Video Article

Reinstatement of Drug-seeking in Mice Using the Conditioned Place Preference Paradigm

M. Carmen Blanco-Gandía[1], María A. Aguilar[1], José Miñarro[1], Marta Rodríguez-Arias[1]

[1]Unidad de Investigación Psicobiología de las Drogodependencias, Departamento de Psicobiología, Facultad de Psicología, Universitat de València

Correspondence to: Marta Rodríguez-Arias at marta.rodriguez@uv.es

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Abstract

The present protocol describes the Conditioned Place Preference (CPP) as a model of relapse in drug addiction. In this model, animals are first trained to acquire a conditioned place preference in a drug-paired compartment, and after the post-conditioning test, they perform several sessions to extinguish the established preference. The CPP permits the evaluation of the conditioned rewarding effects of drugs related to environmental cues. Then, the extinguished CPP can be robustly reinstated by the non-contingent administration of a priming dose of the drug, and by exposure to stressful stimuli. Both methods will be explained here. When the animal reinitiates the behavioral response, a reinstatement of the conditioned reward is considered to have taken place.

The main advantages of this protocol are that it is non-invasive, inexpensive, and simple with good validity criteria. In addition, it allows the study of different environmental manipulations, such as stress or diet, which can modulate relapse into drug seeking behaviors. However, one limitation is that if the researcher aims to explore the motivation and primary reinforcing effects of the drug, it should be complemented with self-administration procedures, as they involve operant responses of animals.

Video Link

The video component of this article can be found at https://www.jove.com/video/56983/

Introduction

The Conditioned Place Preference (CPP) paradigm offers a simple way of assessing the conditioned reward induced by diverse stimuli[1,2], and has been used broadly to study the conditioned rewarding effects of addictive drugs[3]. It is based on Pavlovian conditioning, evaluating the motivational value of drug-associated environmental cues for maintaining addictive behavior[4]. In this model, environmental cues acquire secondary appetitive properties (conditioned rewarding effects) when paired with a primary reinforcer[3]. For example, an initially neutral place (such as the color of one compartment in the CPP cage) is paired with the specific effects of a drug of abuse during some conditioning sessions[5], while another compartment is associated with the injection of a vehicle. Following conditioning, if the animal spends more time in the compartment previously associated with the drug, it is assumed that CPP has developed[3]. The establishment of the preference is achieved when the animal gives a positive value to the environmental cues linked to the drug, which is the primary reinforcer. Consequently, animals will perform behavioral drug-seeking responses in response to those contextual cues[6]. The CPP model permits the evaluation of the rewarding properties of subthreshold doses of the drug, showing whether animals in a specific condition (*e.g.*, having suffered from social defeat previously) are more vulnerable and sensitive to doses that are not effective in naive animals[7].

The CPP model has also been used to evaluate extinction/reinstatement as an animal model to study relapse[3], which is the aim of the present protocol. There are three different phases: acquisition, extinction, and reinstatement (**Figure 1**). In the CPP reinstatement model, animals first acquire the CPP for a drug-paired compartment, and then they perform several extinction sessions. We define extinction as the moment in which the animal reduces its behavioral responses of approximation to a conditioned rewarding stimulus that has been removed (*e.g.*, the drug)[8]. During the extinction sessions, animals explore the compartments in the absence of the drug, so that the acquired preference is gradually attenuated[9]. An important issue to consider is that the behavioral change that the animal exhibits during extinction (the progressive decrease in the time spent in the drug-paired compartment) can be due to new learning processes that compete with the previous learned response, or due to a decrease in the internal motivational state of the subject[3]. Finally, the reestablishment of the place preference through the context or drug cues would be our model of reinstatement[1].

Administering a priming injection of the associated drug can reinstate the preference, which is considered a reestablishment of the approximation to the contextual cues. Drug priming reinstatement occurs due to the persistent memory of the pleasurable effects of the drug, which induced craving and motivates animals to seek the environmental cues related to reward.

Some advantages of the CPP reinstatement model are that the procedure is non-invasive (in contrast with self-administration, which requires surgery), inexpensive, and simple. In addition, this model has a good criterion validity, as it mimics well what occurs in humans[10,11], inducing reinstatement with stimuli that induce relapse, such as re-exposure to the drug[12,13] or stress[14].

There are other techniques such as the extinction – reinstatement model of intravenous self-administration. Here, animals press a lever to self-administer the drug, which permits the evaluation of the operant response of the animal, compulsivity, and motivation[14,15,16]. The main advantage of the CPP over self-administration procedures is that CPP reinstatement is considered to reflect the reactivation of the incentive-motivational value of the context stimuli paired with the drug, consisting of the reappearance of the approach behavior to the context[17]. Moreover, non-drug stimuli, such as stress, can also induce reinstatement[18,19]. For example, one self-administration study described no effects on reinstatement of heroin intake in rats after a foot shock or restraint stress[20]. Authors discussed that it was unsuccessful because those stressors were tested outside the self-administration chamber in a different context. In contrast, when using the CPP model of reinstatement, there was a clear re-establishment of morphine-induced CPP after using the same stressors, and applied in a different context to that of the CPP and at different times (0 and 15 min after stress exposure)[18].

Several studies in the literature have shown different ways of drug and stress-induced reinstatement. On the one hand, drug-induced reinstatement has been reported in rats and mice using morphine[5,21,22,23], cocaine[24,25], amphetamine[26,27], ethanol[28,29], and 3,4-Methylenedioxymethamphetamine (MDMA)[30]. On the other hand, exposure to stress may be a determining factor in vulnerability to drug abuse. Stress is known to increase the rewarding effects of drugs[7,31,32] and their role in relapse is well established[33,34]. For example, defeat in social interactions with a conspecific reinstates morphine and cocaine CPP[18,19]. In addition, animals exposed to repeated social defeat are more vulnerable to the conditioned rewarding effects of subthreshold doses of cocaine, and reinstate the preference with very low doses of cocaine[7].

Application of the CPP reinstatement model is a useful and sensitive way to evaluate vulnerability to relapse in animals, and permits the assessment of different subtle environmental manipulations, which are the main triggers that threaten human relapse, such as drug- or stress-induced reinstatement.

Protocol

All procedures involving mice and their care complied with national, regional, and local laws and regulations, which are in accordance with Directive 2010/63/EU of the European Parliament and the council of September 22, 2010 on the protection of animals used for scientific purposes. The Animal Use and Care Committee of the University of Valencia approved the present protocol.

1. Materials and Set-up for Conditioned Place Preference:

1. Handle mice 3 days prior to testing for 1 - 2 min each. The base of the tail should be grasped. The body should be supported if possible. This is done to minimize pain and distress when handling an animal and to prevent interfering variables, such as stress.
   NOTE: OF 1 male mice 60 days old (young adults) weighing 35 - 40 g were employed in the present experiments. Drug-induced reinstatement can be performed in mice of both sexes. However, regarding stress-induced reinstatement, the social defeat paradigm is designed only for male rodents, as females do no induce an aggressive response in the resident mice.
2. For place conditioning, use identical boxes made with two identical compartments (30.7 cm long × 31.5 cm wide × 34.5 cm high) separated by a smaller central grey area (13.8 long × 31.5 cm wide × 34.5 high).
   NOTE: Compartments have different floor textures and wall colors (a smooth floor in the black compartment and a rough floor in the white one). Each compartment of the CPP box contains four infrared light beams and six in the central compartment. This allows the recording of the crossings between compartments and the position of the animal.
3. Set up the room lights (off or lowered) each day. Use red lights (recommended), as animals do not perceive it and it is attenuated for the experimenter (approximately 40 lux measured at 1 m above floor level).
4. Set up the program to register 15 min for Pre- and Post- conditioning test (see the **Table of Materials** for software) (**Figure 2**)
5. Prepare the cocaine solution required for the whole experiment. Dissolve cocaine hydrochloride in 0.9% NaCl (saline) in a volume of 0.1 mL/10 g body weight. Vortex mixture and store at 4 °C at the end of the day.
   NOTE: Cocaine concentrations may vary depending on the study's purpose. If the aim is to evaluate the subthreshold doses of cocaine, a 1 mg/kg concentration could be used, which is ineffective in control animals, as seen in previous studies[24,35]. If an effective dose is the purpose, a 6 mg/kg concentration can be chosen, which is effective but does not induce reinstatement[24], or a 25mg/kg concentration, which is effective and induces reinstatement of the preference[19].
6. Test the animals during their dark phase and use the same box for each animal across days.
7. Bring animals every day to the testing room in their home cages and leave them undisturbed for 15 min as a habituation period to all the test-related noises.

2. Test

1. **Acquisition**
   NOTE: The procedure of Place Conditioning, unbiased in terms of initial spontaneous preference, consists of three phases: Pre-Conditioning, Conditioning, and Post-Conditioning (**Figure 1**).
   1. **Pre-Conditioning (Pre-C) (3 days) (Figure 2**, **Figure 3**, **Figure 4)**
      NOTE: Here, the neutral value of the compartments in the CPP box is evaluated. Measure the time spent in each compartment and later compare it with that spent in the same compartment in the Post-Conditioning test.
      1. Bring the animals to the test room and leave them habituated for 15 min.
      2. Check that guillotines are removed from the cage.
      3. Set up the computer and the program. Enter animal IDs and press the "Trial" and "Start" command (**Figure 2**).
      4. Place the mouse gently into the middle chamber (grey compartment) and leave the testing room with minimum noise.

5. Bring all the animals back into their home cages and save the data when the trial is finished.
6. Allow mice to access both compartments of the apparatus for 15 min (900 s) per day on 3 consecutive days.
7. On day 3, write down the time spent in each compartment over a 900 s period, and save it for the assignment of compartments in the next phase (**Figure 3**).
8. Assign half the animals in each group to receive the drug or vehicle in one compartment (*e.g.*, black), and the other half in the other compartment (*e.g.*, white) (**Figure 4**).
   NOTE: The final distribution of the animals must ensure that half of the animals are assigned to the initially preferred compartment and the other half to the non-preferred compartment. In addition, half of the animals received the drug in one compartment (for example, the black one) and the other half in the other (in this case, the white one).
9. Balance scores in the group within both compartments as well as across groups (test group differences with an analysis of variance (ANOVA)). As approximate guidance, values should be around 360 - 370 s in the Pre-C test (**Figure 4**). NOTE: no significant differences should be detected between the time spent in the drug-paired and vehicle-paired compartments during the Pre-C test.
10. Exclude animals that exhibit a strong aversion or preference for any compartment.Consider aversion as spending less time than 33% in the compartment, and consider preference when the animal spent more than 67% of the total time in that compartment.

2. **Conditioning (4 days) (Figure 5)**
   1. Weigh the animals and use these weights for cocaine doses during conditioning.
   2. Prepare syringes with cocaine or saline based on body weights.
   3. Inject one mouse at a time intraperitoneally (ip) and immediately place it gently into the assigned black or white compartment of their box.
   4. After an interval of 4 h, inject the drug dose immediately before the mouse is confined to the drug-paired compartment for 30 min (**Figure 5**).
   5. Alternate this procedure each day, beginning with cocaine on days 1 and 3, and saline on days 2 and 4.
   6. Check that confinement is carried out in both cases by closing the guillotine door that separates the two compartments, making the central area inaccessible.
      NOTE: In this phase, there is no need to set up the PC program.

3. **Post-Conditioning (Post-C) (1 day)**
   1. Bring the animals to the test room and leave them habituated for 15 min.
   2. Remove the guillotine door separating the two compartments.
   3. Set up the computer and the program. Enter animal IDs and press the "start" command.
   4. Record the time spent by the untreated mice in each compartment during a 900 s observation period.
   5. When the trial is finished, bring all the animals back into their home cages and save the data.
   6. Consider that the drug has induced place preference if the time spent in the drug-paired compartment during the Post-C is significantly greater than in the Pre-C test. If the opposite occurs, consider an aversion for the drug-paired compartment.

2. **Extinction**
   1. Conduct a weekly extinction session in all groups/animals that developed a preference for the drug-paired compartment.
   2. Place the animal in the apparatus (without guillotines) for 15 min (same as the Pre- and Post-C tests) (**Figure 1**).
      NOTE: If the dose employed in the CPP is high (25 mg/kg), perform 1 extinction session per week. If a subthreshold dose is used (1 mg/kg), then perform 2 extinction sessions per week (*e.g.* Monday and Thursday).
   3. Consider the preference extinguished when data from the extinction test do not show differences with respect to the Pre-C test, but they do show differences with respect to the Post-C test.
   4. Repeat the test 24 h later in order to confirm the extinction.

3. **Reinstatement of CPP**
   1. **Priming-induced reinstatement**
      1. Evaluate the effects of a priming dose of cocaine 24 h after extinction has been confirmed.
         NOTE: Priming doses are half the previous dose that the animal has received during conditioning. The reinstatement test undergoes the same protocol as in Pre-C and Post-C tests, except that animals receive the dose of cocaine in a non-contingent place 15 min before the test. The efficacy of drug priming in inducing reinstatement can be enhanced after repeated extinction/reinstatement experiences[23], although the order of the doses seems not to play a role in the results obtained[36].
      2. Bring the animals to a different room from the testing room (non-contingent place) (**Figure 6**).
      3. Inject them with half the previous dose of cocaine (*e.g.*, if animals were conditioned with 25 mg/kg cocaine, a priming dose would be 12.5 mg/kg).
      4. Bring them back to their vivarium for 15 min.
      5. Take them into the testing room and place the animals in the apparatus immediately (without the guillotine doors separating the compartments) for 15 min (same as the Pre- and Post-C tests).
      6. Consider the difference in seconds between the time spent in the drug-paired compartment during the last Extinction session and the Reinstatement test as a measure of the degree of reinstatement of the preference induced by the drug.
      7. Repeat the extinction-reinstatement procedure every time with decreasing doses until it is confirmed to be ineffective.
         NOTE: A saline injection control can be used to control the capacity of cocaine priming (and not of injection itself) to induce reinstatement.

   2. **Stress-induced reinstatement**
      1. Evaluate the effects of a stress-induced reinstatement 24 h after extinction has been confirmed
         NOTE: Reinstatement tests are the same as those carried out in Post-C (free ambulation for 15 min), except that animals are tested 15 min after the social defeat in an agonistic encounter (**Figure 6**)
      2. Take the animals to a different room from the CPP room.

3. Place the experimental mouse with an aggressive opponent sex matched of equal age and body weight in the plastic cage (**Figure 6**).
   NOTE: The agonistic encounter consists of a 10 min test in a neutral transparent plastic cage (23x13.5x13cm) with a defeat result for the experimental mouse. To prepare the aggressive opponent, first isolate different animals that match in age and body weight as the experimental mice for 3 weeks. Train them to acquire fighting experience and test them to have high levels of aggressive territorial behavior. To obtain aggressive conspecifics, mice must be isolated for at least 3 weeks, and then trained in aggressive and attack behaviors. Animals are trained in a neutral environment, facing them in pairs. We let them bite the opponent 2 or 3 times and get them out of the cage, always ending the training before one of the two mice shows submission, so that the experience of the encounter reinforces aggressive behavior. After suffering aggression (threat and attack from the aggressive conspecific), experimental mice should present avoidance/flee and defensive/submissive behavior. The criterion is to adopt a specific posture of defeat, characterized by an upright submissive position, limp forepaws, upwardly angled head, and retracted ears (**Figure 6**). According to Burke *et al.*, 2016, we consider that the social encounter is terminated earlier if the intruder displays a submissive supine posture for >8 s or if 13 attack bites occurred. Another option to screen animals without risk of significant injury could be to pace the animal in a mesh enclosure with a hole, not large enough for either animal to pass. Aggression could be detected without subjecting animals to direct aggressive behavior. If an animal sustains an injury with a wound 1cm in diameter and/or direct exposure of muscle, it is excluded from the study and should be euthanized.
4. After 10 min of agonistic encounter, take the mice into the testing room and place the animals in the CPP apparatus immediately (without the guillotine doors separating the compartments) for 15 min (same as the Pre- and Post-C tests).

3. Statistical Analysis

NOTE: Ideal sample size should be a minimum of 15 animals per group.

1. Analyze the mean time spent in the drug-paired compartment of the group by means of a mixed ANOVA with one between variable, treatment, with two levels (Control group, Experimental group, example of two groups is in **Figure 7c**), and one within variable – Days, with 2 levels (Pre-C and Post-C).
   NOTE: In the control group, animals receive the drug but have no environmental manipulations. On the other hand, in the experimental group animals are exposed to different environmental conditions. Compare the time spent in the drug-paired compartment on the Post-C day with respect the Pre-C test to see if there are any significant differences in sensitivity to the drug.
2. In addition, perform Bonferroni post-hoc analyses to check either of the significant scores.
3. Analyze data related to extinction and reinstatement values in the groups that developed preference by means of Student´s t-tests to continue with the extinction-reinstatement procedure during the course of the experiment, but at the final moment, analyze them with an ANOVA with a within variable (Days) with the number of levels depending on each group.
   NOTE: When the number of comparisons is increased, the number of those that will be significant by chance also increased. As such, to avoid getting false-positives (Type 1 Errors) we 'correct' the p-value thereby making the test more conservative with the Bonferroni correction for multiple t-tests. However, for the reinstatement test, the comparison should be made between the reinstatement test and the last extinction test. Therefore, there is no need for correction.

Representative Results

Firstly, representative results from the priming- and stress-induced reinstatement are shown in **Figure 7** using adult OF1 male mice.

The data in **Figure 7a-b** representing time spent in the drug-paired compartment(s) in the Pre- and Post-C test were analyzed with a repeated measures ANOVA with a within-subjects variable Days (comparing Pre-C and Post-C). The results showed a significant effect of the variable days [$F(1,34) = 51.179$; $p < 0.001$], as all the animals developed a preference in the Post-C day compared to the Pre-C. All animals were conditioned with 25 mg/kg cocaine and when extinction was achieved, one group received a priming dose of 12.5 mg/kg cocaine to test the reinstatement (**Figure 7a**) and the other was subjected to a social interaction in which the experimental animal was defeated by an aggressive opponent (**Figure 7b**). 15 min after the two events, animals were tested in their corresponding CPP box.

The priming-induced reinstatement (**Figure 7a**) was subjected to subsequent decreasing priming doses (half the previous one) until a priming dose is confirmed to be ineffective. The doses were: 12.5, 6.25, 3.125, and 1.56 mg/kg cocaine. The Student´s t-test revealed a significant effect on the 12.5 mg/kg cocaine reinstatement with respect to the extinction session (t statistic (t) = 20.589, degrees of freedom (d.f.) = 17; $p < 0.001$) and subsequent doses (6.25 mg/kg: t = 18.356; 3.125 mg/kg: t = 14.260; 1.56 mg/kg: t = 16.934 d.f. = 17; $p < 0.001$). Priming with 0.78 mg/kg did not induce the reinstatement.

Regarding the stress-induced reinstatement (see **Figure 7b**), being exposed to an aggressive animal and defeated 15 min before the test leads to reinstating the previously extinguished preference (t = 26.810; d.f. = 15; $p < 0.001$)[19].

Other environmental manipulations, such as suffering repeated social defeat before the CPP procedure, modify the rewarding effects of subthreshold doses of cocaine (**Figure 7c**). A mixed ANOVA with the between variable "stress" and the within variable "days" was performed, and there was a significant effect of the interaction Stress x Days [$F(1,29) = 4.144$; $p < 0.05$]. Only socially defeated animals exhibited preference for the drug-paired compartment with a subthreshold dose of cocaine, which has been shown to be ineffective in standard animals[7]. In addition, after extinction of the preference, socially defeated animals reinstated their preference with only 0.5 mg/kg cocaine priming (t = 25.484 d.f. = 17; $p < 0.001$).

Secondly, as a negative result, we can see how reinstatement can be prevented by a positive social interaction with a non-aggressive conspecific (**Figure 8a**). Therefore, it is important to evaluate whether animals are defeated or not after a social encounter[37].

Another example of negative results that prevent reinstatement can be nutritional manipulations, such as a high-fat palatable diet intake. First, ANOVA analysis revealed an effect of the variable Days [$F(1,26) = 21.527$; $p < .0.001$], as both groups developed preference for the drug-paired compartment ($p < 0.01$) (**Figure 8b**). After extinction, our results show that animals switched to the high-fat diet after the Post-C test decreased their sensitivity to drug-priming induced reinstatement, as they did not reinstate the preference with a 6.25mg/kg cocaine dose[38].

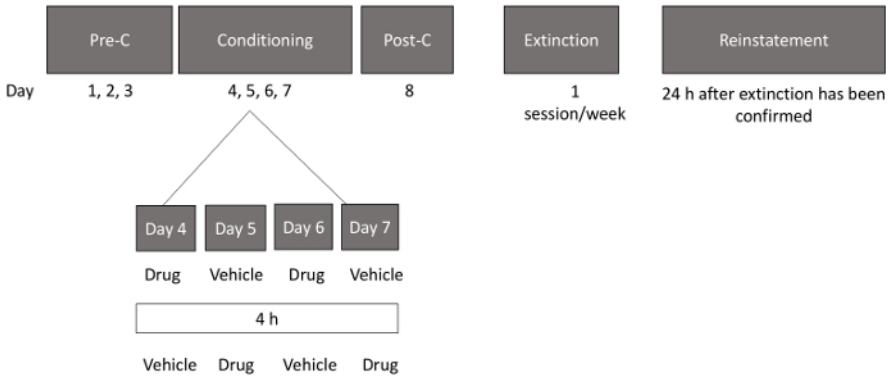


Figure 1: Experimental procedure of the Conditioned Place Preference. It consists of three phases: Pre-Conditioning, Conditioning, and Post-Conditioning. There are 4 conditioning days in which the procedure is alternated each day, beginning with cocaine on days 1 and 3 and with vehicle on days 2 and 4. Then, extinction and reinstatement sessions are performed. Please click here to view a larger version of this figure.

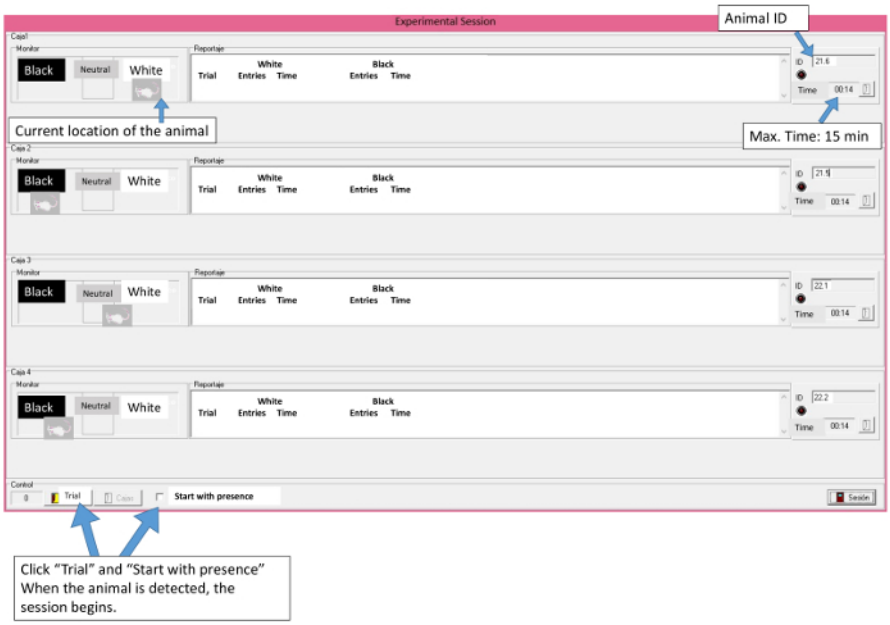


Figure 2: Software usage sessions for Pre-C and Post-C tests where time spent in each compartment and number of entries is recorded. Please click here to view a larger version of this figure.

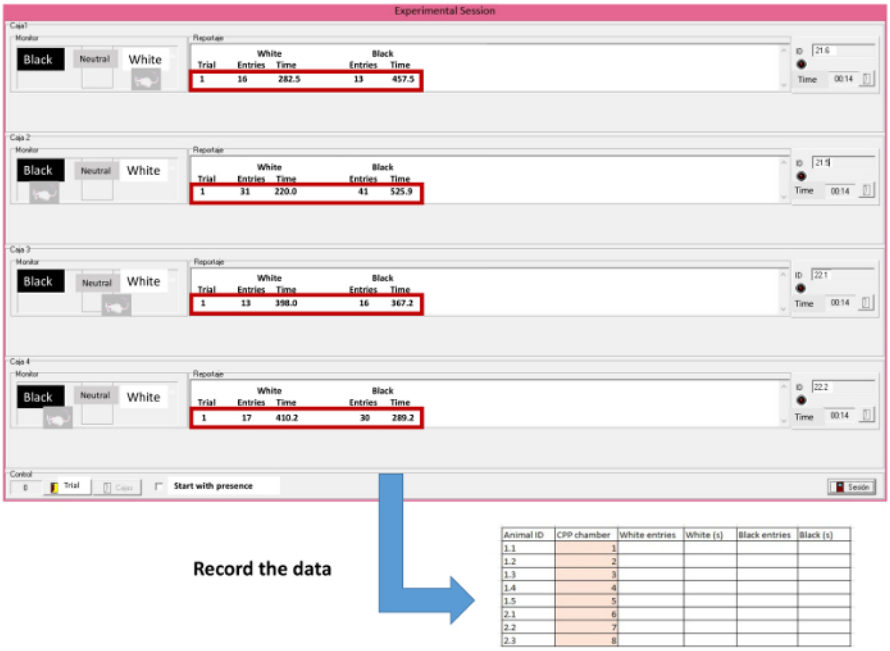
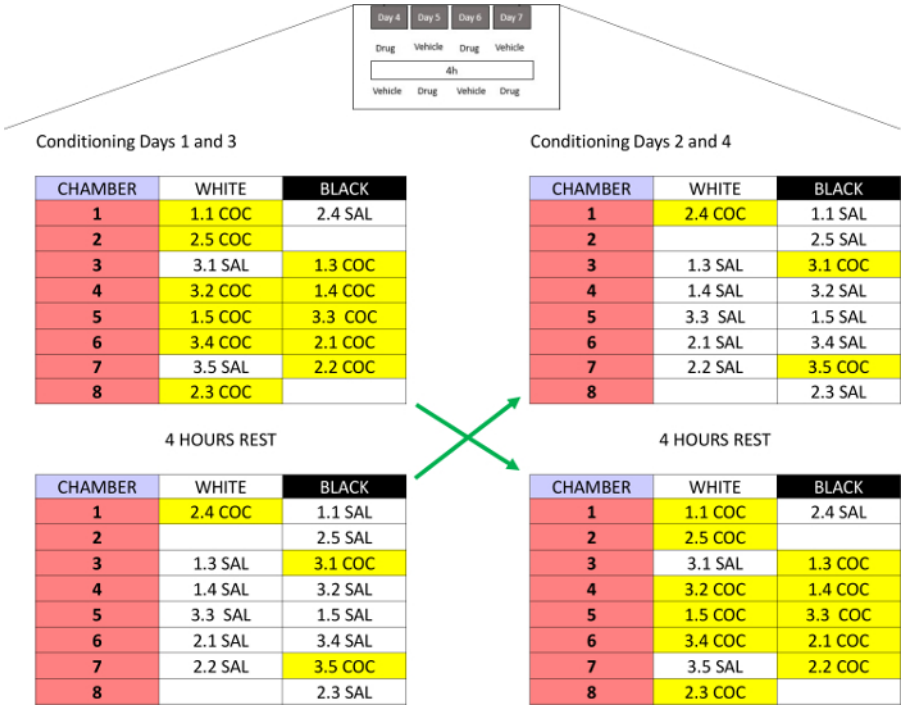


Figure 3: Data after the Pre-C and Post-C test. Here, record the data obtained during the test. Please click here to view a larger version of this figure.

Figure 4: Unbiased assignment of animals to the compartments for the next phase of conditioning. Take the data of the last Pre-C test day in the white and black compartments. Half the animals are assigned to receive the drug or vehicle in one compartment (black or white), and the other half in the other compartment. Exclude animals that show strong aversion or preference. Please click here to view a larger version of this figure.



CHAMBER	WHITE	BLACK
1	1.1 COC	2.4 SAL
2	2.5 COC	
3	3.1 SAL	1.3 COC
4	3.2 COC	1.4 COC
5	1.5 COC	3.3 COC
6	3.4 COC	2.1 COC
7	3.5 SAL	2.2 COC
8	2.3 COC	

CHAMBER	WHITE	BLACK
1	2.4 COC	1.1 SAL
2		2.5 SAL
3	1.3 SAL	3.1 COC
4	1.4 SAL	3.2 SAL
5	3.3 SAL	1.5 SAL
6	2.1 SAL	3.4 SAL
7	2.2 SAL	3.5 COC
8		2.3 SAL

CHAMBER	WHITE	BLACK
1	2.4 COC	1.1 SAL
2		2.5 SAL
3	1.3 SAL	3.1 COC
4	1.4 SAL	3.2 SAL
5	3.3 SAL	1.5 SAL
6	2.1 SAL	3.4 SAL
7	2.2 SAL	3.5 COC
8		2.3 SAL

CHAMBER	WHITE	BLACK
1	1.1 COC	2.4 SAL
2	2.5 COC	
3	3.1 SAL	1.3 COC
4	3.2 COC	1.4 COC
5	1.5 COC	3.3 COC
6	3.4 COC	2.1 COC
7	3.5 SAL	2.2 COC
8	2.3 COC	

Figure 5: Final assignment of Vehicle and Cocaine paired sessions. This figure shows alternation on the order of pairings. On days 1 and 3, an animal (*e.g.*, 1.1) is first exposed to the cocaine-paired compartment (the white one) and, after four hours, is exposed to the vehicle-paired compartment (the black one). On days 2 and 4, the order of pairings is inverted, and the animal is first exposed to vehicle-paired (black) compartment and, after four hours, to the cocaine-paired (white) compartment. As this procedure is unbiased and counterbalanced, simultaneous conditioning sessions can be conducted with two animals using both compartments of the same box (*e.g.*, animals 1.1 and 2.4, 3.1 and 1.3), which allows us to reduce time devoted to the experimental procedure. Note that these pairs of animals follow the reversed order of pairings through the conditioning phase. Please click here to view a larger version of this figure.

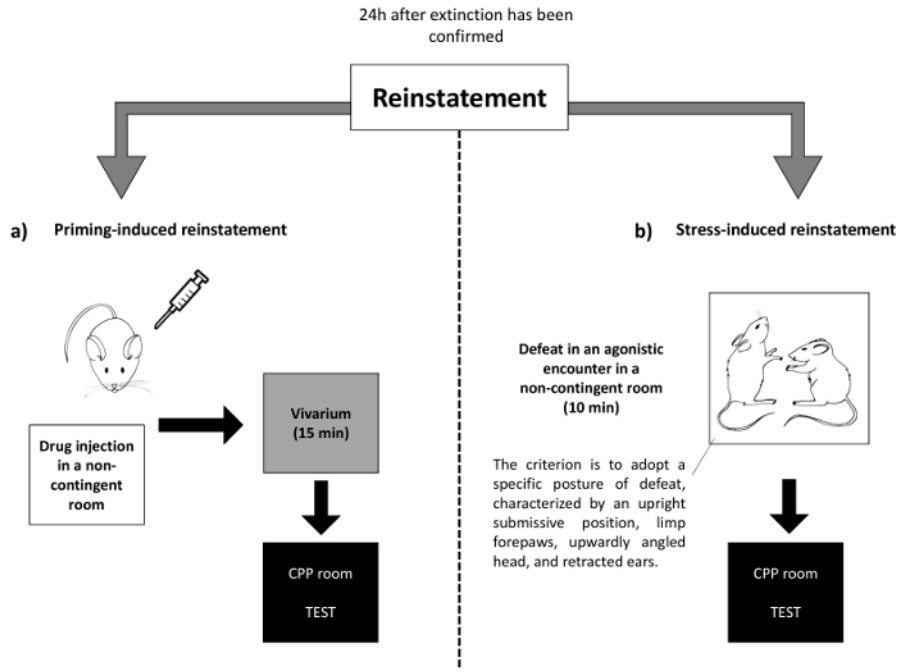


Figure 6: Two models of reinstatement. (**a**) Priming-induced reinstatement and (**b**) stress-induced reinstatement. (**a**) Animals receive a drug injection in a non-contingent room and are brought to the vivarium; 15 min later they are tested in the CPP box. (**b**) Animals are defeated in an agonistic encounter in a non-contingent room for 10 min and, afterwards, they are tested in the CPP box. Please click here to view a larger version of this figure.

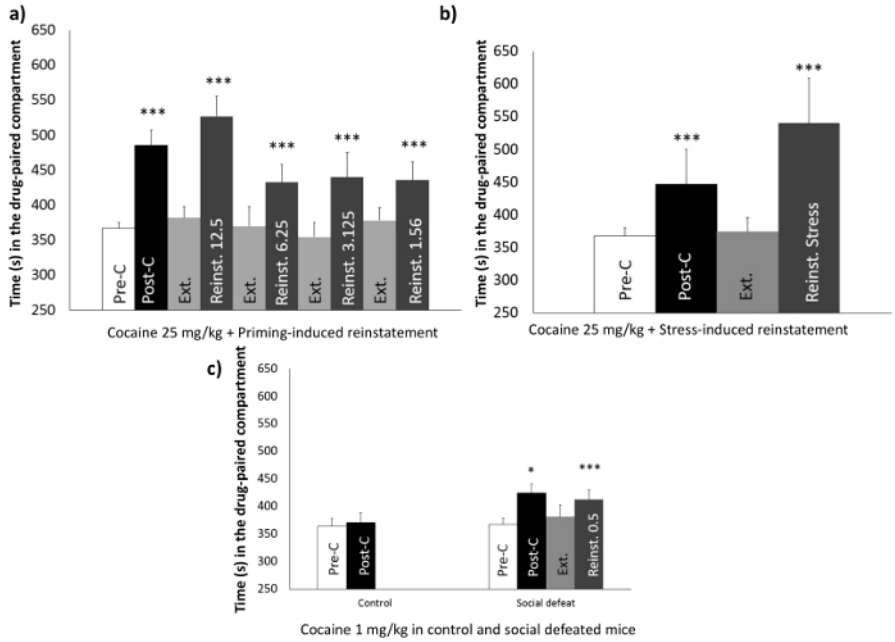


Figure 7: Representative positive results. Priming (**a**) and stress-induced (**b**) reinstatement after 25 mg/kg Cocaine CPP. (**c**) Effects of social defeat on the rewarding effects of subthreshold doses of cocaine (1 mg/kg). Bars represent the mean (± SEM) time in seconds spent in the drug-paired compartment during pre-conditioning (white), post-conditioning (black), the last extinction session (light grey), and reinstatement (dark grey). The reinstatement test was evaluated 15 min after a priming dose of half the previous dose received. *p <0.05; **p <0.01; ***p <0.001 significant difference vs Pre-C (ANOVA with a within-subjects variable Days (comparing Pre-C and Post-C) or the previous extinction session (Student's t test). These figures have been modified from [7,19]. Please click here to view a larger version of this figure.

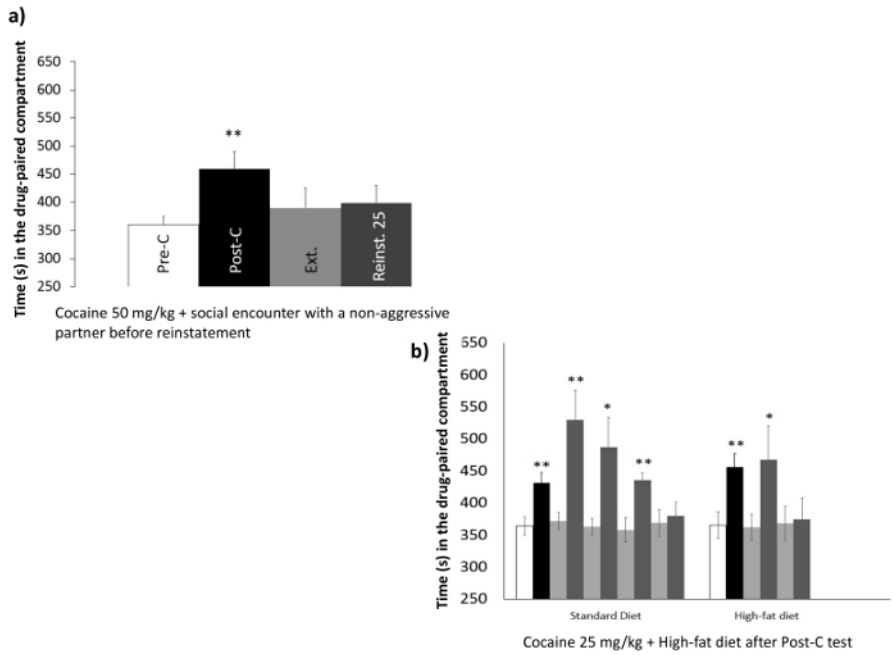


Figure 8: Representative negative results. Effects of protective environmental manipulations on the rewarding effects of cocaine. (**a**) Effects of a positive social interaction before the reinstatement test. (**b**) Effects of a high-fat diet during the whole extinction period and reinstatement. The control group was fed with a standard diet (13% of kcal from fat, 67% kcal from carbohydrates, and 20% kcal from protein; 2.9 total kcal/g) and the high-fat diet group (45% kcal from fat, 36% kcal from carbohydrates, and 19% kcal from protein; 4.6 total kcal/g). Bars represent the mean (± SEM) time in seconds spent in the drug-paired compartment during pre-conditioning (white), post-conditioning (black), the last extinction session (light grey), and reinstatement (dark grey). The reinstatement test was evaluated 15 min after a priming dose of half the previous received dose. *p<0.05; **p<0.01; significant difference vs Pre-C (ANOVA with a within-subjects variable Days (comparing Pre-C and Post-C) or the previous extinction session (Student's t test). These figures have been modified from [37,38]. Please click here to view a larger version of this figure.

Discussion

The key point of drug addiction research is the development of treatments that diminish craving and, consequently, reduce the vulnerability to relapse. Thanks to the reinstatement model of the CPP paradigm, it is possible to study the influence of different procedural and environmental factors that modulate relapse, which is a priority of future research. There are some important points to consider, as the CPP paradigm is a test that is highly sensitive to environmental factors.

Modifications and troubleshooting: The CPP version of the reinstatement model should be modified depending on the drug of interest. There are different factors that may influence the magnitude of conditioned preference, such as duration and number of conditioning sessions. The number of associations usually ranges from one to six, with four being the most common. The duration of the conditioning tests is established according to the characteristics of the drug and its pharmacokinetics.

CPP studies usually involve many trials, so it is useful to use protocols that reduce the time required. One way to achieve this is to reduce the number of trials and another way is to perform two sessions of conditioning per day, as is shown in the present protocol. In this case, it is necessary to ensure that the effects of the drug from the previous session do not drag on to this second session[39], which is why the interval of four hours between the cocaine - vehicle session was chosen. For example, the timing of the pairing in the case of cocaine during the conditioning phase should be every 24 h for 4 days with a pairing duration of 30 min. On the contrary, the time course of the conditioning procedure of ethanol should be every 48 h, a total of 8 days, 1 conditioning session per day, and alternating vehicle and EtOH, finally having a total of 4 EtOH pairings. Ethanol pairing should have a short duration, such as 5 min, as more time could produce aversive states. Effective doses range from 1 - 2 g/kg and subthreshold doses are 0.75 g/kg[40]. Priming-induced reinstatement should be half the previous dose received, and the apparatus should be the same.

However, choosing to do 1 or 2 sessions a day can affect the results in some special drugs of abuse. For example, in the case of MDMA-induced CPL with the two-session protocol, the conditioned preference in post-conditioning is not observed, whereas this can be obtained by performing a single association each day[30], so MDMA should also be administered every 48 h, but duration of the conditioning session should be 30 min.

There are two main methods of extinction that could be run after the establishment of CPP, either daily injections of vehicle confining the animal in both the previously drug and vehicle conditioned compartments[41,42,43], or by repeating the CPP test, letting animals explore the three compartments without the guillotine until preference is no longer observed[7,18,30,38]. In the present protocol, we employed the latter, because it is a type of spontaneous extinction without any pharmacological or behavioral manipulation related to conditioning (more similar to that occurring in human addicts), but both methods are acceptable and valid, as literature has shown for years.

Limitations of the technique: The most important limitation of the CPP paradigm and the reinstatement model is that they are very sensitive to any environmental change. Therefore, room and vivarium conditions should stay stable and constant during the whole procedure. Special care should be taken to aspects such as living in the same room with mates of the opposite sex, cages with artifacts like wheels that make a specific and constant noise, or light conditions. In addition to controlling these variables, the role of the researcher is also critical, as they can induce an increase in animals' stress levels and interfere with the results.

Significance with respect to existing methods: Regarding other methods, in the CPP version of the reinstatement model, the researcher is the one who administers the drug to the animal, in contrast with self-administration paradigms that evaluate the animal´s motivation to take the drug. It is important that we take this into account. This means that we are not evaluating motivation through an operant response nor the primary reinforcing effects of the drug. As we mentioned, CPP is a useful model to assess the critical role that environmental cues play on relapse in substance abuse disorders, which are in fact the main factors that modulate relapse in humans.

Future applications: Future applications of the CPP reinstatement model should be oriented to the so-called incubation of craving, which consists of a progressive increase in cue reactivity after abstinence of drugs, mimicking craving in humans. Nowadays, little is known about this in the CPP paradigm. A few years ago, only one study suggested that CPP could also measure this incubation period[44]. More recently, it was assessed whether or not environmental cues paired to morphine in the CPP could increase the behavioral response over the withdrawal period[45]. This study found that the number of entries to the CPP compartments were increasing over time, reflecting the incubation of craving and suggesting that reward memories and drug craving could be distinguished in the CPP paradigm.

Critical steps: There are several critical steps in the protocol. In order to affirm that animals reinstated their preference, extinction data should reflect a real extinction of the preference, being confirmed before the reinstatement test. Data from the extinction test should not show differences with respect to the Pre-C test but with respect to the Post-C test.

Regarding the social stress-induced reinstatement version, we should have confirmed that the animal has been actually defeated, since a positive social encounter that does not end in defeat shows protective effects blocking the reinstatement of the place preference of cocaine. If the experimental animals have not been defeated, they will be excluded from the study. We recommend recording and evaluating the agonistic encounter, measuring the latency of the aggressive behavior or submission behavior, the number of attacks, and the time spent in each of those behaviors. We consider that the experimental animal is defeated when it receives a minimum of 2 attacks (bites) and shows signs of submission (as illustrated in **Figure 6**). In all cases, the encounter lasts 10 min.

Disclosures

The authors have nothing to disclose.

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