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Extinction of conditioned opiate withdrawal in rats in a two-chambered place conditioning apparatus

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Abstract

Conditioned opiate withdrawal contributes to relapse in addicts and can be studied in rats using the opiate withdrawal-induced conditioned place aversion (OW-CPA) paradigm. Attenuation of conditioned withdrawal through extinction may be beneficial in the treatment of addiction. Here we describe a protocol for studying OW-CPA extinction using a two-chambered place conditioning apparatus. Rats are made dependent on morphine through subcutaneous implantation of morphine pellets and then trained to acquire OW-CPA through pairings of one chamber with naloxone-precipitated withdrawal and the other chamber with saline. Extinction training consists of re-exposures to both chambers in the absence of precipitated withdrawal. Rats tested following the completion of training show a decline in avoidance of the formerly naloxone-paired chamber with increasing numbers of extinction training sessions. The protocol takes a minimum of seven days; the exact duration varies with the amount of extinction training, which is determined by the goals of the experiment.

INTRODUCTION

Conditioned opiate withdrawal

People use drugs initially for their positive reinforcing effects, but as dependence progresses, negative reinforcement (alleviation of withdrawal symptoms) is thought to take precedence as a primary motivator of continuing drug use¹. Even after abstinence has been achieved and acute withdrawal has subsided, addicts are prone to relapse. One of the factors thought to be responsible for this vulnerability is conditioned withdrawal, defined as elicitation of somatic and affective withdrawal signs in the presence of cues previously paired with drug

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AUTHOR CONTRIBUTION STATEMENTS

K.M.M. developed the concept, designed the experiments, collected data, analyzed the data, and wrote the paper; A.J.B.-G. designed the apparatus, gathered pilot data, and edited the manuscript; B.R.C. collected data; and W.A.C., Jr., developed the concept and edited the manuscript.

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use or withdrawal itself ^{2,3}. Addicts rate drug cues as one of the biggest contributors to drug craving, even more so than negative mood states or a desire to get high ⁴.

There are at least two ways in which drug cues can come to elicit conditioned withdrawal. First, to the extent that drug-taking occurs to alleviate withdrawal symptoms, withdrawal can become associated directly with cues such as drug paraphernalia. This type of conditioned withdrawal was emphasized in early accounts of addiction ⁵. Second, withdrawal can be elicited by drug cues as a conditioned compensatory response; that is, a conditioned homeostatic mechanism that counters the effects of drugs about to be introduced into the system ⁶. In either case, conditioned withdrawal motivates drug seeking to alleviate those symptoms, just as acute withdrawal motivates continuing drug use in active drug users.

Conditioned withdrawal can be modeled in animals

Morphine-dependent animals exposed to a novel cue or context while undergoing withdrawal subsequently exhibit conditioned withdrawal in the presence of that cue or context, which can be measured in several ways. These include modulation of operant responding for food, morphine, heroin, or brain stimulation reward ⁷⁻⁹ and avoidance of the withdrawal-paired cue or context ¹⁰⁻¹³. Avoidance of the withdrawal-paired cue is often demonstrated using opiate withdrawal-induced conditioned place aversion (OWCPA), which is a popular measure because of its simplicity. In this paradigm, a morphine-dependent rat is exposed to a two- or three-chambered apparatus for a period of time and its exploration of the chambers is recorded. Sometime later the rat is confined in one of the chambers, which are distinct from one another in features such as wall color or flooring, while undergoing withdrawal. Confinement in another chamber for the same amount of time in the absence of withdrawal is done to control for differences in preference based on exposure, such as effects of familiarity. When later given the opportunity to explore the apparatus freely once again, rats trained in this way tend to avoid the previously withdrawal-paired context, spending significantly less time there than in the other context. Conditioned withdrawal is defined operationally in terms of this difference in time spent in the withdrawal-paired chamber vs. the opposite chamber.

Studies of the neural substrates of acute opiate withdrawal in animals have identified important roles for the extended amygdala (central nucleus of the amygdala, bed nucleus of the stria terminalis, and nucleus accumbens shell) and basolateral amygdala ¹⁴⁻¹⁶. Conditioned opiate withdrawal in animals re-exposed to an environment previously paired with acute withdrawal engages much of this same circuitry ^{15,17}. The basolateral and extended amygdala are substrates of fear, anxiety, stress, and aversive motivation ^{18,19}, and the nucleus accumbens is involved in dysphoria and anhedonia associated with addiction ²⁰. Recruitment of this “anti-reward” system ²¹ during the development of addiction ¹ results in a withdrawal state characterized by anxiety, dysphoria, and drug craving. By re-engaging this circuitry, conditioned withdrawal produces many of these same effects. In this way, drug- and withdrawal-paired cues contribute to relapse even in long-abstinent addicts, just as acute withdrawal is a powerful motivator of continuing drug use in active drug users.

What is extinction?

From a clinical standpoint, alleviating conditioned opiate withdrawal could benefit addicts seeking to achieve or sustain abstinence. Conditioned withdrawal is a Pavlovian conditioned response (CR), a type of response that is acquired through exposure to contingent pairings of an initially neutral cue (called a conditioned stimulus or CS) with a biologically significant event (called an unconditioned stimulus or US). One method of reducing all types of Pavlovian conditioned responses is extinction. In extinction, repeated or prolonged exposure to the CS in the absence of the US it previously predicted results in a decline in the conditioned response (CR) ²². Extinction is a phenomenon distinct from forgetting, as it requires exposure to the CS in the absence of the US as opposed to the simple passage of time. Extinction also is different from “unlearning,” as indicated by recovery of extinguished CRs under some circumstances ²³. Instead, extinction involves a suppression or inhibition of the CR ²⁴.

In the OW-CPA paradigm, extinction occurs either when animals are tested repeatedly or when they are confined repeatedly in the formerly withdrawal- and saline-paired chambers and then tested in a separate session. Extinction of OW-CPA is complete when animals no longer avoid the previously withdrawal-paired compartment.

Why study extinction of opiate withdrawal-induced conditioned place aversion?

Clinical researchers have examined the possibility of using extinction as a component of addiction treatment. Called cue exposure therapy, the protocol involves exposing addicts to drug cues in the absence of drug administration or acute withdrawal in an attempt to weaken the association between the cues and these states. Early studies of the efficacy of cue exposure therapy in reducing conditioned opiate withdrawal in addicts were promising ², but subsequent studies have met with limited success, and a meta-analysis of the literature through 2001 revealed no significant effect²⁵. It has been suggested that basic laboratory research on extinction might lead to improvements in cue exposure protocols that will increase the success rate of this form of therapy ²⁵. Consistent with this, research on the neurobiology of fear extinction in animals has led to advances in the use of exposure therapy to treat fear and anxiety disorders ²⁶. Analogous studies on drug cue extinction could have a similar effect ^{27,28}.

There already is some evidence that this is the case. For example, drawing from the aforementioned literature on fear extinction, we recently examined the effect of systemic, pre-extinction training administration of the NMDA receptor partial agonist D-cycloserine (DCS) on OW-CPA extinction in rats and found that it facilitates extinction learning ²⁸. Others have reported similar effects of DCS on extinction of cocaine conditioned place preference ^{29,30}. Intriguingly, a clinical study examining the utility of DCS as an adjunct to exposure therapy for nicotine addiction in cigarette smokers reported that participants who were given DCS prior to cue exposure therapy sessions showed facilitated extinction of two separate measures of cue-elicited craving as compared to a placebo-treated control group ³¹.

Our opiate withdrawal-induced conditioned place aversion extinction protocol

There are a number of possible ways of conducting OW-CPA extinction experiments^{32,33}. Our protocol has evolved since our initial studies²⁸ as we have learned more about the behavior, identified certain technical problems in measuring it, and optimized our experimental conditions accordingly.

The most significant change we have made is in our choice of place conditioning apparatus. In our earlier work we used a three-chambered apparatus in which two equally-sized conditioning chambers (which serve as the withdrawal-paired environment and the neutral environment) are separated by a smaller “start box” into which a rat is placed at the beginning of a test session. A problem inherent in the use of this type of apparatus is that rats spend some of the test session in the start box rather than the conditioning chambers. In OW-CPA experiments we have found that rats will spend as much as 50% of a test session in the start box after conditioning, even though the area of this box comprises only about 20% of the total area of the apparatus. We have found this “start box effect” to be episodic, appearing in some cohorts of animals but not others. This effect may be related to stress, since its magnitude is increased following restraint stress in animals that had undergone OW-CPA extinction (Myers and Carlezon, unpublished observations). The list of potential sources of stress that may wax and wane is long and includes often-uncontrollable factors that may be related to the commercial supplier, shipping, season, and local conditions (e.g., noise or smells during construction, renovation, or repairs; fire alarms, etc.). The start box effect also tends to become more pronounced with repeated testing, which is particularly troublesome in OW-CPA extinction experiments involving multiple tests (e.g., pretest, post-acquisition test, and post-extinction tests). Regardless, it is problematic because it artificially decreases the differential in time spent between the withdrawal-paired and neutral environments, since so much of the session time is spent outside of either chamber. It is possible to minimize its prominence by modifying the way in which the data are expressed; for example, by expressing the time spent in the withdrawal-paired chamber as a percentage of the total time spent in the withdrawal-paired and neutral chambers, but this is unsatisfactory, particularly if the start box effect dwarfs effects noted in these chambers, increases in magnitude across tests, or differs in magnitude between or among experimental groups.

To circumvent the problem altogether, we have begun using a different place conditioning apparatus modeled after one used extensively by Cunningham and colleagues³⁴. These boxes differ from our three-chambered boxes in two major respects: they are two-chambered boxes that lack a start box, and the chambers are distinguished from one another solely on the basis of floor texture (Figure 1). The apparatus is unbiased in the sense that there is no overall preference for one floor texture vs. the other in a group of naïve rats, although individual rats do tend to spend more time on one or the other floor type (Supplementary Figure 1A). In the large majority of rats, this initial apparent preference is not consistent when the rats are tested repeatedly (Supplementary Figure 1B). The lack of a consistent preference indicates that pre-testing rats prior to conditioning (a common first step in place conditioning experiments) is unnecessary, since any preference is observed in each rat during this test is not likely to be meaningful. Because the initial preference is not consistent

across tests inclusion of these data in the analyses actually increases (rather than decreases) the variability of the data.

Another modification we have made to our experimental protocol is in the parameters of OW-CPA acquisition training. Specifically, we have increased the intensity of training by running two sessions (each involving one pairing of one floor type with saline and the other floor type with naloxone) rather than just one. Rats trained in this way show robust, consistent place aversions that are relatively resistant to extinction, allowing us to titrate the pace of extinction through appropriate adjustments in the extinction training parameters. Our dose of naloxone is quite low (15 µg/kg) and was chosen because it elicits affective/motivational symptoms of withdrawal with few somatic symptoms¹⁶ and conditions significant place aversions in morphine-dependent rats but not in nondependent controls³⁵.

For OW-CPA extinction training, we repeatedly confine rats in the formerly saline- and naloxone-paired boxes in the absence of withdrawal. We prefer this method compared to testing rats repeatedly, for two reasons. First, we are able to control the amount of exposure to the formerly saline- and naloxone-paired environments, whereas with repeated testing the amount of exposure differs from rat to rat based on each rat's behavior. Second, we have found that in the two-chambered apparatus, there is a steady increase in the magnitude of the preference for one floor over the other with repeated testing (Supplementary Figure 1C). In other words, rats continue to show apparently random behavior with regard to which floor is preferred in any individual test, but the magnitude of that preference (measured as the amount of time spent on the preferred floor minus the amount of time spent on the non-preferred floor) increases over test sessions, suggesting that rats become less apt to explore as they are tested repeatedly. Hence, we have adopted a protocol in which rats are trained to acquire OW-CPA, then are exposed to a set number of extinction training sessions or no further training, and finally are given a single test session. The experimental design is entirely between-groups, thereby eliminating any measurement artifact associated with repeated testing.

MATERIALS

REAGENTS

- Sprague-Dawley rats (theoretically, any strain of rats can be used, although if using an automated scoring system, the color of the chambers may need to be altered to provide adequate contrast with the natural coloring of the strain; see below).

CAUTION: Experimenters must follow national and institutional guidelines for the care and use of laboratory animals, including local requirements for surgical anesthetic.

CRITICAL: Use of adult animals weighing 225-250 g upon arrival is recommended, since temporal changes in naloxone-induced withdrawal indices and plasma concentrations of morphine following implantation of two, 75-mg pellets are well-characterized in rats of this size³⁶.

- Isoflurane (Abbott Laboratories; Butler Schein Animal Health, Albany, NY; catalog number 037375)
- Morphine pellets (75 mg base/pellet; NIDA Drug Supply Program, National Institute on Drug Abuse, Bethesda, MD)
- Naloxone hydrochloride dihydrate (Sigma-Aldrich, St. Louis, MO; catalog number N7758)

EQUIPMENT

- Four identical place conditioning boxes (50 × 15 × 18 cm) without attached floors or lids, constructed of 1/2" black Plexiglas (Figure 1, panel A1). Two strips of black Plexiglas are adhered to the interior center of each long side ~3/8" apart (Figure 1, panel A4).
- Eight distinctively-textured floors mounted to Plexiglas frames (27 × 19 × 2 cm) (Figure 1, panel A2). Grid floors (four total) consist of equally-spaced (7 mm center-to-center) 1/8" stainless steel rods. Hole floors (four total) consist of perforated (16-gauge) stainless steel with 13-mm round holes on 19-mm staggered centers. All floors are painted black.
- Four lids (53 × 17 cm) constructed of 1/2" clear Plexiglas (Figure 1, panel A3).
- Four dividers (15 × 18 cm) constructed of 1/4" black Plexiglas (Figure 1, panel A5).
- A single sheet of 1/4" black Plexiglas (35" × 23")
- Video camera
- Television
- DVD recorder
- Optional: computer and software to automate scoring (e.g., Ethovision, Noldus, Wageningen, the Netherlands)
- Veterinary anesthesia machine (e.g., Paragon Medical; Coral Springs, FL)
- Anesthesia induction chamber (e.g., Paragon Medical; Coral Springs, FL)
- Surgical supplies (alcohol wipe prep pads, antibiotic cream, hemostat, scalpel, scalpel blades, surgical stapler, surgical staples, wound spreader); depending upon the institution, sterile supplies and post-operative analgesics may be required
- Nylon stocking
- Surgical silk suture thread (4/0; e.g., Fine Science Tools, Inc.; Fisher Scientific, Pittsburgh, PA; catalog number NC9483190)
- Syringes, 1 cc (e.g., BD Biosciences; Fisher; catalog number 309602)
- Needles, 26 gauge (e.g., BD Biosciences; Fisher; catalog number 305115)

EQUIPMENT SETUP

Morphine pellet preparation—Prepare pellets for implantation by wrapping them in strips of nylon stocking in packets of two and tying the packets closed with surgical thread. This process is helpful in quickly and simultaneously placing two pellets in a single rat. It also facilitates the removal of the pellets if required for experimental designs other than those described here.

Chambers—Assemble the place conditioning chambers on a cart atop a single sheet of black Plexiglas. Position the cart beneath a video camera affixed to the ceiling of the room. Place each box on top of two floors such that one half of the total floor space is of one texture (grid) and the other half is of the other texture (hole). The orientation of the floor types alternates from one chamber to the next (Figure 1B, 1C). For OW-CPA acquisition and extinction training, slide the dividers into the tracks created by the strips of Plexiglas adhered to the inside of the boxes. The dividers should cleanly divide the boxes into two equally-sized compartments, each with its own floor type. The dividers are not used during testing. Use the clear Plexiglas lids to cover the chambers once the rats have been placed inside the boxes.

Room setup—Run experiments involving Sprague Dawley rats under red light conditions to maximize the contrast of the white animals against the black background and minimize glare (Figure 1D). (For experiments involving darkly-colored strains against a light background, standard room lighting may be preferable.) Output from the video camera may be split via a BNC T-adapter splitter and inputted to both a DVD recorder and a computer. Couple the DVD recorder with a television such that the camera's view is visible on the screen.

Software—Use any of several commercially-available software packages for automated scoring of test sessions. We use Ethovision, which tracks the rats' trajectories and computes the amount of time spent on each floor type. To accomplish this, we input the following information into the software: (1) Whether the animals are dark against a light background or light against a dark background. There is a contrast setting that can be adjusted to maximize contrast. (2) The number of trials. (3) The number of animals to be tracked. (4) The location of the tracking "arenas." Under our conditions, there are four arenas, each one corresponding to one of the place conditioning chambers. Each arena is scored separately. The arena locations are set by capturing a screen shot of the overhead view of the chambers from the video camera, then using a tool in the software to outline the chambers on the computer screen. (5) The location of the floor types. These "zones" (regions of interest within arenas) are outlined on the computer screen in much the same way as are the arenas. (6) Identification of the background. By capturing another image of the chambers with the lids in place and the room lighting set as it would be for running animals, the software can be instructed to identify this as background, facilitating discrimination of the rats from the background while tracking. (7) How to collect the data. The software is able to track any number of variables; of most interest in OW-CPA extinction experiments is in the time spent on each of the two floor types (i.e., in the two zones of each arena) by each animal.

PROCEDURE

Preliminary procedures—1. Following arrival of the animals at the colony, leave them to acclimate. We recommend acclimation of 7-10 d prior to morphine pellet implantation.

Pellet implantation surgery—2. Anesthetize each rat individually with isoflurane anesthesia administered via a vaporizer into a small rodent anesthesia induction chamber. Monitor the level of anesthesia via toe pinch and re-anesthetize as necessary. The anesthesia protocol should be tailored to meet institutional requirements.

3. Shave a patch of fur about 1" × 1" between the scapulae of the rat.
4. Sanitize the area using betadine and alcohol wipe prep pads.
5. Using a scalpel, make a small (~1/2") incision between the scapulae.
6. Insert a wound spreader into the incision, oriented towards the tail of the rat, and create a subcutaneous pocket ~2" away from the incision site. Remove the wound spreader.
7. Using a hemostat, insert the pellet packet deep into the pocket created in step 6. Using a free hand, gently hold the packet in place through the skin and remove the hemostat. The nylon wrapping of the pellet packet (see REAGENT SETUP) facilitates the process of pellet implantation.
8. Using a wound stapler, close the incision with 2-3 surgical staples. Attach the staples firmly to prevent the rats from pulling them out.
9. Cover the incision site with topical antibiotic cream.
10. Return the rats to their living environment. Rats can be group-housed after surgery.
11. Allow the animals to recover for 3 d following pellet implantation. During this time, monitor recovery according to institutional requirements.

CRITICAL STEP: Recovery time should be no less than 3 d to allow plasma concentrations of morphine to peak ³⁶. The "lifespan" of the pellets is ~2 wks, beginning at implantation ³⁶.

TROUBLESHOOTING

OW-CPA acquisition training—12. Randomly assign rats to chambers, grid+ and hole + conditions, and experimental groups. Each rat should be run in a single chamber for the duration of the experiment. Rats in the grid+ condition are trained to associate the grid floor with morphine withdrawal, and rats in the hole+ condition are trained to associate the hole floor with morphine withdrawal. Experimental groups should contain equal numbers of rats assigned to each chamber and to grid+ and hole+ conditions (Supplementary Figure 2). Keep rats in their home cages in a room separate from the testing room when not being trained.

CRITICAL STEP: Inclusion of a “no extinction” control group in every experiment is recommended to establish that rats acquired OW-CPA prior to extinction training.

CRITICAL STEP: Training occurs over two successive days, each involving one exposure to the saline-paired floor of the assigned chamber and one exposure to the naloxone-paired floor.

13. Put the chamber dividers in place. Inject two rats (one from each floor type group) subcutaneously (SC) with 0.9% saline and place immediately and simultaneously into the chambers (Supplementary Figure 3). Put the chamber lid in place immediately after placing the rats in the chambers. Continue injecting rats in pairs and placing them into the chambers until all chambers are loaded.

14. Leave rats exposed to the saline-paired floor for 1 hr. After the exposure is complete, remove rats from the chambers and return to their home cages in a room separate from the testing room.

15. Two to three hrs later, inject rats SC with 15 µg/kg naloxone HCl and place immediately on the naloxone-paired floor of their assigned chamber for 1 hr, following the same general protocol as was just described for the saline-paired floor (step 13).

CRITICAL STEP: Because the saline and naloxone pairings are separated by only 2-3 hrs, it is important to do the saline pairing first and the naloxone pairing second on each day. Otherwise, there is the risk that the rats will be experiencing residual symptoms of morphine withdrawal during their exposure to the saline-paired floor. This could interfere with conditioning.

16. Return rats to their home cages overnight.

OW-CPA extinction training—17. Put the chamber dividers in place. Inject rats SC with 0.9% saline and place immediately into the chambers, following the same general protocol as described in step 13 for OW-CPA acquisition (Supplementary Figure 3).

CRITICAL STEP: Extinction training occurs over as many successive days as is optimal for the purposes of a particular experiment. For example, one day of extinction training (leading to suboptimal extinction) may be used in experiments involving a manipulation predicted to facilitate extinction, whereas multiple days of extinction training (leading to complete extinction) may be used in experiments involving a manipulation predicted to impair extinction.

CRITICAL STEP: Unlike OW-CPA acquisition training, in OW-CPA extinction training the order of exposures to the formerly saline-paired and naloxone-paired floors is counterbalanced across rats and reversed on each successive extinction training day.

CRITICAL STEP: As with OW-CPA acquisition training, rats should be kept in their home cages in a room separate from the testing room when not being extinction trained.

18. Leave rats exposed to the saline-paired floor for 30 min. After the exposure is complete, remove rats from the chambers and return them to their home cages in a room separate from the testing room.

19. Two to three hrs later, inject rats again SC with 0.9% saline and place immediately on the opposite floor of their assigned chamber, for 30 min.

20. Return rats to their home cages overnight.

Testing—21. Remove the chamber dividers. Place rats into the chambers at the junction of the two floor types, facing away from the experimenter. Put the lids in place. Allow rats to explore the entire chamber freely for 30 min.

CRITICAL STEP: All rats are tested in a single session.

TROUBLESHOOTING

22. Score the test session in real time through the use of a dedicated software package such as Ethovision, as described in Equipment setup. Alternatively, record the session on a DVD-R for later automated or manual scoring. Even if using a real time automated scoring system, it is useful to record the session for backup data storage in the event of computer failure or to have the option of adding additional measures of potential interest such as distance traveled or number of entries into each side of the chamber).

TIMING

Step 1, preliminary procedures: 7-10 d

Steps 2-11, pellet implantation: ~5 min per rat, plus 3 d recovery

Steps 12-16, OW-CPA acquisition training: 2 hr per day per rat, for 2 d; up to eight rats can be run concurrently with four place conditioning chambers

Steps 17-20, OW-CPA extinction training: 1 hr per day per rat; number of days varies by experiment (generally 1-3 d); up to eight rats can be run concurrently with four place conditioning chambers

Steps 21-22, testing: 30 min per rat; up to four rats can be tested concurrently with four place conditioning chambers

TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

ANTICIPATED RESULTS

Express the raw data as time (seconds) spent on each floor type (grid or hole) by each rat. To analyze the data, these times should be converted into time spent on the formerly naloxone- and saline-paired floors for each individual rat based on its assignment to grid+ or hole+

conditions. Aversion scores can then be calculated by subtracting the time spent on the saline-paired floor from the time spent on the naloxone-paired floor for each rat. Negative aversion scores indicate that the rats spent less time on the formerly naloxone-paired floor than on the formerly saline-paired floor; positive aversion scores indicate that the rats spent more time on the formerly naloxone-paired floor than on the formerly saline-paired floor; and aversion scores near zero indicate that the rats spent approximately equal amounts of time on the two floors.

Typical behavior of normal (unmanipulated) rats trained as described is shown in Figure 2. There were eight groups in this experiment: two that were trained to acquire OW-CPA but not extinguished, and six that were trained to acquire OW-CPA and received differing amounts of extinction training (1-6 d) (Figure 2A). In general, group sizes for this type of experiment should be pre-determined based on power analyses, but n's of 8-12 are recommended.

The data expressed as seconds spent on the formerly naloxone- and saline-paired floors are presented in Figure 2B. Both no extinction groups spent less time on the formerly naloxone-paired floor than on the formerly saline-paired floor. Importantly, this indicates that rats do not forget the significance of the naloxone-paired floor in the week following OW-CPA acquisition in the absence of extinction training, implying that any behavioral changes seen in the extinction groups are due to extinction training^{28,37}. The behavior of the extinguished groups differed depending on the amount of extinction training they received, such that the 1 d and 2 d ext groups continued to show aversions whereas the 3 d ext group did not. No further behavioral changes were seen in the 4, 5, or 6 d ext groups. This indicates that 3 d of extinction training is necessary and sufficient to extinguish OW-CPAs acquired using the parameters we have described. Data presented in this format can be analyzed using a repeated measures ANOVA with Floor as a repeated measure and Group as a between-groups factor; significant interactions of Floor and Group can then be followed up with planned comparisons or post-hoc tests of the time spent on the two floor types within each group (see figure caption for details). All statistical analyses should be two-tailed with an alpha no greater than .05.

In Figure 2C the data are presented as aversion scores. Because there was no difference in the behavior of the two no extinction groups, their data are pooled. Here it can be seen, again, that extinction was complete following 3 d of extinction training. Aversion scores are advantageous in that they are amenable to between-groups comparisons using one-way ANOVAs, planned comparisons, and post-hoc tests (see figure caption for details).

Earlier we noted that it is important to include a no extinction group in every experiment to verify that the animals acquired OW-CPA prior to extinction training. In light of this, it is important to point out that OW-CPA acquisition under the parameters we have described is quite robust. Figure 2D shows the aversion scores of the individual rats (n = 18) in the pooled no extinction group. There were two rats that showed no evidence of having acquired **OW-CPA**, but the remainder showed robust place aversions. Hence, the small percentage of non-learners in any given sample of rats is not a major concern because the expectation

would be that with random assignment of rats to groups, the few non-learners would be distributed equally.

We acknowledge that we are not the first to use a two-chambered apparatus to study conditioned opiate withdrawal in rats ^{e.g., 38, 39}, but our scoring method differs somewhat from that used by other investigators. Typically, place aversions are quantified as the change in time spent in the withdrawal-paired chamber in a post-conditioning test versus a pre-conditioning test. In our protocol we have omitted the pretest and focus instead on the difference in time spent in the withdrawal- and saline-paired chambers in the posttest. Among the benefits of the former method are that it allows the experimenter to eliminate animals showing an overly strong preference for one or the other chamber prior to conditioning, and/or to assign one or the other chamber as the withdrawal-paired chamber on an individual animal basis in a biased or unbiased experimental design. These benefits are predicated on the assumption that preferences shown during the pre-test are stable; that is, that untrained animals exhibit a consistent preference for one or the other chamber across repeated tests. As described earlier, we have found this not to be the case in our apparatus, as most rats exhibit remarkably unstable preferences from test to test (Supplementary Figure 1). We have also found that the magnitude of the preference that they do exhibit increases across repeated tests, suggesting the introduction of an extraneous variable with repeated testing. For this reason we have chosen to omit the pretest, assign rats randomly to groups and conditions, and use the differential in time spent in the withdrawal- and saline-paired chambers in the posttest as an operational definition of conditioned withdrawal. We believe that this measure represents the cleanest way of assessing OW-CPA in our apparatus under our testing conditions.

It is simple to introduce experimental manipulations such as systemic or localized drug administration into this experimental protocol. Like other extinction paradigms ⁴⁰, OW-CPA extinction lends itself to investigations of the effects of a drug on the development, consolidation, or retention of extinction through variation of the time of drug administration relative to extinction training and/or test. Hence, to examine the development of extinction, administer the drug prior to extinction training; i.e., substitute drug administration for the saline injections that otherwise would precede placement of the animals into the chambers for each exposure. To examine consolidation of extinction, inject the rats with saline prior to placing them into the chambers for each exposure and administer the drug immediately or at a delay after each exposure is complete. To examine retention of extinction memory, follow the standard extinction training protocol and then administer the drug prior to the test session. In each case, the interval between drug administration and extinction training or test should be determined by the pharmacokinetics of the drug.

Another consideration when designing a mechanistic experiment is the number of extinction training sessions. The choice here should be guided by the behavior of unmanipulated animals (Figure 2B, 2C) and the predicted effect of the manipulation (impairment or facilitation of extinction). If an impairment of extinction is expected, then the experiment should involve no less than 3 d of extinction training, whereas if a facilitation is expected, then the experiment should involve no more than 2 d of extinction training. In either case,

the goal is to maximize the possibility of detecting the effect by choosing an appropriate point along the extinction curve.

Two hypothetical mechanistic experiments are shown in Figure 3. The first experiment (Figure 3A) is one in which a drug administered prior to extinction training is expected to impair OW-CPA extinction. The experimental design involves four groups: extinction plus vehicle or drug, and no extinction plus vehicle or drug. The drug is expected to have no effect in nonextinguished animals, and therefore the group sizes of the no extinction groups are halved in anticipation of pooling their data. (An anticipated null effect in nonextinguished rats should be based on previous findings, and pooling of data should occur only after the absence of a group difference is confirmed statistically via an independent samples t-test. In the absence of evidence upon which to base a prediction of a drug effect or lack thereof upon CPA memory, experiments should be designed such that the no extinction groups are as large as the extinction groups, and the data from the no extinction groups should be analyzed separately rather than being pooled.) Three extinction training sessions are included because this is a point on the extinction curve (Figure 2) in which the extinction/vehicle group is expected to show complete extinction. The second experiment (Figure 3B) shows an analogous experiment in which a drug administered prior to extinction training is predicted to facilitate extinction. The experimental design is much the same except that only two sessions of extinction are included. At this point on the extinction curve (Figure 2), rats continue to show robust place aversions, but the magnitude of those aversions drops considerably following one more day of extinction training.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The 2-chambered place conditioning apparatus. **(a)** Each chamber consists of (1) a black Plexiglas box without an attached lid or floor; (2) two distinctively-textured floors (painted black) constructed of perforated stainless steel (“hole”) or equally spaced stainless steel rods (“grid”) mounted on Plexiglas frames; and (3) a sheet of clear Plexiglas that serves as a lid. Thin strips of Plexiglas glued to the center of both sides of the box (4) create a track for (5) a removable black Plexiglas partition to be inserted. **(b)** The chambers are assembled on a cart and positioned below a camera affixed to the ceiling of the room. Four chambers are arranged side-by-side on a single sheet of black Plexiglas, with the orientation of the grid and hole floors alternating from one chamber to the next. The chambers are shown without lids or partitions in place. **(c)** The data can be scored automatically using an appropriate software package, such as Ethovision. In Ethovision, prior to placing the rats into the chambers, the experimenter outlines in the data collection file the location of the two floor types (“zones”) within each chamber (“arena”), using an image captured by the video camera with the room lights on. **(d)** Experiments are run under red light conditions to maximize the contrast between the albino rats and the black apparatus for scoring purposes.

Figure 2.

Behavior of normal (unmanipulated) rats trained using the protocol we describe. Error bars indicate SEMs. **(a).** Experimental design. There were eight groups in this experiment: two that were trained to acquire OW-CPA but not extinguished, and six that were trained to acquire OW-CPA and then received differing amounts of extinction training (1-6 d). All extinguished groups were tested the day following their final extinction training session. One of the no extinction groups was tested the day after the second OW-CPA acquisition training session and the other was tested 7 d later. The latter group received no intervening training during this period (i.e., they remained in the colony undisturbed). **(b)** The data expressed as seconds spent on the formerly naloxone- and saline-paired floors during the test session. Overall, there was a significant Group x Floor interaction [$F(7, 64) = 3.503$; $p = .003$]. The aversions shown by the two nonextinguished groups were of a similar magnitude [Floor x Group interaction: $F(1, 16) < 1$; t -tests using the Bonferroni correction (adjusted p value = .00625) revealed the aversions to be statistically significant in both the no ext (1 d test) and the no ext (7 d test) group (p 's = .00006 and .0005, respectively). By contrast, in the extinguished groups, the 1 d ($p = .0008$) and 2 d ext groups ($p = .003$) showed significant place aversions, whereas the 3, 4, 5, or 6 d ext groups did not (p 's = .569, .933, .655, and .659, respectively). **(c)** The data from panel b converted to aversion scores. Overall there was

a significant main effect of Group [$F(7, 64) = 3.503$; $p = .003$]. The 3, 4, 5, and 6 d extinction groups differed significantly from the pooled no extinction group (p 's = .007, .005, .00005, and .0003, respectively) whereas the 1 d and 2 d extinction groups did not (p 's = .306 and .173, respectively), as indicated by t-tests performed using the Bonferroni correction (adjusted p value = .00833). (d) Aversion scores of the individual animals in the pooled no extinction group. The group mean is indicated by the hash mark. All experimental protocols were approved by McLean Hospital's Institutional Animal Care and Use Committee (IACUC). * paired t-test, $p < .00625$ (Bonferroni adjusted p value) † $p < .00833$ vs. no ext (pooled) group (Bonferroni adjusted p value)

Figure 3.

Hypothetical mechanistic experiments. **(a)** In this example experiment, a drug is administered on each of three extinction training days prior to each exposure to the place conditioning chamber. Three days of extinction training are run because the predicted effect is an impairment of extinction. The anticipated outcome in terms of seconds spent on the formerly naloxone- and saline-paired floors, and in terms of aversion scores, is shown. The no extinction group is expected to show a robust place aversion; the extinction/vehicle group is expected to show no place aversion (i.e., complete extinction); and the extinction/drug group is expected to behave similarly to the no extinction group (i.e., to show a blockade of extinction). **(b)** In a second example experiment, a drug is administered on each of two extinction training days prior to each exposure to the OW-CPA chamber. Two days of extinction training are run because the predicted effect is a facilitation of extinction. The no extinction group is expected to show a robust place aversion; the extinction/vehicle group is expected to show a nominally smaller but still significant place aversion as compared to the no extinction group; and the extinction/drug group is expected to show no place aversion (i.e., complete extinction).

Table 1

Troubleshooting table.

Step	Problem	Possible reason	Solution
3	Pellet implant incision opens post-surgery	Rat pulled staples out	Make incision between shoulder blades where it is difficult for rat to access Apply staples firmly during surgery Re-anesthetize rat and re-staple Consider single-housing rats
3	Infection at pellet implant site	Soiled surgical instruments	Use a bead sterilizer to clean instruments between rats
7	Rats do not acquire CPA	Rat pulled staples out Morphine pellets not in place for a minimum of 3 d prior to acquisition Rats are too large; plasma concentrations of morphine inadequate to induce sufficient morphine dependence Inappropriate dose of naloxone	Take measures to prevent this (see above) Allow a minimum of 3 d to elapse between pellet implant surgery and acquisition Use rats no larger than ~225-250 g upon arrival Conduct pilot work to determine optimal naloxone dose
7	Rats do not extinguish CPA	Rats not exposed to saline pairing first and naloxone pairing second on each day of CPA acquisition training Morphine pellets in place longer than 2 wks Insufficient extinction training	Expose rats to saline-paired floor first and naloxone-paired floor second on each day of CPA acquisition training Complete all phases of experiment within 2 wks of implanting pellets Use a more intensive extinction training protocol (i.e., more days)
7	Rat does not explore the apparatus during the test	Insufficient sample size Sick rat Injured rat	Use a minimum of 8-12 rats per group Eliminate rat from experiment Eliminate rat from experiment