS exposure facilitates extinction of ethanol-induced CPP (Cunningham et al. 1995, 1998). This effect was observed after both short (10-min) and long (60-min) extinction sessions, suggesting that facilitated extinction does not depend on a long exposure to the CS under the influence of naloxone. Importantly, the effect was maintained when animals were tested later in absence of NLX.

Experiment 2: Effect of post-test NLX treatment on choice extinction of ethanol-induced CPP

To test whether NLX facilitated extinction of EtOH-induced CPP by altering post-trial memory processes, mice received NLX injections after each extinction trial (Fig. 2A). As shown in Fig. 2B, NLX given immediately after each test did not affect preference during the first 10 min of testing. Regardless of extinction test duration or drug treatment, preference was consistently strong in all groups across three consecutive tests. In support of this conclusion, three-way repeated measures ANOVA of percent time scores showed no significant main effects or interactions involving drug treatment or test duration. Separate three-way analyses of grid times during the first 10 min of each extinction test further supported these findings, showing only a significant main effect of Conditioning Subgroup on each test (see Table 1). Finally, analyses of performance on the final test, after pretreatment with saline only, also showed only a significant main effect of Conditioning Subgroup, both for the first 10 min of testing [$F(1, 88) = 53.3, p < 0.001$; Fig. 2C] as well as for all 60 min [$F(1, 88) = 38.3, p < 0.001$; data not shown]. A separate two-way ANOVA was applied only to data from SAL pretreated mice to address whether the longer test duration had produced more rapid extinction under control conditions. That analysis yielded a significant Duration $\times$ Conditioning Subgroup interaction [$F(1, 44) = 5.0, p = 0.03$], confirming the expectation of more rapid extinction in control mice exposed to 60-min tests. Analyses of test activity rates showed no significant group differences either during the extinction phase (SAL = 43.6 ± 1.6 , NLX = 43.8 ± 1.4) or during the final test (SAL = 46.6 ± 1.9 , NLX = 45.6 ± 1.6).

Experiment 3: Effect of NLX treatment on forced extinction of ethanol-induced CPP

Experiment 3 examined effects of NLX on the extinction of EtOH-induced CPP using a forced extinction procedure (Fig. 3A). After the first six sessions of forced extinction with drug pretreatment (3 CS+ and 3 CS- trials), all groups showed significant preference for the EtOH-paired floor in a saline test (Test 1), albeit at a reduced level in the NLX group (Fig. 3B). After six more extinction trials (Test 2), CPP was slightly weaker in the SAL and NO-EXT groups, but completely eliminated in the NLX group (Fig. 3C). Two-way ANOVAs yielded significant main effects of Conditioning Subgroup on both tests [both $F_s(1, 90) = 20.2, p < 0.001$] as well as significant Group $\times$ Conditioning Subgroup interactions [both $F_s(2, 90) = 3.2, p < 0.05$]. To further clarify the interactions, we also conducted two-way follow-up ANOVAs that compared only two groups at a time. On Test 1, those ANOVAs indicated no difference between the SAL and NO-EXT groups, a non-significant trend between the NLX and SAL groups [interaction $F(1, 60) = 3.3, p = 0.07$], and a significant difference between the NLX and NO-EXT groups [interaction $F(1, 60) = 4.9, p = 0.03$]. On Test 2, those ANOVAs also showed a significant difference between the NLX and NO-EXT

groups [interaction $F(1, 60) = 13.8, p < 0.001$] as well as trends toward significant differences between the SAL and NLX groups [interaction $F(1, 60) = 3.4, p = 0.07$] and between the SAL and NO-EXT groups [interaction $F(1, 60) = 3.6, p = 0.06$]. Bonferroni-corrected comparisons between the G+ and G- subgroups showed significant CPP for all groups on Test 1 ($p < 0.05$), but significant preference only in the SAL and NO-EXT groups on Test 2, indicating that NLX pretreatment facilitated loss in CPP strength during a forced extinction procedure.

Analyses of activity rates during the 30-min test sessions indicated that the NO-EXT group was generally more active (35.3 ± 1.0) than either the SAL (29.8 ± 0.9) or NLX (28.3 ± 1.1) groups [Test 1: $F(2, 88) = 8.8, p < 0.001$; Test 2: $F(2, 93) = 10.8, p < 0.001$], which did not differ. Thus, previous exposure to NLX during forced extinction did not have a residual effect on activity during subsequent testing after saline injections.

Experiment 4: Effect of NLX on extinction of cocaine-induced CPP

Experiment 4 assessed effects of NLX on the choice extinction of cocaine-induced CPP using a design and procedure similar to Experiment 1 (Fig. 4A). Percent time spent on the cocaine-paired floor during the first 10 min of each extinction session showed little effect of NLX on performance during extinction (Fig. 4B). This conclusion was consistent with a three-way repeated-measures ANOVA that showed no significant main effects or interactions involving drug treatment or test duration. A significant main effect of Test Day [$F(1, 87) = 6.4, p < 0.05$] supported the development of extinction across sessions. Separate analyses of grid times during the first 10 min of each extinction session revealed only main effects of Conditioning Subgroup [$F(1, 83) = 17.0, p < 0.001$], indicating significant CPP during each session (Table 1). During the final test after saline pretreatment, CPP was still apparent in both of the 10-min groups, but not in the 60-min groups (Fig. 4C). This observation was supported by a three-way ANOVA of grid times during the first 10 min of Test 3, which showed a significant main effect of Conditioning Subgroup [$F(1, 83) = 10.1, p < 0.005$] and a trend toward a significant Duration $\times$ Conditioning Subgroup interaction [$F(1, 83) = 3.4, p = 0.07$]. A similar analysis of grid times over all 60 min of Test 3 showed a significant main effect of Conditioning Subgroup [$F(1, 83) = 13.6, p < 0.001$] as well as a significant Duration $\times$ Conditioning Subgroup interaction [$F(1, 83) = 7.6, p < 0.01$; data not shown]. Post-hoc analyses applied separately to the 10-min grid times data from each duration condition yielded a significant main effect of Conditioning Subgroup for the 10-min groups [$F(1, 41) = 12.9, p < 0.001$], but not for the 60-min groups. Analyses of activity rates during the first 10 min of each extinction session and the final test showed no significant group differences (Extinction: SAL = 57.6 ± 1.2 , NLX = 53.7 ± 1.6 ; Test 3: SAL = 54.5 ± 1.4 , NLX = 52.2 ± 1.9).

Discussion

These studies showed that repeated administration of NLX, a nonspecific opioid receptor antagonist, facilitated choice extinction of ethanol-induced CPP in DBA/2J mice. This outcome was observed after either 10- or 60-min extinction trials and was maintained in the absence of NLX (Exp. 1). The same manipulation given immediately after each extinction

trial had no impact on CPP extinction (Exp. 2), suggesting that the effect seen in Exp. 1 was not caused by post-trial alterations in memory processing. We also found that NLX facilitated extinction in a forced extinction procedure, indicating that this outcome does not depend on expression of CPP during opioid receptor blockade, but only on exposure to the previously conditioned cues in the absence of ethanol during receptor blockade (Exp. 3). Finally, administration of NLX before choice extinction tests had no effect on the extinction of cocaine-induced CPP, suggesting that NLX's facilitation of extinction may be specific to ethanol-conditioned motivational processes (Exp. 4). Overall, these data support the hypothesis that maintenance of ethanol-induced CPP during extinction is normally dependent on conditioned motivational processes sensitive to opioid-receptor blockade.

The literature offers mixed evidence on the role that activation of the opioid system plays during acquisition of ethanol-conditioned memories underlying CPP. For example, acquisition of ethanol-induced CPP was not affected in mice deficient in β -endorphin neurons due to a neurotoxic lesion (Pastor et al. 2011) or in male μ -opioid receptor knockout mice (Becker et al. 2002). However, another study reported that female mice heterozygous or homozygous for the μ -opioid receptor deficiency failed to develop CPP compared to the strong CPP seen in wild-type female mice (Hall et al. 2001). Pharmacological blockade studies have similarly provided mixed results with one study showing no effect of a low (1.5 mg/kg) or high (10 mg/kg) NLX dose on CPP acquisition (Cunningham et al. 1995), two studies showing no effect at low doses (0.1 or 1.0 mg/kg) but interference at a high (10 mg/kg) NLX dose (Kuzmin et al. 2003; Tseng et al. 2013), as well as a third study that showed interference at low (1 mg/kg) and intermediate (5 mg/kg) NLX doses (Wróbel 2011). Although the reasons behind these discrepancies are unknown, they are likely due to differences in the CPP procedure or mouse strain that will require additional research to resolve. Regardless, whether the opioid system is involved in either the primary rewarding effect of ethanol or learning about that effect in the CPP procedure remains unclear on the basis of these acquisition studies.

In contrast, the literature is much more consistent in showing that opioid receptor blockade reduces the expression of ethanol-induced CPP, either during the first test with an opioid receptor antagonist (Middaugh & Bandy 2000; Kuzmin et al. 2003; Bechtholt & Cunningham 2005; Gremel et al. 2011; Pastor et al. 2011; Wróbel 2011) or during extinction (Cunningham et al. 1995, 1998). Exps. 1 and 3 replicated the latter findings, showing that NLX facilitates extinction of ethanol-induced CPP. Moreover, they extend those findings in two important ways. First, Exp. 1 shows that NLX's ability to facilitate extinction does not depend on the use of long duration (60-min) treatment sessions, but also occurs after a relatively short period (10-min) of CPP testing during receptor blockade. Second, Exp. 3 shows that the NLX-facilitated extinction does not depend on use of a choice extinction procedure, but also occurs when a forced extinction procedure is used. This finding suggests that it is not necessary for mice to actually express CPP during opioid receptor blockade, but only to receive non-rewarded exposures to the previously conditioned cues during receptor blockade. Although the behavioral and neurobiological differences between choice and forced extinction procedures have not yet been well studied (Cunningham et al. 2011), our findings support the idea of a common neural mechanism underlying choice and forced extinction of ethanol-induced conditioned memories.

To explain the greater decrease in magnitude of ethanol-induced CPP during opioid receptor blockade, one could argue that: (a) the antagonist interferes with CPP by altering motor behavior, (b) the antagonist increases aversive motivation, (c) the antagonist influences general extinction-related learning or memory processes, or (d) the antagonist interferes with the conditioned motivational response or state that normally maintains ethanol-seeking behavior during extinction. Although each of the first three explanations might be able to explain some of the previous or current findings, only the last interpretation is consistent with all of the data related to opioid antagonist effects on expression of ethanol-induced CPP.

First, the suggestion that NLX's ability to reduce CPP is a byproduct of its activity suppressing effect is generally at odds with previous data from our lab and others showing an inverse relationship between test activity and CPP magnitude (Cunningham 1995; Gremel and Cunningham 2007), which suggests that NLX-induced suppression of activity should yield stronger rather than weaker CPP (Neisewander et al. 1990). An even stronger argument against the activity hypothesis, however, is the finding from Exp. 3 showing that NLX facilitated forced extinction in the complete absence of any activity differences between groups previously treated with NLX or SAL. Thus, the present findings are quite consistent with previous studies indicating that NLX-induced facilitation of extinction is not related to residual effects of NLX exposure on activity during post-treatment testing (Cunningham et al. 1995, 1998).

Second, the trend toward CPA after NLX pretreatment during extinction (see NLX-60 group in Fig. 1C), together with findings of significant CPA in similarly treated mice in previous studies (Cunningham et al. 1995, 1998), raises the possibility that facilitated extinction of ethanol-induced CPP is related to NLX's ability to induce or enhance an aversive motivational state (Norris et al. 2009). This possibility receives further support from previous studies showing that NLX alone, in the dose range used here (0.1–20 mg/kg), is able to induce CPA to paired contextual cues in a standard place conditioning procedure (e.g., Cunningham et al. 1995; Mucha and Walker 1987; Solecki et al. 2009). However, there are several reasons for dismissing the aversion hypothesis. For example, simply on logical grounds, it is difficult to explain why NLX's aversive effect would selectively alter preference for the floor cue previously paired with ethanol when mice are exposed to both floor cues during the extinction phase (Exps. 1 and 3). More compelling evidence against this interpretation, however, comes from the failure to see a similar effect of NLX on extinction when cocaine was used to induce CPP (Exp. 4) as well as the failure to see any impact on ethanol CPP extinction when a different aversive drug, lithium chloride, was given before extinction sessions (Cunningham et al. 1995, 1998).

A third possible explanation for our results is that NLX facilitates extinction of EtOH-induced CPP by altering extinction-related learning or memory processes. Several lines of evidence indicate that extinction involves new learning, rather than simple forgetting or erasure of original learning (see Bouton 1993; Rescorla 2001; Schroeder and Packard 2004). Thus, NLX might directly enhance that form of inhibitory learning. Alternatively, NLX might enhance consolidation of the extinction memory, perhaps by releasing central β -adrenergic mechanisms from a tonic inhibitory influence of endogenous opiate peptides

released during or immediately after training (Introini-Collison and Baratti 1986; Castellano et al. 1996; Quirarte et al. 1998; Roozendaal et al. 2007). However, our current and previous findings do not support either of these possibilities. For example, the failure of post-test administration of NLX to affect extinction of ethanol-induced CPP (Exp. 2) provides evidence contrary to predictions based on enhanced memory consolidation. Moreover, the failure of NLX to alter extinction of cocaine-induced CPP (Exp. 4) or extinction of ethanol-induced CPA (Bormann and Cunningham 1997; Cunningham et al. 1998) argues against both interpretations. Overall, the data suggest that activation of opioid receptors is only required for some types of extinction learning (e.g., ethanol-induced CPP), but not all forms. Future research must resolve this issue.

The data reported here as well as our previous findings are best explained by the idea that opioid antagonists facilitate extinction of ethanol-induced CPP by interfering with a conditioned motivational response, mediated by endogenous opioid release, that normally reinforces approach and contact with the ethanol-paired cues during extinction (Cunningham et al., 1995). When opioid receptors are blocked, approach behavior rapidly extinguishes because the conditioned motivational response is reduced. By this analysis, there is no need to assume that opioid system is otherwise involved in the inhibitory learning process that normally occurs during the extinction procedure.

The mechanism by which opioid receptor activation mediates ethanol-related motivational effects is hypothesized to involve the proopiomelanocortin (POMC)-derived opioid peptide, β -endorphin (Herz, 1997; Gianoulakis, 2001; Sanchis-Segura et al. 2005; Pastor et al. 2011). β -endorphin binds with high affinity to μ and δ -opioid receptors (Williams et al. 2001), and blockade of these receptors by non-specific or specific antagonists reduces the conditioned effects of ethanol (Cunningham et al. 1995; 1998; Kuzmin et al. 2003; Bechtholt & Cunningham, 2005; Dayas et al. 2007; Marinelli et al. 2009; Gremel et al. 2011; Pastor et al. 2011). NLX, at the doses used in our study, acts as a non-selective opioid receptor antagonist of μ and δ receptors that are expressed in regions innervated by β -endorphin neurons, such as the ventral tegmental area (VTA), anterior cingulate cortex (ACC), nucleus accumbens and amygdala (Gutstein and Akil, 2001; Mansour et al. 1996; Sudakov et al. 2010; Tseng et al. 2013). Previous research from our lab has suggested that the conditioned motivational response that normally maintains cue-induced ethanol-seeking behavior depends on μ -opioid receptors within the VTA (Bechtholt and Cunningham, 2005) and the ACC (Gremel et al. 2011), but not the nucleus accumbens (Bechtholt and Cunningham, 2005). The role of specific opioid receptor subtypes in the conditioned motivational response underlying maintenance of ethanol-induced CPP remains a topic for future research.

The literature's general consistency in showing opioid antagonist effects on the expression (or extinction) of ethanol-induced CPP raises the interesting question of why the literature is less consistent in showing antagonist effects on acquisition of ethanol CPP. If the primary and conditioned rewarding effects of ethanol are both mediated by opioid receptors, antagonists should be similarly effective in reducing both acquisition and expression. However, the opioid system might play little or no role in ethanol CPP acquisition while other systems are much more important (e.g., dopamine D1-like receptors: Pina and Cunningham 2014; Young et al. 2014). Dissociations between the mechanisms underlying

acquisition and expression of drug-induced CPP are well known. For example, although opioid antagonists interfere with the acquisition of heroin-induced CPP, they have little effect on the expression of heroin CPP (Hand et al. 1989). Similarly, although D1-like receptor antagonists interfere with ethanol CPP acquisition, neither D1- nor D2-like receptor antagonists affect ethanol CPP expression (Pina and Cunningham 2014; Young et al. 2014). The suggestion that the opioid system has little involvement in ethanol CPP acquisition, however, raises the question of why some studies have nevertheless reported detrimental effects of opioid antagonists on ethanol CPP acquisition (Kuzmin et al. 2003; Tseng et al. 2013; Wróbel 2011). Since naloxone alone is capable of inducing CPA, we have previously suggested that such effects might be explained in terms of “behavioral antagonism” rather than pharmacological antagonism. According to this analysis, the likelihood of observing antagonist effects on ethanol CPP acquisition would depend on how well the conditioning parameters (e.g., drug dose, trial duration, interstimulus interval) favored the conditioning of ethanol-induced CPP vs. the conditioning of naloxone-induced CPA (see Cunningham et al. 1995).

The outcomes of the present studies are generally consistent with past research supporting use of the opioid receptor antagonist NTX in the treatment of alcoholism (Jonas et al. 2014) and with data suggesting that opioid antagonists reduce alcohol cue reactivity and craving (Miranda et al. 2014; Monti et al. 1999). Moreover, our findings have potentially important implications for the exact manner in which opioid antagonists are used to treat alcoholism. Specifically, our data suggest that opioid antagonists might be most effective at reducing cue-induced craving for alcohol when co-administered in the presence of cues that have previously been associated with alcohol. Indeed, Exp. 2 showed that an identical schedule of exposure to the antagonist in the absence of such cues had no impact on the conditioned motivational response to those cues. Of particular interest, even relatively short exposures to the conditioned stimuli after pretreatment with the antagonist were effective for enhancing the rate of extinction. Our findings parallel results from a study of cue reactivity in alcohol-dependent men that showed no effect of NTX on the initial reaction to a 3-min exposure to alcohol cues, but a more rapid reduction in urge to drink after repeated cue exposures (Rohsenow et al. 2000). Such findings suggest that giving opioid antagonists immediately before cue-exposure therapy for alcoholism might be especially useful for reducing the likelihood that cues previously paired with alcohol would trigger relapse in the future.

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Figure 1.

A). Outline of experimental procedure for Exp. 1 (see text for additional detail). B). Mean percent time ($\pm$ SEM) spent on the ethanol paired floor (collapsed over conditioning subgroup) during the first 10 min of each session in groups given three 10-min (SAL-10, NLX-10) or 60-min (SAL-60, NLX-60) extinction sessions after pretreatment with either saline (SAL) or 10 mg/kg naloxone (NLX). Each group contained 23–24 mice. C). Mean s/min ($\pm$ SEM) spent on the grid floor by each conditioning subgroup ($n = 11–12$ /subgroup) in each treatment group during the first 10 min of the final preference test after pretreatment with saline. * $p < 0.01$

Figure 2.

A). Outline of experimental procedure for Exp. 2 (see text for additional detail). B). Mean percent time ($\pm$ SEM) spent on the ethanol paired floor (collapsed over conditioning subgroup) during the first 10 min of each session in groups given three 10-min (SAL-10, NLX-10) or 60-min (SAL-60, NLX-60) extinction sessions just before post-test treatment with either saline (SAL) or 10 mg/kg naloxone (NLX). Each group contained 24 mice. C). Mean s/min ($\pm$ SEM) spent on the grid floor by each conditioning subgroup ($n = 12$ /subgroup) in each treatment group during the first 10 min of the final preference test.

Figure 3.

A). Outline of experimental procedure for Exp. 3 (see text for additional detail). B–C). Mean s/min ($\pm$ SEM) spent on the grid floor by each conditioning subgroup ($n = 16$ / subgroup) in each treatment group during both 30-min preference tests after pretreatment with saline. Each test was preceded by 3 days of forced extinction training after pretreatment with either saline (SAL) or naloxone (NLX) or after no-extinction (NO-EXT). * $p < 0.05$

Figure 4.

A). Outline of experimental procedure for Exp. 4 (see text for additional detail). B). Mean percent time ($\pm$ SEM) spent on the cocaine paired floor (collapsed over conditioning subgroup) during the first 10 min of each session in groups given two 10-min (SAL-10, NLX-10) or 60-min (SAL-60, NLX-60) extinction sessions after pretreatment with either saline (SAL) or 10 mg/kg naloxone (NLX). Each group contained 22–24 mice. C). Mean s/min ($\pm$ SEM) spent on the grid floor by each conditioning subgroup ($n = 10$ –12/subgroup) in each treatment group during the first 10 min of the final preference test after pretreatment with saline. * $p < 0.001$ for main effect of Conditioning Subgroup in the 10-min groups.

Table 1

Mean ($\pm$ SEM) time spent on the grid floor (sec/min) during the first 10 min of each extinction test in Experiments 1, 2 and 4.

Experiment	Group	Conditioning Subgroup	n	T1 ^{ab}	T2 ^{ab}	T3 ^b
(1) NLX pre-treatment on extinction of ethanol CPP	SAL-10	G+	11	41.6 $\pm$ 2.6 *	46.2 $\pm$ 3.2 *	46.5 $\pm$ 2.0 *
		G-	12	19.1 $\pm$ 2.7 *	16.6 $\pm$ 2.1 *	19.4 $\pm$ 2.6 *
	NLX-10	G+	12	31.3 $\pm$ 3.7 *	31.3 $\pm$ 4.9	22.1 $\pm$ 4.9
		G-	12	19.8 $\pm$ 2.5 *	27.4 $\pm$ 4.3	34.6 $\pm$ 4.7
	SAL-60	G+	12	45.1 $\pm$ 2.7 *	39.8 $\pm$ 4.2 *	32.4 $\pm$ 4.2 *
		G-	12	17.5 $\pm$ 3.1 *	16.7 $\pm$ 3.2 *	18.0 $\pm$ 2.7 *
	NLX-60	G+	12	38.3 $\pm$ 2.1 *	19.9 $\pm$ 4.3	19.0 $\pm$ 4.1
		G-	12	19.1 $\pm$ 3.3 *	27.8 $\pm$ 4.4	32.2 $\pm$ 4.8
Experiment	Group	Conditioning Subgroup	n	T1 ^a	T2 ^a	T3 ^a
(2) NLX post-treatment on extinction of ethanol CPP	SAL-10	G+	12	41.8 $\pm$ 3.6	38.3 $\pm$ 3.9	36.7 $\pm$ 4.1
		G-	12	16.0 $\pm$ 3.8	16.2 $\pm$ 3.9	13.1 $\pm$ 3.8
	NLX-10	G+	12	38.2 $\pm$ 4.3	34.2 $\pm$ 4.0	37.8 $\pm$ 4.5
		G-	12	16.5 $\pm$ 3.7	13.8 $\pm$ 3.7	14.0 $\pm$ 3.7
	SAL-60	G+	12	39.5 $\pm$ 4.2	35.9 $\pm$ 4.3	32.8 $\pm$ 4.1
		G-	12	15.0 $\pm$ 3.3	15.8 $\pm$ 3.8	11.7 $\pm$ 1.8
	NLX-60	G+	12	45.8 $\pm$ 2.8	42.8 $\pm$ 2.2	39.5 $\pm$ 2.8
		G-	12	16.6 $\pm$ 1.5	17.8 $\pm$ 3.8	16.9 $\pm$ 2.6
Experiment	Group	Conditioning Subgroup	n	T1 ^a	T2 ^a	--
(4) NLX pre-treatment on extinction of cocaine CPP	SAL-10	G+	12	38.4 $\pm$ 1.5	34.7 $\pm$ 1.4	
		G-	10	23.7 $\pm$ 2.4	22.5 $\pm$ 2.2	
	NLX-10	G+	12	36.3 $\pm$ 1.9	35.3 $\pm$ 2.9	
		G-	11	25.3 $\pm$ 3.4	27.7 $\pm$ 3.4	
	SAL-60	G+	10	37.1 $\pm$ 2.3	29.7 $\pm$ 2.6	

Experiment	Group	Conditioning Subgroup	n	T1 ^{ab}	T2 ^{ab}	T3 ^b
	NLX-60	G-	12	27.7 ± 2.4	24.5 ± 2.2	
		G+	12	38.0 ± 2.7	31.8 ± 2.8	
		G-	12	22.9 ± 1.7	26.4 ± 2.7	

^a significant main effect of Conditioning Subgroup (p < 0.001)

^b significant Treatment × Conditioning Subgroup interaction (p < 0.05)

* significant difference between G+ and G- subgroups within a given treatment group (shown only when the overall ANOVA yielded a significant interaction)