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A conditioned place preference protocol for measuring incubation of craving in rat

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TITLE:
A Conditioned Place Preference Protocol for Measuring Incubation of Craving in Rats

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KEYWORDS:
Morphine, Balanced biased design, Conditioned place preference, Incubation of craving, Long-term reward memory, Somatosensory cues

SUMMARY:
Here, a morphine conditioned place preference (CPP) protocol is described to measure incubation of craving in rats.

ABSTRACT:
A major cause of repeated relapses is a craving for the drug. Drug craving increases progressively during the abstinence period, a phenomenon termed incubation of drug craving. Here, we describe a morphine conditioned place preference (CPP) protocol for measuring the incubation of craving in rats. In this protocol, a CPP paradigm mainly employing somatosensory cues is used to establish a long-term reward memory of morphine. A three-chamber CPP box that differs in the texture of the chamber floor is constructed. First, the animals are tested for their baseline preference to the two side chambers for three consecutive days. Then, they are injected intraperitoneally with morphine/saline and put into their non-preferred/preferred chamber for 45 min. After 6 days of conditioning, their preference to the side chambers is tested for 15 min at different time points after the last conditioning session. With this paradigm, the reward memory of morphine could last for at least 18 days. To test whether the above-mentioned protocol can model increased craving, the number of entrances into the two side chambers are counted during the abstinence period. The results show that the entrances increased, suggesting that the CPP paradigm could mimic the incubation of craving. Future

studies can employ this model to study neural mechanisms underlying long-term memory and incubation of craving.

INTRODUCTION

Drug addiction is a chronic disease[1] with several different stages[2]: binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation. In the initial binge/intoxication stage of the drug addiction cycle, subjects take drugs due to the reinforcing effect[2]. In the withdrawal/negative affect stage, subjects experience negative somatic and emotional states of drug withdrawal, which promote negative reinforcement mechanisms associated with the development of addiction[3]. In the preoccupation/anticipation stage, subjects reinstate drug-seeking after extinction, which could be triggered by drug-associated cues or stressors. In animal studies of drug addiction, conditioned place preference (CPP) paradigms are used to mimic the positive reinforcing effect of drugs[4-6] and, thus, it is used to model the initial stage of the drug addiction cycle. Morphine, a member of the opioid family, produces a positive reinforcing effect by activating the opioid receptors in the brain[7,8].

The CPP paradigm is based on the principle of classical (Pavlovian) conditioning and commonly used to measure the rewarding effect of drugs of abuse, such as morphine[4,5,9,10], due to its easy availability across laboratories. A typical CPP paradigm consists of three phases: pre-conditioning, conditioning and test phases. During the conditioning phase, the rewarding properties of a drug (unconditioned stimulus) are paired with one or more neutral contextual stimuli, which gain rewarding properties after several conditioning pairings and serve as conditioned stimuli. During the test phase, the animals' preference for drug-paired compartments are measured and reflected as their reward memory. For this reason, the CPP paradigm is also used to investigate the mechanisms of learning and memory. To establish a long-term reward memory of morphine, variables that affect the establishment of CPP have been adjusted and optimized with the below described protocol[11]. Contextual stimuli are a combination of visual, tactile and odor cues. Variations of contextual stimuli and testing time points are made according to different research purposes. For example in our previous study[12], the somatosensory cues (different floor textures) were mainly used as contextual stimuli to investigate the role of the caudal part of the insular cortex, a brain region with somatosensory function, in the acquisition and maintenance of long-term reward memory. The design of the apparatus and the assignment of rats to preferred/non-preferred sides affect the establishment of CPP. A three-chamber apparatus instead of a two-chamber apparatus is used in the below protocol to avoid forced choices. The balance of two side chambers and the initial assignment of animals into them affect the acquisition of CPP[4,13]. Thus, a balanced design of the side chambers and biased assignment of animals are applied in this protocol to acquire a strong place preference. Maintenance of long-term reward memory requires more conditioning trials during the conditioning phase. To reduce the time cost during conditioning phase, two conditioning trials per day are carried out and morphine/saline injections are counterbalanced to avoid the acute withdrawal effect of morphine in the saline paired trials[4].

Reinstatement animal models[14] have been established to model the preoccupation/anticipation stage of drug addiction cycle. CPP is also utilized to study reinstatement that models the relapse

to addictive behavior[5]. Relapse is one of the barriers in the treatment of drug addiction. Craving is one cause of repeated relapse. The craving for drugs increases constantly during the abstinence period, a phenomenon termed incubation of craving[15,16]. Incubation of craving is widely studied in the drug self-administration paradigm but not the CPP paradigm. The use of the CPP paradigm to reflect incubation of craving was first proposed by Li *et al.*[17]. Their results[17] showed that the CPP scores of low doses of morphine (1 mg/kg and 3 mg/kg) but not a high dose (10 mg/kg) increased progressively during the first 14 days of abstinence. Similarly, mice injected with a low dose of cocaine showed increased CPP scores over time[18]. The increased CPP scores of the low doses of morphine were used to reflect incubated craving[17]. Both duration spent in and number of entries into the drug-associated environment are suggested to reflect the reinforcing conditioned responses in the CPP paradigm[19]. Most of the recent studies use CPP scores to directly or indirectly reflect time spent in drug-paired chambers while the number of entries to side chambers as a parameter is often neglected. For these reasons, a morphine dose of 10 mg/kg was used and the number of entries was measured in our study[11]. The results show that entrances of rats to the side chambers increased significantly during the abstinence period[11]. This indicates that both CPP scores and number of entries are useful parameters in assessing increased craving for drugs and the CPP paradigm is applicable to model the incubation of craving phenomenon. However, to reduce the number of animals required, a group of morphine-experienced animals is tested repeatedly; thus, the effect of extinction could not be excluded with the current within subject design. A between subject design with the same protocol can overcome this limitation.

The following describes the CPP protocol that established long-term reward memory and modeled increased craving. Briefly, after three pre-conditioning days, rats go through six conditioning days with two conditioning trials per day and then are tested at different time points after the last conditioning trial. In this CPP paradigm, a three-chamber polyvinyl chloride (PVC) box used as the CPP apparatus is constructed and the floor of the apparatus is balanced. The apparatus consists of two large black side chambers (30 cm × 25 cm × 30 cm, L×W×H) and one middle white chamber (11 cm × 25 cm × 30 cm, L×W×H) with white smooth PVC floor. The two side chambers have different floor textures as somatosensory cues: one with a grid plexiglass floor, the other with a rough PVC floor.

PROTOCOL:

The experimental procedures were performed in accordance with the guidelines for the National Care and Use of Animals and the experiments were approved by the Institutional Animal Care and Use Committee at Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences.

1. Animal Habituation

1.1. Obtain male adult rats from animal laboratory and maintain them under a 12 h light/dark cycle (light on from 7:00 to 19:00) with free access to food and water.

1.2. Handle the animals by playing with them or patting them twice per day for two weeks before the experiments.

1.3. Randomly assign animals into saline or morphine groups.

2. Drug Preparation

2.1. Prepare morphine hydrochloride as 10 mg/mL and inject rats intraperitoneally (*i.p.*) at a volume of 1 mL/kg.

2.2. As a control, inject rats with saline at a volume of 1 mL/kg.

3. CPP Apparatus

3.1. Construct a three-chamber CPP apparatus with different tactile (floor) cues and test rats' initial preference to two side chambers. The apparatus consists of two large black side chambers (30 cm × 25 cm × 30 cm, L×W×H) and one middle white chamber (11 cm × 25 cm × 30 cm, L×W×H) with white smooth PVC floor. The two side chambers have different floor textures as somatosensory cues: one with a grid plexiglass floor, the other with a rough PVC floor.

3.2. Vary the combination of tactile cues to make sure that the apparatus is unbiased, since a biased apparatus is susceptible to yielding false positive results[4].

Note: An important methodological issue in the design of the CPP apparatus is whether the apparatus is "biased" or "unbiased"[4]. If one group of untrained rats shows no initial preference for one side chamber over another, the apparatus is considered "unbiased". Otherwise, consider it as "biased"[13].

4. CPP Procedure

4.1. Establish the morphine-induced CPP paradigm mainly according to previous studies[21,22] with modification of conditioning time points, number of conditioning sessions, conditioning trial period and testing time points[20]. A short description is given below, of which the experimental schedule is depicted in **Figure 1**.

4.2. Pre-conditioning phase

4.2.1. During this phase (day 1 to 3), remove guillotine doors to allow free access to the entire apparatus and place the animal in the middle chamber.

4.2.2. Record the activity of the animals for 15 min.

4.2.3. Calculate the time spent in the two side chambers on day 3 as the baseline and refer this as T0.

4.2.4. Remove rats that enter less than -four times to either of the side chambers from the experiment[23] as these rats are likely to have problems in general well-being (*e.g.*, dirty fur and having tumors). Check the health state of the animals during the whole experimental period.

4.3. Conditioning phase

Note: Conditioning phase includes 6 days (day 4 to 9).

4.3.1. For morphine-conditioned subjects, inject rats *i.p.* with morphine (10 mg/kg) or saline (1 mL/kg) alternately in the morning (9:00) and evening (19:00) sessions (10 h apart).

4.3.2. Confine rats immediately after each injection to its drug-paired (non-preferred) or saline-paired (preferred) chamber for 45 min (**Figure 1B**).

4.3.3. Keep the video recording system on.

Note: According to our experience, around 5% of rats have the respiratory depression after the first injection of morphine (10 mg/kg). In such a case, to increase the survival rate of respiratory depressed rats, get the rat out of the conditioning chamber, pull its tongue out and put it on a white tissue paper in its home cage.

4.3.4. For saline-conditioned controls, give rats saline injections (1 mL/kg) prior to exposure to one side chamber of the CPP apparatus during the morning session and to the other side during the evening session for 45 min/session in line with morphine group (**Figure 1C**).

4.4. Testing phase

4.4.1. During this phase, remove guillotine doors to allow free access to the entire apparatus and place the animal in the middle chamber to let them freely explore the whole apparatus for 15 min.

4.4.2. Record their activity during this 15 min.

4.4.3. Carry out three post-conditioning tests, *i.e.,* test 1 (T1), test 2 (T2) and test 3 (T3), 2 days, 10 days and 18 days after the last conditioning session in a morphine-free state, respectively (**Figure 1A**).

4.4.4. Vary testing time points for different experimental goals.

Note: To minimize the number of rats required, a within-subject design is employed. However, to exclude the extinction effect, a between subject design is required.

[Place **Figure 1** here]

5. Data Analysis

5.1. Calculate the CPP score in morphine(saline) group as: CPP score = the time spent in the drug-paired (non-preferred) chamber/ the total time spent in both conditioning chambers.

5.2. Perform data analysis of CPP scores and entrances to side chambers with appropriate statistical methods using SPSS.

5.3. Present all behavioral data as mean ± SEM, where SEM is the standard error of mean. Consider values of $p<0.05$ as statistically significant and indicate by asterisks (*) and octothorpesin (#) in the figures.

REPRESENTATIVE RESULTS

The following representative results show that a morphine CPP can be successfully established by the protocol described above and long-term morphine reward memory (**Figure 2**) can be maintained. The number of entries to side chambers, analogous to number of active responses in the self-administration paradigm, reflects the reinforcing property of morphine. The increased number of entries (**Figure 3**), indicating the incubation of craving, was observed using this morphine CPP paradigm.

CPP memory could last at least 18 days after the last conditioning session.

Twenty-one rats in the morphine group and 10 rats in the saline group were used to test the expression of the long-term rewarding memory. A mixed ANOVA revealed significant differences in the interaction between group and test ($F(3,87) = 4.973$, $p = 0.003$), test ($F(3,87) = 18.237$, $p< 0.001$) and group ($F(1,29) = 11.413$, $p = 0.002$). Significant differences among tests ($F(3,60) = 46.628$, $p < 0.001$) in the morphine group but not saline group($F(3,27) = 1.576$, $p = 0.218$) were detected by a one-way repeated ANOVA. The multiple comparison analysis with Bonferroni adjustment showed significant differences between the three tests and T0 in the morphine group. As shown in **Figure 2**, the morphine reward memory lasted at least 18 days and decreased in a time-dependent trend. Data are obtained from a previous published study[11].

Entrances to side compartments in morphine group increased progressively

Morphine group

To test the trend of entrances, group (saline-paired and morphine-paired) as between-subjects factor and test (T0, T1, T2 and T3) as within-subjects factor were set for a mixed ANOVA. No significant differences in the interaction between group and test ($F(2.311,92.449) = 1.915$, $p = 0.147$) and group ($F(1,40) = 0.898$, $p = 0.349$), but significance in test ($F(2.311,92.499)= 24.243$, $p < 0.001$), were detected. The multiple comparison analysis with least significant difference (LSD) test showed significant differences between T1 and T0 ($p<0.001$), T2 and T0 ($p < 0.001$) and T3 and T0 ($p < 0.001$) in the morphine group in morphine-paired entrances. Concerning saline-paired compartments, statistical significances existed between T2 and T0 ($p = 0.002$) and T3 and T0 ($p = 0.003$) but not T1 and T0 ($p = 0.246$), which is similar to the pattern of incubation

of heroin craving[24]. In general, entrances to side chambers in the morphine group increased time-dependently.

Rats entered more frequently to the morphine-paired compartment than saline-paired compartment at T1 ($t(1,21) = -2.833$, $p = 0.010$) and T2 ($t(1,21) = -4.458$, $p = 0.0002$) but not T3 ($t(1,21) = -0.471$, $p = 0.642$) (two-tailed paired t-tests).

Saline group

Similar with the morphine group, a mixed ANOVA was applied and revealed no significant differences in the interaction between group and test ($F(3,54) = 0.345$, $p = 0.793$), test ($F(3,54) = 1.793$, $p = 0.159$) and group ($F(1,18) = 0.151$, $p = 0.702$).Thus, entrances to side chambers in the saline group were stable over time.

FIGURE AND TABLE LEGENDS

Figure 1. Experimental procedures. (A) Experimental procedures of the whole CPP process. (B) Experimental schedule of the conditioning phase of morphine group. (C) Experimental schedule of the conditioning phase of saline group.

Figure 2. CPP memory can last at least 18 days but declines with time. Memory retention of CPP in the morphine group (n = 21) and the saline group (n = 10) was assessed by measuring the CPP score for several time points after last conditioning (T1, 2 days; T2,10 days and T3, 18 days). All values of CPP scores are mean ± SEM. ### indicates $p<0.001$ and ## means $p < 0.01$ compared with saline group; *** indicates $p < 0.001$ compared with T0 in the morphine group. This figure has been modified from Sun *et al.*,2017[11].

Figure 3. Entrances to side-chambers in morphine and saline groups. Entrances to two side chambers in the morphine group (n = 21) and the saline group (n = 10) were assessed at several time points after the last conditioning (T1, 2 days; T2,10 days and T3, 18 days) and compared with T0. All values of entrances are mean ± SEM. In the morphine group (A), entrances to the morphine-paired side increased very significantly from T1 to T3 while entrances of saline-paired chamber only increased significantly from T2. Paired t tests with morphine-paired compartment and saline-paired compartment showed very significant increases in both T1 and T2. In the saline group (B), there are no significant changes in entrances to both sides. This figure has been modified from Sun *et al.*,2017[11].

DISCUSSION

Here, a method using a morphine CPP paradigm is described to establish long-term reward memory in rat with somatosensory cues as conditioned context. To build such a long-term reward memory, a previous protocol[21] with a balanced CPP apparatus is modified and the saline and morphine sessions are set at least 10 h apart. In addition, more conditioning sessions are added during the conditioning phase to enhance the associative learning. Apart from the CPP scores, the entrances to two side chambers are measured and the pattern of increased

entrances to side chambers is similar with that of the increased active responses in the self-administration paradigm.

Several factors influence the establishment of the CPP paradigm mentioned above. One key factor is the design of the apparatus: rats in general should have similar preference to both side chambers to ensure the apparatus is balanced. An unbalanced apparatus can mask the acquisition of CPP due to the initial high preference to one side chamber[13]. Therefore, extreme preference to one side chamber (more than 90% of the total time in any of the two side chambers) should be avoided when choosing the texture of the floor or other contextual cues. For different experimental purposes, experimenters can balance the CPP apparatus by modifying the contextual stimuli. For balanced apparatus, two ways of experimental design are commonly used[4]. One biased way is to pair drugs with the non-preferred chamber with all subjects; an unbiased way is to pair half of the animals with drugs in the non-preferred chamber, the other half with the preferred chamber. Both ways can establish CPP[13] but studies[17,25] with the latter design always exclude many subjects due to their strong preference to one side chamber. As individual vulnerability to drug addiction[26-28] exists, a biased design was chosen in this study to avoid exclusion of healthy subjects. One limitation of biased design is the effect of inconsistent preference to side chambers. As shown in **Figure 2**, rats in the saline group have consistent low CPP scores for the initial non-preferred chamber. Thus, the possibility that inconsistent preference to side chambers could affect the CPP scores in the morphine group is excluded by setting the saline controls. To avoid the ceiling effect of the preferred chamber, we use the initial non-preferred chamber as the morphine paired chamber. To avoid the effect of novelty and stress on the baseline preference to side chambers, three pre-conditioning sessions are performed, and the third session is used as the baseline. The fourth factor is the interval between morphine and saline sessions. In our paradigm, we choose intervals of at least 10 hours to avoid the withdrawal effect in the saline conditioning period. The dosage of morphine is also an important factor. Extremely low doses of morphine, such as 0.01 mg/kg[6], did not induce place preference. A dose of 10 mg/kg induced the highest CPP score[17] in rats and is commonly used in rat morphine CPP studies[17,21,23]. However, 10 mg/kg morphine (*i.p.*) could induce respiratory depression in around 5% of rats. Lower doses of morphine, such as 5 mg/kg, could be used in future studies. For other species, such as tree shrew, tolerance to drugs should be considered when building the CPP paradigm. Since tree shrew develops fast tolerance to morphine, increased doses of morphine should be used during the conditioning phase[29,30].

With the modified protocol, a long-term reward memory is established for at least 18 days after 6 days of conditioning[11] (**Figure 2**). Future studies should measure whether CPP could be maintained for a longer time. Repeated relapse is the major barrier in treating drug addiction. Stress, environmental cues and drugs could induce relapse. After extinction of the CPP memory, this model could be used to model relapse induced by stress or drugs.

The entrances to the drug-paired chambers in the morphine group increased in the abstinence period[11] (**Figure 3**), which is similar with the increased active responses in the self-administration paradigm. However, entrances to the saline-paired chamber also increased with

the CPP paradigm in both rats and tree shrews[11], which is different from the inactive responses of the self-administration paradigm. This difference might due to the different underlying principles of the two paradigms: one is classical conditioning, the other is operant conditioning. Besides, tree shrews with food CPP experiences did not show such a pattern of increased entrances[30]. Therefore, the increased entrances to both side chambers in morphine-experienced animals are thought to represent incubated craving of drugs. Although the limitation of using repeated measures in one subject cannot be excluded, the pattern of increased entries was conserved in rats[11], tree shrews[11,30] and monkeys[31]. Nonetheless, a between subject design during the abstinence period is highly recommended for future studies to exclude the extinction effect of repeated measurements.

Apart from the increased entrances, CPP scores with low doses of morphine (1 mg/kg and 3 mg/kg) increased progressively during the abstinence period[17]. These results suggest that the CPP paradigm could also model the incubation of craving phenomenon. However, an increased CPP score might also represent an increased memory for the conditioned stimulus. In this aspect, entrances to the side chambers in a three-chamber apparatus, which are similar to active responses in the self-administration paradigm, more closely resemble the search for drugs or the rewarding effect of drugs. It would be interesting to check the trends of entrances to side chambers during the abstinence period in rat CPP paradigms of lower doses of morphine. Future studies with other drugs and mice should be done to verify whether this phenomenon is conserved across drugs of abuse/species.

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DISCLOSURES

The authors have nothing to disclose.

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Figure 1

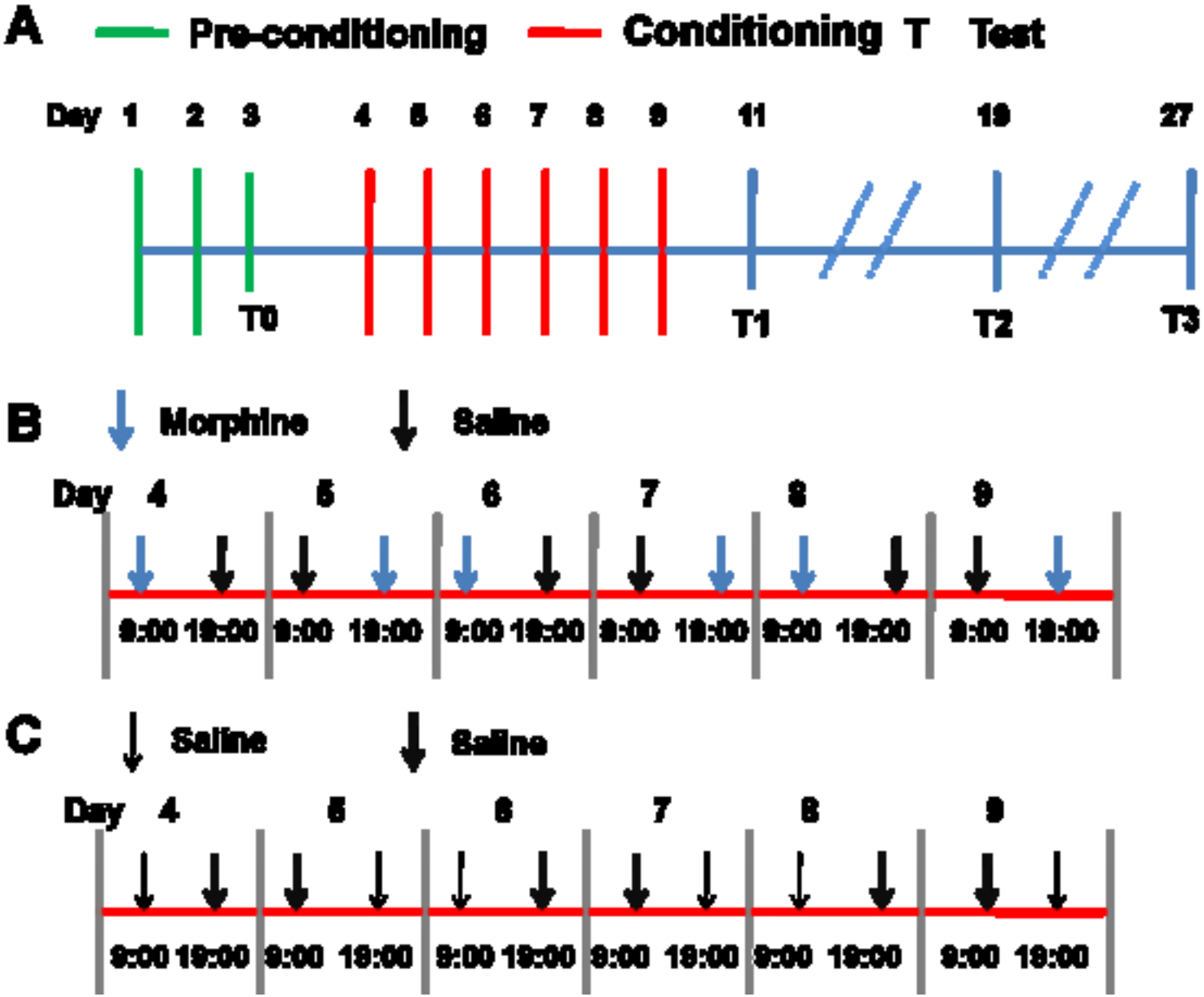


Figure 2

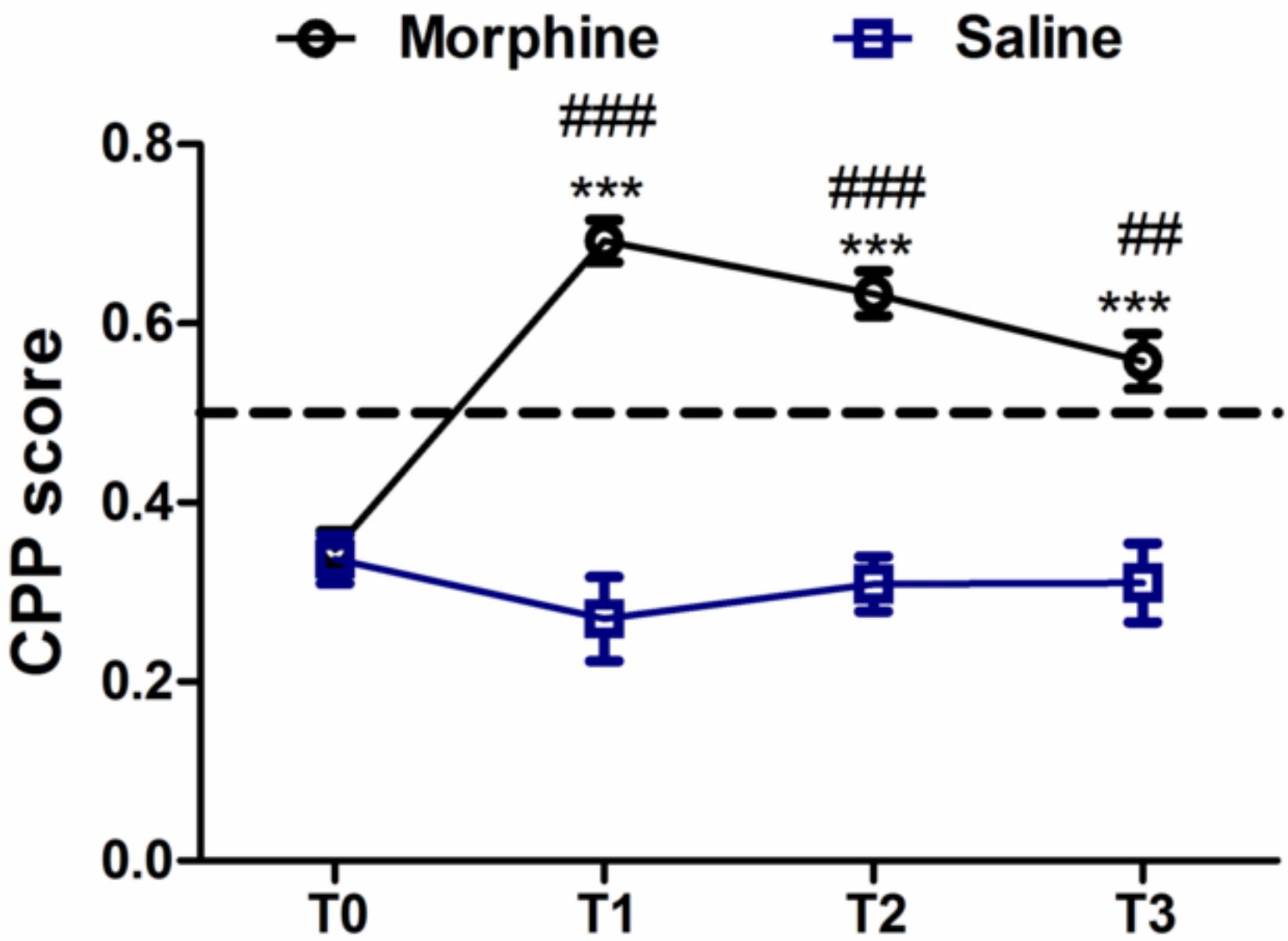
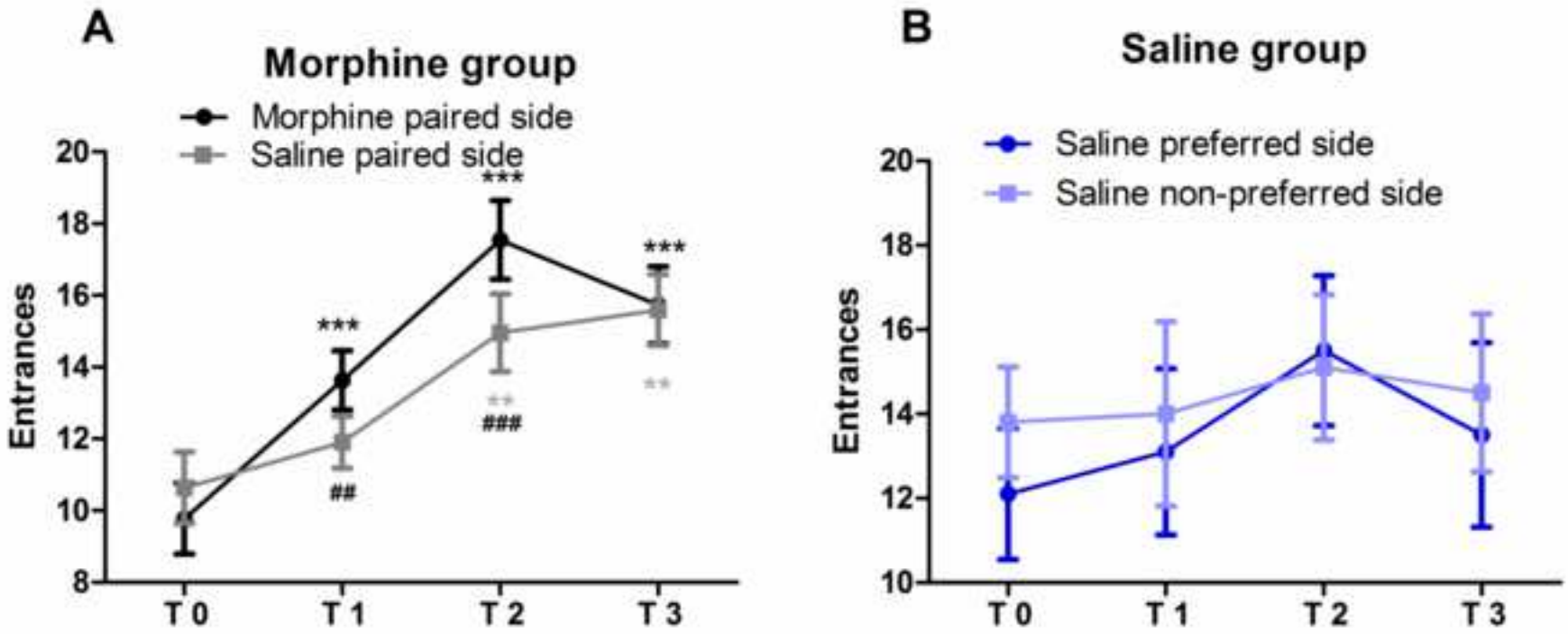


Figure 3



Materials Table

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Morphine	Shenyang First Pharmaceutical Factory		
CPP apparatus/Camera	Anlai Technology Company		
SPSS	IBM Inc		

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Title of Article: Establishment of a morphine-induced conditioned place preference paradigm to measure long-term reward memory and incubation of craving in rat

Author(s): Yongmei Sun, Gaowei Chen, Kuikui Zhou, Yingjie Zhu

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Date:	28.04.2018

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Dear Dr. DSouza,

On behalf of other co-authors and myself, I would like to thank you for giving us the opportunity to revise our manuscript JoVE58384. Based on the editorial and reviewers' comments, changes have been made and highlighted in the manuscript. For the details of our rebuttal letter, please see them in a separate document.

With kind regards,

Yongmei Sun, Ph.D

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

--Thank you for this comment. The manuscript has been thoroughly proofread by a service company. Please see the certificate of this company in the uploaded file.

2. Unfortunately, there are a few sections of the manuscript that show text overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 56-61, 95-106, 163-173, 179-183, 187-188, 197-199, 208-210, 214-216.

-- All of the overlapped sessions have been revised substantially.

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4. Figure 2 legend: Please spell out SEM.

-- We spelled out SEM, which is Standard Error of Mean, in part 5.3 of the revised manuscript.

5. Please shorten the title if possible.

--The title has been changed to **A conditioned place preference protocol for measuring incubation of craving in rat.**

6. Keywords: Please provide at least 6 keywords or phrases.

-- One more key word, *i.e.* incubation of craving, was added as suggested.

7. Please expand the Short Abstract to also describe the applications of the protocol.

-- The applications of the protocol was added and highlighted as suggested.

8. Please rephrase the Long Abstract to more clearly state the goal of the protocol.

--The Long Abstract part was re-written to make the goal of the protocol clearer and the future applications of this model.

9. Please revise the Introduction to include all of the following:

a) A clear statement of the overall goal of this method

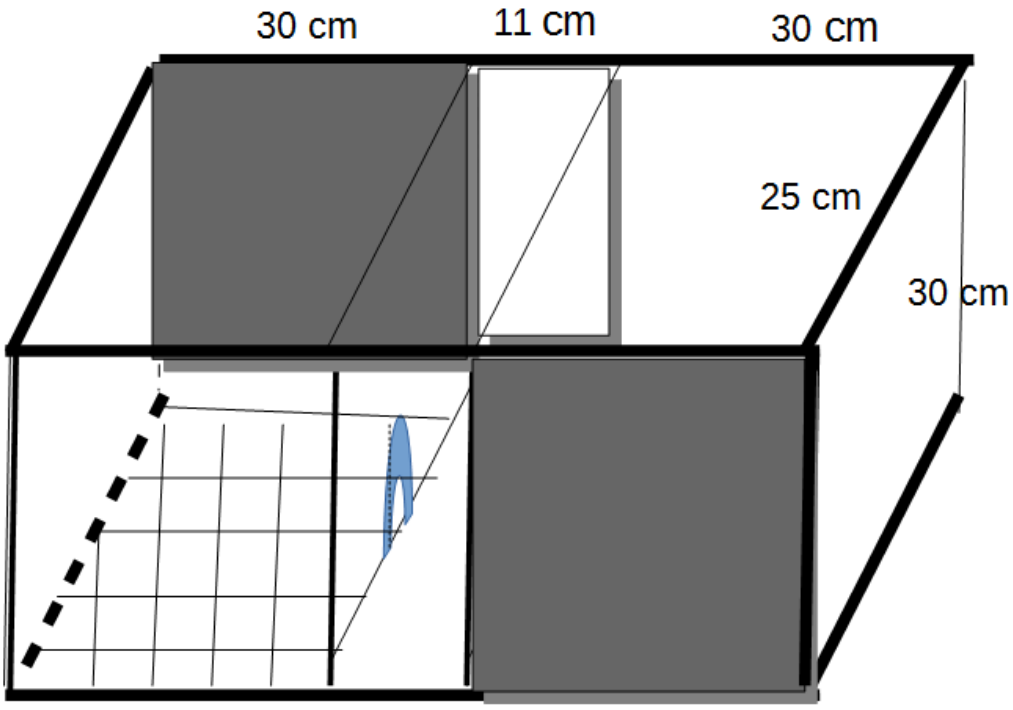
b) The rationale behind the development and/or use of this technique

c) The advantages over alternative techniques with applicable references to previous studies

d) A description of the context of the technique in the wider body of literature

e) Information to help readers to determine whether the method is appropriate for their

application
--- The introduction part has been thoroughly revised and highlighted on pages 1-3.
10. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.
--An ethics statement has been added under Protocol on page 3 as suggested.
11. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
-- The whole protocol section has been revised to avoid the use of personal pronouns.
12. 1.2: This is unclear. What does “handle the animals” mean?
--In order to decrease the stress level of animals during the experiment, experimenters usually " handle the animals" by playing with them or patting them to let them get used to human touch or manipulation later on. For an example, please see the video of one new published JOVE protocol (https://www.jove.com/pdf/55864/) and description part line 169-170 on page 3 of this protocol.
13. 2: Please remove commercial language: Shenyang First Pharmaceutical Factory.
-- The company information has been removed.
14. 3: A schematic of the apparatus and the chamber setup as Figure 1 would greatly aid in the protocol.
-- Although we made the following schematic of the apparatus, we think maybe it is better to show it vividly in the video. Besides, we think it is also very important that other researchers can vary the apparatus according to their research purposes following the rules of apparatus design. Therefore, we think it is better to add it in the video part instead of the manuscript part.



15. 3/4/5: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please revise the protocol to contain only action items that direct the reader to do something. The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”
-- The Protocol has been revised to discrete steps.

16. Please revise to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.
-- One paragraph has been added in the result session to explain the representative

results on page 6 (line 277-282).
17. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
-- The discussion part has been sufficiently revised based on the editorial and reviewers' comments and highlighted in the manuscript on pages 8-10.
18. References: Please do not abbreviate journal titles.
-- The journal titles are abbreviated referring to the author guidelines and other publications of JOVE (one example is the recent published one https://www.jove.com/pdf/55864/). We are confused by this comment. We appreciate if you could provide us the new version of reference format.

Reviewers' comments:

Reviewer #1:
In this manuscript, Sun and colleagues presented a behavioral procedure on measuring morphine conditioned place preference. In this procedure, they used a biased design and found that CPP to morphine-conditioned chamber peaked 2 days and lasted 18 days after the last conditioning session. In the meanwhile, the entrance to morphine-conditioned side progressively increased after the last conditioning session, which authors stated that this resembles incubation of craving observed with self-administration procedure in rats. Overall, the manuscript is clearly-written and the steps are easy to follow. It will be helpful for others who want to establish this procedure in their own research groups. However, I have a few minor concerns.

-- Thank you for your comments on this manuscript. We have carefully revised the manuscript based on editorial and reviewers' comments and suggestions.

1. Please cite the historical paper Mucha et al., 1982 when introducing CPP procedure.
-- This reference was added in the first paragraph of the introduction on page 1(lines 81-82) as suggested.
2. The Introduction lacks citations for "incubation of craving".
-- References of "incubation of craving" (line 125, line 128) have been added.
3. The Introduction also lacks information on the premises of establishing CPP procedure on morphine, the advantage and differences of current procedure vs previous procedures (such as 2008 JN paper from Lin group). These should also be included in the discussion.
-- The premises of establishing morphine CPP (lines 78-82 on page 1) have been added. And the advantage and differences of different procedures have been

discussed in the discussion part (line 424-434, page 9).

4. Here authors used the with-in subject design to study the different time points, which is acceptable. However, when interpreting the entrance data, the with-in subject design may be a confounding factor. During the first test (2 days), while the CPP score was the highest, the rats already went through extinction. During the following tests, rats may enter the chamber more often because of weakened reward memory/extinction learning, instead of increased craving, especially that the animal enhanced the entrance to both the saline and morphine-paired side. Unless the same results are obtained from a between-subject design, I am not convinced that the time-dependent increase of entrance resemble increased craving in these animals.

-- We agree with you that a between subject design makes the conclusion more solid. It is possible that rats entered more often to the side chambers due to weakened reward memory/extinction learning. However, craving/increased craving for the rewarding feeling after repeated testing might also be one of the driving forces for the increased entrances. Although entrances to both side chambers increased, rats entered more frequently to morphine-paired side than saline-paired side at T1 and T2, indicating that rats could clearly distinguish these two side chambers. In our and others' studies, morphine-experienced rats[1], tree shrews[1,2] and monkeys[3] showed increased entries to both side chambers during the morphine-free tests[1]. No such increase of entries during abstinence of food was found in tree shrew food CPP paradigm[2], indicating that it is a drug-specific phenomenon. As the underlying principles of CPP and self-administration paradigms are different: one is classical conditioning, another is operant conditioning. Taken together, we tend to think the increased entrances to both side chambers represent the incubated craving in these three species. We think it is an interesting point for discussion so we add this part in the discussion.

5. Step 4.2.2, Note: Did the rats that had respiratory depression continue their study after they returned to normal behavior? How long did it take? Or they were excluded for the study on that particular day?

-- In our pilot study, one rat with respiratory depression died after putting back to the home cage. Then we learned this way to manage the respiratory depression. According to our experience with 5 depressed rats out 100 morphine experience rats, it took them 15-60 minutes to recover. In this manuscript, 1/21 rat experienced respiratory depression and this rat was excluded for the first morphine conditioning but continued with the rest of experiments.

6. For Figure 1, it would be helpful to show the procedure timeline for saline group, which reminds the readers that there were two groups in this study.

-- The procedure timeline for saline group has been added in Figure 1 as suggested.

7. Analysis of entrances in morphine group: authors used a mixed ANOVA with group (saline-paired and morphine-paired) as the between-subject factor, which is confusing to me. If I understand this correctly, authors analyzed in the morphine rats, the entrances to saline side vs morphine side. If this is the case, isn't the side (saline vs morphine) supposed to be with-in the same subject? The same question also applies

to saline control group.
-- For a subject like rat, the two side chambers are different chambers. Therefore, chamber is used as a group factor with two levels. Same with the saline group.
8. As mentioned before, it would be beneficial to the readers if author could briefly discuss different procedures previous literatures used to study morphine CPP. In addition, one important factor for CPP is the dose of morphine, which should be discussed.
-- We have added this part in the discussion as suggested.
Reviewer #2:
Manuscript Summary:
This manuscript had been tried to establish a rat conditioned place preference (CPP) paradigm that mainly employed somatosensory cues. The authors have tried to investigate the neural mechanisms underlying long-term reward memory and incubation of craving in the future studies. However, this manuscript suffers from several major concerns, some of which are commented forthwith.
-- Thank you for your comments on this manuscript. We have carefully revised the manuscript based on editorial and reviewers' comments and suggestions.

Major Concerns:
1. The paper is lack of novelty because this protocol has been previously used in other studies.
-- Although CPP is commonly used, the number of entrances as a parameter is often neglected. Here we showed that number of entries to side chambers is an interesting parameter to reflect the craving of morphine-experienced animals and observed increased entrances to side chambers with this protocol.
We modified several variables of this protocol from previous studies: conditioning time points, length of conditioning period, number of conditioning days, contextual stimuli and testing days. And we found that the reward memory could last at least 18 days.
2. The aim of study is not clear. In the first sentence of abstract, the authors have emphasized on assessing reward induced by somatosensory cortex while it is not main purpose of this study.
-- We have revised the abstract and introduction to clearly express the aim of this protocol.
3. Introduction of this manuscript is short and it is not coherent. It looks like several unrelated paragraphs.
-- Introduction has been revised based on your and other reviewers' comments.
4. What is reason for using dose of Morphine (10 mg/kg)?
-- According to a previous study[4], 10 mg/kg morphine induced the highest CPP score , which maintained at the same level for at least 14 days.
5. In page 3, the authors of the manuscript mentioned that "around 5% of rats have the respiratory depression after the first injection of morphine (10 mg/kg)." It would be better to test a lower dose like 5 mg/kg to prevent of respiratory depression.
-- Lower doses of morphine (1 mg/kg and 3 mg/kg) could induce morphine CPP but the CPP score increased during the abstinence period using an unbiased design as

mentioned above[4].To exclude the impact of increased CPP score, we used a 10 mg/kg dose. However, we agree with you that it is interesting to check a lower dose, such as 5 mg/kg. We added this point in the discussion part.
6. It would be better to continue the experiment until extinction time (more than 18 days).
-- We agree with this point and add it in the discussion part.
7. The quality of English writing is not good enough, there are several grammatical errors in the manuscript. Therefore, the whole manuscript should be revised and re-written to correct language errors.
-- The whole manuscript has been thoroughly proofread.

Reviewer #3:
Manuscript Summary:

The present study was designed to measure the long-term reward memory and participation of somatosensory cortices in morphine-induced conditioned place preference paradigm. The design protocol was described to study the relapse model in addictive rats. However, few concerns need to be justified so that the efficacy of the protocol can be demonstrated.
-- Thank you for your comments on this manuscript. We have carefully revised the manuscript based on editorial and reviewers' comments and suggestions.

Major Concerns:
1)Section 4.1.3:
Note: Remove rats that enter less than 4 times to either of the side chambers from the experiment as this correlated 100% with problems in general well-being (e.g., dirty fur and having tumors).
How do the authors claim that the no. of entry correlates 100% with general health such as tumors?
-- We monitored the healthy condition of each animal. The healthy condition of animals can be judged by their fur quality: animals that had dirty fur are unhealthy. We dissected the animals with clean fur but less than 4 times entries and found tumors in their organs. To avoid misunderstanding of 100% correlation, we revised this sentence as " as these rats are likely to have problems in general well-being (e.g., dirty fur and having tumors)" Besides, we added one reference[5] that used the same exclusion standard.
2) What is the rational of injecting twice a day (morphine and saline) instead of daily injection with alternate morphine and saline?
-- Increasing conditioning trials (twice a day instead of once per day) could enhance CPP learning and memory. In our pilot studies, we tried the protocol with daily injection with alternate morphine and saline, but the CPP memory was weaker and did not last at T2.
3)Section 4.2.2:
To overcome the respiratory depression, the author suggests get the rat out of the

chamber, pull its tongue out and put it on a white tissue paper in their home cages. These do not make sense since the respiratory depression can only be cured by injection of naloxone.

-- Naloxone and nalmefene hydrochloride, are clinically used together with oxygen inhalation to treat respiratory depression caused by morphine. In our pilot study, one rat with respiratory depression died after putting back to its home cage. Then we learned this way to manage the respiratory depressed rats. The purpose of our management is to let rats breathing more oxygen and avoid breathing in small particles. According to our observation and personal communication with Prof. Taco J. De Vries at VU medical center, the respiratory depressed rats can spontaneously recover to normal behavior after 15-60 minutes without any naloxone injections. These difference between rats and human might due to that these two species have different reactions to the side effects of morphine.

4) It is confusing when the CPP score in the saline group was calculated by dividing the spent in the non-preferred chamber by the total time spent in both conditioning chambers.

-- The saline group is the control group. In the morphine group, the morphine paired side is the non-preferred side. For this reason, the CPP score of the saline group was calculated in the same way.

Minor Concerns:

English proofreading is required in this manuscript.

--- The whole manuscript has been thoroughly proofread.

The F values in Morphine group is incorrect.

A mixed ANOVA with group (saline-paired and morphine-paired) as between-subjects factor and

198 test (T0, T1, T2 and T3) as within-subjects factor revealed that there were no significant

199 differences in the interaction between group and test ($F(2.311,92.449)=1.915$, $p=0.147$) and

200 group ($F(1,40)=0.898$, $p=0.349$), but significance in test ($F(2.311,92.499)=24.243$, $p<0.001$).

-- When performing a mixed ANOVA, the Mauchly's Test was carried out to test the Sphericity of the data. As the data of the number of entrances did not meet the Sphericity assumption, we reported the Greenhouse-Geisser results. For details, please see the table below.

Tests of Within-Subjects Effects

Measure: Entrances

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
test	Sphericity Assumed	915.548	3	305.183	24.243	.000	.377
	Greenhouse-Geisser	915.548	2.311	396.133	24.243	.000	.377
	Huynh-Feldt	915.548	2.523	362.948	24.243	.000	.377

	Lower-bound	915.548	1.000	915.548	24.243	.000	.377
test * group	Sphericity Assumed	72.310	3	24.103	1.915	.131	.046
	Greenhouse-Geisser	72.310	2.311	31.286	1.915	.147	.046
	Huynh-Feldt	72.310	2.523	28.665	1.915	.142	.046
	Lower-bound	72.310	1.000	72.310	1.915	.174	.046
Error(test)	Sphericity Assumed	1510.643	120	12.589			
	Greenhouse-Geisser	1510.643	92.449	16.340			
	Huynh-Feldt	1510.643	100.901	14.972			
	Lower-bound	1510.643	40.000	37.766			

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