Published in final edited form as:

Neurosci Lett. 2013 April 29; 541: 39–42. doi:10.1016/j.neulet.2013.01.049.

Extinction of conditioned opiate withdrawal in rats is blocked by intracerebroventricular infusion of an NMDA receptor antagonist

Brian R. Coleman^{a,b}, William A. Carlezon Jr.^{a,b}, and Karyn M. Myers^{a,b}

^aBehavioral Genetics Laboratory, McLean Hospital, 115 Mill Street, Belmont, MA, USA

^bDepartment of Psychiatry, Harvard Medical School, 115 Mill Street, Belmont, MA, USA

Abstract

Maladaptive conditioned responses (CRs) contribute to psychiatric disorders including anxiety disorders and addiction. Methods of reducing these CRs have been considered as possible therapeutic approaches. One such method is extinction, which involves exposure to CR-eliciting cues in the absence of the event they once predicted. In animal models, extinction reduces both fear and addiction-related CRs, and in humans, extinction-based cue exposure therapy (CET) reduces fear CRs. However, CET is less effective in drug addicts, for reasons that are not clear. Increased understanding of the neurobiology of extinction of drug-related CRs as compared to fear CRs may help illuminate this issue. Here, we examine the N-methyl-D-aspartate (NMDA) receptor-dependence of extinction of conditioned opiate withdrawal in rats. Using a place conditioning paradigm, we trained morphine-dependent rats to associate an environment with naloxone-precipitated withdrawal. We then extinguished that association by returning the rats repeatedly to the environment in the absence of acute withdrawal. In some rats we administered the NMDA receptor antagonist D,L-2-amino-5-phosphovaleric acid (AP5) intracerebroventricularly immediately prior to extinction training. In a subsequent test session, these rats avoided the formerly naloxone-paired environment, similar to rats that had not undergone extinction training. By contrast, rats that received vehicle prior to extinction training did not avoid the formerly naloxone-paired environment. This finding indicates that extinction of a drug-related CR (conditioned opiate withdrawal) is dependent on NMDA receptors, similar to extinction of conditioned fear. The locus of the critical NMDA receptors is unclear but may include basolateral amygdala and/or medial prefrontal cortex.

Keywords

addiction; extinction; glutamate; morphine; NMDA receptor; withdrawal

Corresponding author: Brian R. Coleman, Behavioral Genetics Laboratory, McLean Hospital, Mailman Research Center Room 001 (Mail Stop 146), 115 Mill Street, Belmont, MA 02478, Tel: +001 1 617 855 2157, Fax: +001 1 617 855 2023, bcoleman@mclean.harvard.edu.

The authors report no biomedical financial interests or potential conflicts of interest.

Introduction

In addicts, conditioned drug craving and withdrawal elicited by drug-related cues, such as drug paraphernalia and environments in which drugs were taken, contribute to ongoing drug use and relapse after periods of abstinence [1]. Methods of reducing these responses have been considered as possible therapeutic approaches. One such method is extinction, which involves exposure to a cue (called a conditioned stimulus or CS) in the absence of the event with which it was once paired (called an unconditioned stimulus or US) until the conditioned response (CR) declines in frequency and/or amplitude [2].

Extinction is a useful clinical tool for the treatment of anxiety disorders. Called cue exposure therapy (CET), it involves exposing a patient to a feared object or situation (CS) in the absence of any aversive event (US) until the fear response (CR) declines [3]. Basic research on fear extinction in animal models has revealed that it is dependent on N-methyl-D-aspartate (NMDA) receptors. Fear extinction is blocked by pre-extinction training administration of an NMDA receptor antagonist and facilitated by pre- or immediate post-extinction training administration of the NMDA receptor partial agonist D-cycloserine (DCS) [for review see 4]. Likewise, in humans, coupling DCS with CET enhances the efficacy of this form of treatment [5].

By contrast, CET for addiction (in which addicts are exposed to drug-related cues in the absence of drug administration or acute withdrawal) generally seems to be ineffective [6]. The possibility of increasing its efficacy by coupling it with DCS has been explored in a number of recent studies, but the results have been disappointing [7]. This is despite the fact that DCS enhances extinction of drug-related CRs in animal models [7]. The reasons for the apparent disconnect between the human and animal addiction literatures, and between the human anxiety and addiction literatures, are unclear.

Understanding of this issue likely would be enhanced by increased understanding of the mechanisms underlying extinction of drug-related CRs as compared to fear CRs. To this end, we have begun an investigation of the neurobiology of extinction of conditioned opiate withdrawal in rats. To study conditioned opiate withdrawal, we first induce opiate dependence in rats by implanting slow-release, subcutaneous morphine pellets. We then use a place conditioning paradigm to teach the rats to associate a cue (a chamber with a distinctive floor texture) with naloxone-induced withdrawal and another cue (a chamber with a different floor texture) with no elicited state. To extinguish the association we expose the rats to both chambers repeatedly without inducing withdrawal. Finally, we test the rats by allowing them access to both chambers and recording the amount of time they spend in each one. Nonextinguished rats avoid the naloxone-paired floor (i.e., show a conditioned place aversion or CPA), spending more time on the opposite floor. By contrast, extinguished rats spend approximately equal amounts of time on the two floors [8, 9]. Opiate withdrawal-induced CPAs are long-lasting in the absence of extinction training [9, 10], indicating that the loss of CPA in extinguished rats is due to learning, not forgetting.

In the present study we examined whether extinction of conditioned opiate withdrawal could be blocked by pre-extinction training administration of the NMDA receptor antagonist 2-

amino-5-phosphonovalerate (AP5). Because AP5 does not cross the blood-brain barrier, it must be administered intracerebroventricularly (i.c.v.). We trained rats to acquire a CPA as just described, then administered AP5 or vehicle prior to re-exposing the rats to the place conditioning boxes (extinction groups) or in the absence of re-exposure (no extinction groups). Finally, we tested the rats for CPA. We predicted that AP5 would block extinction of conditioned opiate withdrawal.

Material and Method

Animals

Male Sprague-Dawley rats (250 g upon arrival; Charles River Laboratories, Raleigh, NC) were housed in a climate-controlled animal facility on a 12 h light:dark schedule (lights on at 0700 h) with *ad libitum* access to rat chow and water. Rats were housed in groups of four in standard plastic tub cages.

Cannula Implantation

After 6–8 d of habituation to the animal colony, rats were implanted with a single guide cannula targeting the right lateral ventricle. Rats were anesthetized with a ketamine (80 mg/mL)/xylazine (12 mg/mL) cocktail (Sigma-Aldrich, St. Louis, MO) administered intraperitoneally (i.p.) at a volume of 1 mL/kg and were mounted in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). A 22-ga guide cannula (Plastics One, Roanoke, VA) was implanted (coordinates relative to Bregma: AP –0.5 mm, ML –1.5 mm, DV –2.7 mm) and secured to the skull with dental cement anchored by skull screws. To maintain guide cannula patency, a stainless steel wire (014BSH-2.5; Plastics One) cut flush with the guide was inserted. To permit group housing of rats post-surgery, a stainless steel nylock nut (6–32; Small Parts, Inc., Logansport, IN) was screwed over the exposed portion of the cannula pedestal. Rats recovered for 5–7 d prior to behavioral training.

Morphine Pellet Implantation

Three d prior to behavioral training, rats were implanted subcutaneously (s.c.) with two 75-mg morphine pellets (National Institute on Drug Abuse [NIDA], Bethesda, MD) under isoflurane anesthesia [9]. These pellets slow-release morphine continuously for a period of 14 d [11].

Apparatus

The place conditioning apparatus has been described in detail elsewhere [9]. Briefly, the apparatus consisted of four black Plexiglas boxes (50 × 15 × 18 cm), each of which could be subdivided widthwise into two equally-sized chambers by a removable partition. The chambers were distinguished by floor texture: perforated (16-ga) stainless steel with 13-mm round holes on 19-mm staggered centers (“hole”) or 1/8” stainless steel rods spaced 7 mm apart (“grid”). The boxes were placed on a cart and positioned below a video camera mounted to the ceiling of the testing room. Each box had a clear Plexiglas lid. The testing room was illuminated by red light.

Behavioral Training and Testing

The behavioral training and testing protocol has been described in detail elsewhere [9]. Briefly, rats underwent 2 d of acquisition, 3 d of extinction training, and a single test. Rats were assigned pseudorandomly to extinction/vehicle, extinction/AP5, no extinction/vehicle, and no extinction/AP5 groups, with the restriction that the hole floor was naloxone-paired for half the rats in each group.

On each of the 2 d of acquisition, rats were injected s.c. with saline and immediately placed in one of the chambers of a place conditioning box for 1 h. Two to 3 h later, rats were injected s.c. with naloxone (15 µg/kg; saline vehicle; Sigma-Aldrich) and placed in the opposite chamber for 1 h. Assignment of grid and hole floors as saline- and naloxone-paired was counterbalanced across rats.

On each of the 3 d of extinction training, the extinction/vehicle and extinction/AP5 groups received an infusion of AP5 or vehicle (see below) and were immediately placed in one of the chambers of a place conditioning box for 30 min. Two to 3 h later, they received a second infusion of AP5 or vehicle and were placed in the opposite chamber for 30 min. The order in which the rats were exposed to the chambers (formerly saline- or naloxone-paired first) was counterbalanced across rats and reversed on each day. The no extinction/vehicle and no extinction/AP5 groups received infusions at the same times as the extinction/vehicle and extinction/AP5 groups but were returned to their home cages immediately thereafter.

On the testing day the partitions were removed from the place conditioning boxes. Rats were placed in the center of a box at the junction of the two floor types and freely explored for 30 min. Their behavior was recorded by a video camera and scored using EthoVision software (Noldus Information Technology, Wageningen, The Netherlands). The measure of interest was the time (sec) spent on the formerly saline- and naloxone-paired floor types.

Drug Infusion

AP5 (5 µg/3 µL) was dissolved in artificial cerebrospinal fluid (ACSF) and the pH was adjusted to ~7.3. Immediately prior to infusions, the nut and wire were removed from the guide cannula and a 28-ga injector (Plastics One) cut to extend 1 mm beyond the tip of the guide was inserted. The injector was attached via PE-20 tubing to a Hamilton syringe (Hamilton, Reno, NV) mounted in an infusion pump (World Precision Instruments, Sarasota, FL). AP5 or vehicle was infused at a volume of 3 µL and a rate of 3 µL/min. After the infusion was complete, the injectors were left in place for 1 min, then the rats were immediately placed in the place conditioning boxes.

Histology

After the experiment was complete, the rats were deeply anesthetized with sodium pentobarbital (Sigma-Aldrich) and perfused transcardially with saline followed by 4% paraformaldehyde. The brains were removed and stored overnight at 4°C in 4% paraformaldehyde. The brains were then transferred to a 30% sucrose solution and stored at 4°C for a minimum of 2 d prior to sectioning on a microtome. Fifty micrometer sections were taken through the region of interest, mounted on Superfrost Plus slides (Fisher

Scientific, Waltham, MA), and air dried. The sections were stained with cresyl violet and coverslipped with Permount (Fisher). Placement of cannula tips in the ventricle was confirmed under a microscope. Rats with inaccurate cannula placements were excluded.

Data Analysis

The data are presented in two ways: as time (sec) spent on the formerly naloxone- and saline-paired floors during the 30-min test session, and as change scores, defined as time spent on the formerly naloxone-paired floor minus time spent on the formerly saline-paired floor. The time spent data were analyzed with a mixed-model ANOVA with group as a between-subjects factor and floor (formerly naloxone- or saline-paired) as a repeated measure. Post-hoc t-tests were used to compare the time spent on the formerly naloxone- and saline-paired floors within each group. The change score data were analyzed with a one-way ANOVA followed by post-hoc t-tests. Statistical significance was set at $p < 0.05$.

Results

Cannula placements were verified in all rats. A photomicrograph showing cannula placement in a representative rat is presented in Figure 1.

As expected, the test performance (change scores) of the no extinction/vehicle and no extinction/AP5 groups was similar [$t(6) = 1.53$; $p = 0.177$]. The data from these groups were pooled for further analyses.

Figure 2 presents the data from the test session. As can be seen in the top panel of Figure 2, the no extinction group ($n = 8$) showed a robust CPA whereas the extinction/vehicle group ($n = 6$) did not, indicating that extinction was successful. The extinction/AP5 group ($n = 9$) showed a strong CPA similar to that seen in the no extinction group. Consistent with these observations, there was a significant floor $\times$ group interaction [$F(2,20) = 3.942$; $p = 0.036$]. The main effect of floor was also significant [$F(1, 20) = 12.14$; $p = 0.002$] but the main effect of group was not ($F < 1$). Paired t-tests comparing the time spent on the naloxone- and saline-paired floors within each group indicated that the no extinction group [$t(7) = -3.275$; $p = 0.014$] and the extinction/AP5 group [$t(8) = -5.171$; $p = 0.001$] showed significant CPAs but the extinction/vehicle group did not [$t(5) = .217$; $p = 0.837$].

The magnitudes of the place aversions shown by the groups were compared through an analysis of change scores, as shown in the bottom panel of Figure 2. A one-way ANOVA on these data revealed a significant main effect of group [$F(2) = 3.942$; $p = 0.036$]. Post-hoc t-tests indicated that both the no extinction group [$t(12) = 2.29$; $p = 0.041$] and the extinction/AP5 group [$t(15) = -1.04$; $p = 0.320$] showed a significantly larger place aversion than did the extinction/vehicle group. The place aversions shown by the no extinction and extinction/AP5 groups were of similar magnitude [$t(15) = -1.04$; $p = 0.320$].

Discussion

We found that i.c.v. administration of the NMDA receptor antagonist AP5 prior to extinction training blocks extinction of conditioned opiate withdrawal as measured in a rat

CPA paradigm. This finding is consistent with a previous observation from our laboratory that pre-extinction training, systemic administration of the NMDA receptor partial agonist DCS facilitates extinction in this paradigm [8]. It also is consistent with reports from other groups that extinction of conditioned drug-seeking behavior and extinction of conditioned fear are NMDA receptor-dependent [for review see 4].

It is unclear where the critical NMDA receptors are located, but the basolateral amygdala (BLA) and infralimbic region of medial prefrontal cortex (IL) are likely candidates. The roles of these structures in fear extinction have been studied extensively. BLA is a critical locus of plasticity underlying fear memory, and IL contributes to the consolidation and expression of fear extinction, perhaps by activating BLA GABAergic interneurons and GABAergic intercalated cell masses lying between BLA and the central nucleus of the amygdala [for review see 4]. NMDA receptors within both BLA and IL are critical for fear extinction, as indicated by findings that pre- or immediate post-extinction training infusion of AP5 or DCS directly into BLA impairs and facilitates fear extinction, respectively, and that pre-extinction training infusion of the NR2B-subunit-containing NMDA receptor antagonist ifenprodil directly into IL impairs fear extinction [for review see 4]. The neurobiology of extinction of drug-related CRs is not nearly as well understood as that of fear extinction, but there is some evidence that this same general framework is relevant. For example, immediate post-extinction training, intra-BLA infusion of AP5 impairs extinction of cue-induced reinstatement of cocaine-seeking behavior [12]; immediate post-extinction training, intra-BLA infusion of DCS facilitates extinction of a cocaine conditioned place preference [13]; and pre-extinction training infusion of AP5 into medial prefrontal cortex impairs extinction of an amphetamine conditioned place preference [14]. To determine the contribution of NMDA receptors within these two regions in extinction of conditioned opiate withdrawal in our paradigm, future experiments involving localized infusions of AP5 into BLA and IL will be useful.

A possible alternative interpretation of our findings is that AP5 does not modulate extinction *per se*, but instead counterconditions a competing CR (approach towards the formerly naloxone-paired floor) due to an inherent rewarding effect. Indeed, there is some evidence that NMDA receptor antagonists can be rewarding [e.g., 15]. However, we do not believe that this mechanism is likely to explain our observations because we administered AP5 prior to exposures to both chambers, rather than just the formerly naloxone-paired chamber, during extinction training.

The NMDA receptor-dependence of extinction of drug-related CRs, including conditioned opiate withdrawal, may have clinical implications. The finding that fear extinction is NMDA receptor-dependent led to the development of DCS as an adjunct to CET for anxiety disorders, which has been used with much success [16]. There has been interest in using a similar strategy to target drug-related CRs contributing to relapse in addicts, although the results of clinical studies on DCS-coupled CET for addiction generally have not been supportive [for review see 7], and other approaches, such as memory reconsolidation blockade, are being explored actively as well [17]. Improved understanding of the behavioral features and neurobiological underpinnings of extinction of drug-related CRs as

compared to fear CRs may lead to enhanced CET protocols and/or more effective pharmacological adjuncts to CET for addiction.

Conclusions

Extinction of conditioned opiate withdrawal is NMDA receptor-dependent, similar to extinction of conditioned drug-seeking behavior and conditioned fear. BLA and IL are possible loci of the critical NMDA receptors.

Acknowledgments

This work was supported by the National Institute on Drug Abuse (DA027752 to K.M.M.) and the National Institute of Mental Health (MH063266 to W.A.C.). We thank Sabina Berretta, Rebecca Misra, Harry Pantazopoulos, and Ashlee Van't Veer for their assistance.

References

1. Weiss F. Neurobiology of craving, conditioned reward and relapse. *Curr Opin Pharmacol.* 2005; 5:9–19. [PubMed: 15661620]
2. Pavlov, IP. *Conditioned Reflexes: an investigation of the physiological activity of the cerebral cortex.* Oxford University Press; London: 1927.
3. Rothbaum BO, Hodges LF. The use of virtual reality exposure in the treatment of anxiety disorders. *Behav Modif.* 1999; 23:507–25. [PubMed: 10533438]
4. Myers KM, Carlezon WA Jr, Davis M. Glutamate receptors in extinction and extinction-based therapies for psychiatric illness. *Neuropsychopharmacology.* 2011; 36:274–93. [PubMed: 20631689]
5. Norberg MM, Krystal JH, Tolin DF. A meta-analysis of D-cycloserine and the facilitation of fear extinction and exposure therapy. *Biol Psychiatry.* 2008; 63:1118–26. [PubMed: 18313643]
6. Conklin CA, Tiffany ST. Applying extinction research and theory to cue-exposure addiction treatments. *Addiction.* 2002; 97:155–67. [PubMed: 11860387]
7. Myers KM, Carlezon WA Jr. D-cycloserine effects on extinction of conditioned responses to drug-related cues. *Biol Psychiatry.* 2012; 71:947–55. [PubMed: 22579305]
8. Myers KM, Carlezon WA Jr. D-cycloserine facilitates extinction of naloxone-induced conditioned place aversion in morphine-dependent rats. *Biol Psychiatry.* 2010; 67:85–7. [PubMed: 19782965]
9. Myers KM, Bechtholt-Gompf AJ, Coleman BR, Carlezon WA Jr. Extinction of conditioned opiate withdrawal in rats in a two-chambered place conditioning apparatus. *Nat Protoc.* 2012; 7:517–26. [PubMed: 22362157]
10. Stinus L, Caille S, Koob GF. Opiate withdrawal-induced place aversion lasts for up to 16 weeks. *Psychopharmacology (Berl).* 2000; 149:115–20. [PubMed: 10805605]
11. Gold LH, Stinus L, Inturrisi CE, Koob GF. Prolonged tolerance, dependence and abstinence following subcutaneous morphine pellet implantation in the rat. *Eur J Pharmacol.* 1994; 21:45–51. [PubMed: 8013548]
12. Feltenstein MW, See RE. NMDA receptor blockade in the basolateral amygdala disrupts consolidation of stimulus-reward memory and extinction learning during reinstatement of cocaine-seeking in an animal model of relapse. *Neurobiol Learn Mem.* 2007; 88:435–44. [PubMed: 17613253]
13. Botreau F, Paolone G, Stewart J. D-cycloserine facilitates extinction of a cocaine-induced conditioned place preference. *Behav Brain Res.* 2006; 172:173–8. [PubMed: 16769132]
14. Hsu E, Packard MG. Medial prefrontal cortex infusions of bupivacaine or AP-5 block extinction of amphetamine conditioned place preference. *Neurobiol Learn Mem.* 2008; 89:504–12. [PubMed: 17905604]

15. Carlezon WA Jr, Wise RA. Microinjections of phencyclidine (PCP) and related drugs into nucleus accumbens shell potentiate medial forebrain bundle brain stimulation reward. *Psychopharmacology (Berl)*. 1996; 128:413–20. [PubMed: 8986012]
16. Davis M. NMDA receptors and fear extinction: implications for cognitive behavioral therapy. *Dialogues Clin Neurosci*. 2011; 13:463–74. [PubMed: 22275851]
17. Taylor JR, Olausson P, Quinn JJ, Torregrossa MM. Targeting extinction and reconsolidation mechanisms to combat the impact of drug cues on addiction. *Neuropharmacology*. 2009; 56(Suppl 1):186–95. [PubMed: 18708077]

Highlights

- The neurobiology of extinction of cue-induced drug withdrawal is poorly understood.
- We studied morphine withdrawal-induced conditioned place aversion in rats.
- We found that extinction in this paradigm is dependent upon brain NMDA receptors.
- The specific locus of the critical NMDA receptors is not yet known.

Figure 1. Photomicrograph showing guide cannula placement in a representative rat. The right lateral ventricle was targeted. Abbreviation: lv, lateral ventricle.

Figure 2.

Top panel. Time (sec) spent by each group on the formerly naloxone- and saline-paired floors in the 30-min test session. *Bottom panel.* Test session data represented as change scores, defined as time spent on the formerly naloxone-paired floor minus time spent on the formerly saline-paired floor. Abbreviations: ext, extinction; nalox, naloxone; veh, vehicle. * $p < 0.05$, ** $p < 0.01$.