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Original Article

The reinstatement of the expression phase of morphine-induced conditioned place preference in male Wistar rats under ventral tegmental area stimulation and brief inactivation

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Abstract

Background and purpose: Previous research has found that the electrical stimulation of the ventral tegmental area (VTA) is involved in drug-dependent behaviors and plays a role in reward-seeking. However, the mechanisms remain unknown, especially the effect of electrical stimulation on this area. Therefore, this study aimed to investigate how the electrical stimulation and the temporary inactivation of VTA affect the morphine-dependent behavior in male rats.

Experimental approach: The adult Wistar male rats were anesthetized with ketamine and xylazine. The stimulation electrode (unilaterally) and the microinjection cannula (bilaterally) were implanted into the VTA, stereotaxically. Then, the rats underwent three-day of repeated conditioning with subcutaneous morphine (0.5 or 5 mg/kg) injections, in the conditioned place preference apparatus, followed by four-day forced abstinence, which altered their conditioning response to a morphine (0.5 mg/kg) priming dose on the ninth day. On that day, rats were given high- or low-intensity electrical stimulation or reversible inactivation with lidocaine (0.5 µL/site) in the VTA.

Findings/Results: Results showed that the electrical stimulation of the VTA with the high intensity (150 µA/rat), had a minimal effect on the expression of morphine-induced place conditioning in rats treated with a high dose (5 mg/kg) of morphine. However, the reversible inactivation of the VTA with lidocaine greatly increased place preference in rats treated with a low dose (0.5 mg/kg) of morphine. Additionally, the reinstatement of 0.5 mg/kg morphine-treated rats was observed after lidocaine infusion into the VTA.

Conclusion and implications: These results suggest that VTA electrical stimulation suppresses neuronal activation, but the priming dose causes reinstatement. The VTA may be a potential target for deep brain stimulation-based treatment of intractable disorders induced by substance abuse.

Keywords: Deep brain stimulation; Dopamine; Drug addiction; Rat; Ventral tegmental area.

INTRODUCTION

Drug addiction is a compulsive pattern of drug-seeking and drug use behavior with recurrent episodes of abstinence and relapse and a loss of control despite negative consequences. Addictive drugs promote reinforcement by increasing dopamine (DA) in the mesocorticolimbic system, which modifies excitatory glutamate transmission within the reward circuitry, causing reward processing to be hijacked (1). Similar to many chronic disorders, addiction is characterized by relapse and remission cycles (2).

The first motivation for using drugs of abuse comes from their rewarding qualities. Repeated drug exposure leads to sensitivity to specific behavioral effects of drugs, which may aid in the development of addiction (3). Sensitization is a significant issue that contributes to opioid drug addiction. The behavioral and rewarding effects of morphine may become less noticeable (tolerance) or more noticeable (sensitization) with repeated treatment (4).

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Reinforcement, reward, and aversion are fundamental processes that guide appropriate behaviors. Dopaminergic neurons in the ventral tegmental area (VTA) and the descending pathways of the limbic system have long been thought to be important systems for modulating these behaviors (5).

Morphine sensitization is characterized by increasing DA release and alternations in the sensitivity of mesolimbic dopaminergic D1 receptors, which include those in the striatum, nucleus accumbens (NAc), VTA, hippocampus, and prefrontal cortex (6). Additionally, behavioral sensitization necessitates the activation of the D1-dopaminergic receptor in the VTA, as well as glutamatergic transmission mediated by α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) in the NAc, which correlates with a sustained hyper-reactivity of noradrenergic and serotonergic systems in the locus coeruleus (LC) and dorsal raphe, respectively (7).

Dopaminergic neurons in the VTA are important components of the reward pathway, and their activity is strongly influenced by inhibitory GABAergic inputs. Local VTA interneurons and the neurons of the rostromedial tegmental nucleus are two important sources of GABAergic nerve terminals within the VTA. Furthermore, it has been demonstrated that nitric oxide-induced potentiation of GABAergic synapses on VTA dopaminergic cells is lost following exposure to drugs of abuse or acute stress and electrical stimulation simultaneously activates the numerous GABAergic afferents (8).

In recent investigations, the local anesthetic and voltage-sensitive sodium channel blocker, lidocaine hydrochloride 2%, has been employed to disclose other aspects of brain function when specific target effects are absent (9,10). The inactivation of the VTA pathway by lidocaine infusion into the VTA bi-laterally relieved transient inhibition established hyperalgesia and anti-nociceptive tolerance (11), increased the pain threshold of the adult colorectal distension group (12), effectively relieved symptoms of stress (13), significantly potentiated nicotine-induced conditioned place preference (CPP) reduction (14), and decreased DA release by 50% (15).

It seems that among these studies, deep brain stimulation (DBS) is the least prominent system studied in morphine sensitization. Chronic high-frequency stimulation induces a range of functional changes, from quick physiological to slower metabolic effects, and eventually contributes to the structural reorganization of the brain, so-called neuroplasticity (16). Several brain regions, including the lateral hypothalamic area (LHA) (17) and NAc (18,19) have been examined in this regard. However, further investigations are necessary to clarify the effects of DBS during withdrawal and prevent future relapse (19). Moreover, some evidences have indicated the role of high-frequency DBS on Morphine-induced conditioned place preference (CPP) in the LHA (17) and the orbitofrontal cortex (20) for preventing morphine reinforcement.

The present study investigates the effects of intra-VTA administration of DBS and reversible inactivation by lidocaine on the expression phase of morphine-induced place conditioning in morphine-sensitized rats.

MATERIALS AND METHODS

Animals

The experiments were conducted on adult male Wistar rats, which were an outbred rat strain, weighing 250 - 300 g, obtained from the Royan Institute. All animals were housed in groups of 2 - 3 per cage on wood shavings bedding, in an environmentally controlled room with a 12-h light/dark cycle (temperature of 22-25 °C, the humidity of 60-70%), and access to laboratory chow and tap water *ad libitum*. The experiments were performed between 7:00 a.m. and 7:00 p.m. Before surgery, the rats were allowed to acclimate to the laboratory environment for one week. Behavioral studies were performed during the light phase under dim light in semi-dark conditions. The experimental design is illustrated in Fig. 1.

All animal use protocols and procedures were reviewed and approved by the Ethical Committee of Isfahan University of Medical Sciences (IR. MUI. RESEARCH. REC. 1397. 360). Also, the study was carried out in accordance with the ARRIVE guidelines, the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, and the recommendations of the International Associations for the Guide for the Care and Use of Laboratory Animals (21). We made all possible efforts to minimize animal suffering, reduce the number of animals used, and replace *in vivo* procedures with alternatives.

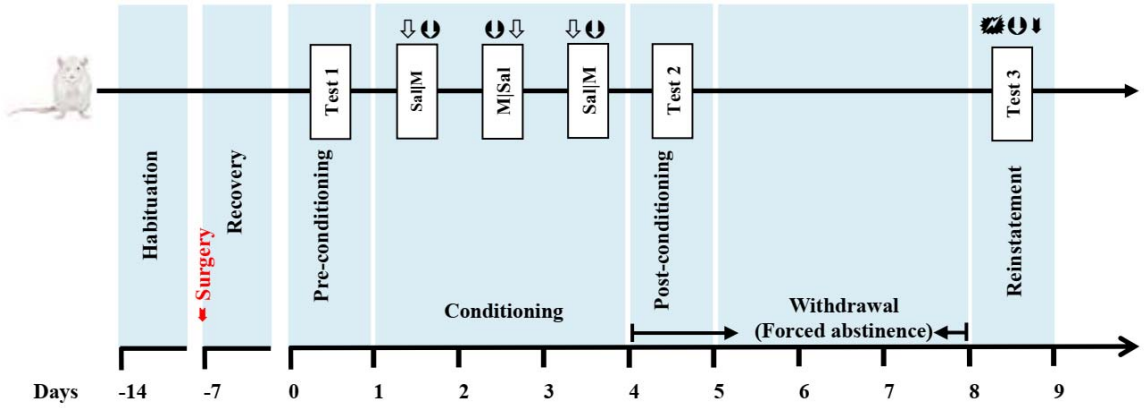


Fig 1. Experiment protocol timeline in the current study. After one week habituation animals underwent stereotaxic surgery (day -7). Then, those were recovered for one week. After recovery, a pre-conditioning test (test 1) was performed to determine baseline preference on day 1. Next, the animals received morphine or saline during the conditioning phase (days 2-4). Then, a post-conditioning test (test 2) was performed on day 5. After that, the rats were faced with a forced abstinence phase. Finally, the animals received electrical stimulation or lidocaine on day 9. Then a priming dose of morphine was injected before the reinstatement test (test 3). Sal, Saline; M, morphine; ⇩, saline injection; ❶, morphine injection; ✸, electrical stimulation; ⬇, lidocaine microinjection.

Drugs

The following drugs were utilized: morphine hydrochloride (Temad Co., Tehran, Iran), sodium chloride 0.9% (Iranian parenteral and pharmaceutical Co., Tehran, Iran), Ketamine (50 mg/mL, Trittau, Germany), Xylazine (Interchemi, Holland), Gentamicin (40 mg/mL, Alborz Darou Co., Tehran, Iran), Ketorolac (30 mg/mL, Exir Pharmaceutical Co., Boroujerd, Iran), Chlorpheniramine (10 mg/mL, Darou Pahksh Pharmaceutical Co., Tehran, Iran), Lidocaine hydrochloride (Lignodic 2%, Caspian Tamin Pharmaceutical Co., Rasht, Iran). All drugs were dissolved in sterile saline (0.9%) just before the experiments. Saline was administered to the control groups.

Experimental groups

Ninety-eight rats were randomly assigned into 17 experimental groups presented in Table 1.

Surgical procedures

Surgery for VTA cannulation

All surgical procedures were performed under a mixture of ketamine and xylazine anesthesia (100/10 mg/kg, intraperitoneal (i.p.)). The animals were placed in a stereotaxic apparatus (RWDLife Science, China) after confirming the absence of a reflexive reaction through the pinch test and shaving their heads to expose the skin. A rostral-caudal incision was made to expose the skull and, the three-dimensional stereotaxic coordinates for the VTA were determined according to the Paxinos and Watson atlas: the incisor bar -3.3 mm, -5.8 mm posterior to the bregma, ± 0.8 mm lateral to the sagittal suture, and -9.3 mm down from the top of the skull (22). Two 23-gauge stainless steel guide cannulas were bilaterally implanted in each rat for microinjection (23). The cannulas were secured with dental cement and affixed to two stainless steel jewelers' screws fastened to the exposed skull. After ending surgery, guide cannulas were obstructed by stainless steel thin rods to keep away from debris or unwanted particles during the recovery period. Then, the rats were housed individually and allowed to recover for 7 days before any behavioral tests.

Implanting the stimulating electrodes

Unipolar stimulation stainless steel wire electrodes with a 0.125 mm diameter bare wire (Polytetrafluoroethylene insulated, Advent, England) uncoated tip were used as the negative polarity stimulating pole. Previous studies have shown that unilateral DBS can be sufficiently effective to generate a desirable result (24,25). The copper electrode was coiled around the screw several times, then covered with dental acrylic cement, as previously described (23).

Based on prior work, a stimulation electrode was implanted exclusively into the right VTA since there is no functional lateralization in the VTA for the acquisition (AQ) and/or expression of morphine-induced CPP in rats (10). After surgery, the animals received gentamicin to prevent infection. In addition, ketorolac (a nonsteroidal anti-inflammatory drug) and chlorpheniramine (an antihistamine) at the dose of 1 mg/kg/day (i.p, for consecutive 3 days) were administered to attenuate pain and allergic conditions, respectively. Also, the animals received saline solution (1 mL/rat/day, i.p. for consecutive 3 days) to prevent dehydration. Animals were then recovered separately in plexiglass cages for 7 days until behavioral trials.

Microinjection method

The steel rods were gently removed from the guide cannulas for drug infusion and replaced with a 30-gauge injection needle (1 mm below the tip of the guide cannula) attached to a Hamilton syringe through a narrow polyethylene tube. Saline or lidocaine infused slowly in a total volume of 1 μL/rat (0.5 μL into each side) over a 60-s period. To promote the diffusion of solutions, the injection needles were left in place for an additional 60 s (26).

Table. 1. Description of experimental groups.

Group	n	Description
Sal	11	Animals received saline (1 mg/kg, s.c.) during the conditioning phase.
M 0.5	5	Animals received morphine (0.5 mg/kg, s.c.)
M 5	6	Animals received morphine (5 mg/kg, s.c.)
Sal + sham-operated	6	Animals with electrode implantation received saline (1 mg/kg, s.c.) during the conditioning phase without electrical stimulation in the reinstatement phase.
Sal + ES 25	5	Animals with electrode implantation received saline (1 mg/kg, s.c.) during the conditioning phase and electrical stimulation of 25 μA in the reinstatement phase.
Sal + ES 150	5	Animals with electrode implantation received saline during the conditioning phase and electrical stimulation of 150 μA in the reinstatement phase.
M 0.5 + sham-operated	9	Animals with electrode implantation received morphine (0.5 mg/kg, s.c.) during the conditioning phase without electrical stimulation in the reinstatement phase.
M 5 + sham-operated	5	Animals with electrode implantation received morphine (5 mg/kg, s.c.) during the conditioning phase without electrical stimulation in the reinstatement phase.
M 0.5 + ES 25	5	Animals with electrode implantation received morphine (0.5 mg/kg, s.c.) during conditioning and electrical stimulation of 25 μA in the reinstatement phase.
M 0.5 + ES 150	5	Animals with electrode implantation received morphine (0.5 mg/kg, s.c.) during the conditioning phase and electrical stimulation of 150 μA in the reinstatement phase.
M 5 + ES 25	5	Animals with electrode implantation received morphine (5 mg/kg, s.c.) during the conditioning phase and electrical stimulation of 25 μA in the reinstatement phase.
M 5 + ES 150	5	Animals with electrode implantation received morphine (5 mg/kg, s.c.) during the conditioning phase and electrical stimulation of 150 μA in the reinstatement phase.
M 0.5 + Sal injection	5	Animals with cannula implantation received morphine (0.5 mg/kg, s.c.) during the conditioning phase and an intra-VTA infusion of saline (1 μL) in the reinstatement phase.
M 5 + Sal injection	5	Animals with cannula implantation received morphine (5 mg/kg, s.c.) during the conditioning phase and an intra-VTA infusion of saline (1 μL) in the reinstatement phase.
M 0.5 + L injection	6	Animals with cannula implantation received morphine (0.5 mg/kg, s.c.) during the conditioning phase and an intra-VTA infusion of lidocaine (0.5 μL/site) in the reinstatement phase.
M 5 + L injection	5	Animals with cannula implantation received morphine (5 mg/kg, s.c.) during the conditioning phase and an intra-VTA infusion of lidocaine (0.5 μL/site) in the reinstatement phase.
Sal + L injection	5	Animals with cannula implantation received saline (1 mg/kg, s.c.) during the conditioning phase and an intra-VTA infusion of lidocaine (0.5 μL/site) in the reinstatement phase.

Sal, saline; M 0.5, morphine of 0.5 mg/kg; M 5, morphine of 5 mg/kg; s.c., subcutaneous; ES, electrical stimulation; VTA, ventral tegmental area; L, lidocaine.

Electrical stimulation procedure

Electrical stimulation was performed for 10 min using a stimulus isolator (World Precision Instruments, R A360 model, USA). Square pulses were delivered through a cable connected to a 2-pin PCB male connector. One of the ends of the electrode line was attached to the port linked to the rat's head, while the other end was connected to an external pulse generator, a stimulus isolator. Electrical stimulation was turned on during both the saline- and morphine-pairings to establish a full stimulus circuit. The stimulation parameters included 25 and 150 µA pulse amplitudes, 25 Hz pulse frequency, and 100-µs pulse width (9). The pulses and the time points were chosen based on a previous study that led to an increase in the released levels of DA and its major metabolites in the anterior cingulate (27) and membrane depolarization in presumed pyramidal cells in the medial prefrontal cortex (mPFC) *via* DA D1-like receptors (28).

Using behavioral tests to assess seeking of the drug

A CPP test was used to measure the positive affective properties of morphine. Although morphine decreases neuronal activity (29), the rewarding effects of morphine are inferred by comparing the time spent in a specific context previously paired with morphine with another context that was never paired with morphine.

CPP apparatus

The CPP test was conducted in a three-compartment (A, B, and C) CPP box apparatus (23,30) in an unbiased paradigm. The apparatus consisted of two equal-sized (30 × 40 × 30 cm) conditioning chambers (A and B) with distinct tactile and visual cues and separated by a removable guillotine door. These chambers were linked by a smaller neutral chamber C (28 × 12.5 × 40 cm), where rats were placed at the start of a test session. The apparatus was made of metal, and to provide the tactile differences between compartments A and B, one of the compartments had a smooth floor, while the other had a rough floor. Prior to the behavioral test of CPP, the animals were allowed to acclimate with the apparatus. Cameras were installed above the chambers to record the time spent by rats in each chamber by video tracking software (ANY-maze, Stoelting Co., USA) during all test sessions.

Measurement of morphine-induced CPP

The CPP test consisted of a 9-day schedule with five distinct phases: pre-conditioning (pre-test or baseline), conditioning, post-conditioning (PC or test), forced abstinence, and reinstatement (20,31,32) (Fig. 1). The time spent by each rat in each compartment was recorded during all test sessions.

Pre-conditioning phase

On day 1, each rat was placed into chamber C, while the middle door was opened, and the rat was allowed to move freely in all chambers for 15 min to determine the baseline preference side. The time spent in each chamber was recorded and analyzed to verify the absence of preconditioning chamber preference. The morphine-paired side was selected as the compartment where the rat spent the least amount of time, ensuring a minimal net difference in baseline time between groups.

Conditioning phase

The conditioning phase consisted of a 3-day (days 2 - 4) schedule of conditioning sessions. In this phase, all gates were closed from 7:00 - 12:00 every day. During this phase, which included six sessions (three with saline and three with morphine), the rats received s.c. morphine hydrochloride at the doses of 0.5 or 5 mg/kg dissolved in saline solution once per day and restricted to one chamber of the device (23,32,33). Then, the rats were placed in the morphine-paired compartment for 30 min. Next, the rats received the same dose of saline at 14:00–18:00 on the same day and then immediately put in the non-morphine-paired compartment for 30 min. The chamber associated with morphine and the presentation order of morphine and saline were reversed each day and counterbalanced across subjects in each group. Each group received alternative injections of either saline or morphine every 6 h. Groups 1, 4-6, and 17 received saline as a

vehicle instead of morphine. In other words, these groups received saline in both chambers, but the non-preferred side was designated as the reference context for each of them. At the end of the test, the animals returned to the housing facility overnight (31,32,34).

PC phase

On the fifth day (the preference test day), the test procedure was similar to the pre-test procedure. The gates were removed, allowing the rats to have full access to the entire apparatus. Both morphine and saline injections ceased. The amount of time that each rat spent in either compartment during a 15-minute period was determined as the preference criteria. The CPP indicates a preference score, which is defined as the time spent on the drug-paired side on the fifth day minus the time spent on the same side on the first day. A positive score indicates an improved PC time spent in the morphine-paired chamber.

Reinstatement phase

Four days after morphine withdrawal (forced abstinence), the reinstatement phase was conducted to assess if the expression of morphine reward was affected by DBS in the electrical stimulation groups. In the temporary inactivation group of rats, lidocaine (2%; 0.5 µL/site) was infused into VTA. The place-conditioning paradigm was induced by an ineffective dose of morphine (0.5 mg/kg, s.c.) on the back of the rats' necks as a priming dose just once after electrical stimulation or lidocaine infusion, and they were immediately placed in the neutral chamber C for 30 min with unrestricted access to all apparatus compartments, similar to days 1 and 5. High dosages of morphine were not tested, as they could produce CPP in non-sensitized rats.

Locomotor activity

Overall locomotion was assessed during the pre-conditioning, PC, and reinstatement phases using the ANY-maze video tracking system by quantifying the total distance traveled and compartment entered (30). The traveled distance was measured as the distance traveled in each compartment for 15 min and calculated as a CPP score.

Histological procedures for the verification of cannula position

After the completion of all experiments, all animals were deeply anesthetized with urethane (1 g/kg, i.p.) and received transcardiac perfusion with 0.9% normal saline followed by 10% buffered formalin, and then they were decapitated. The brains were removed and coronally cut into 60 µm slices through the cannula placements using a freezing microtome (LEICA, Germany). Serial coronal slices were obtained at the VTA level. Brain slices with implanted cannula locations were selected and pasted onto glass slides (coated with 2% gelatin). The slices were examined using light microscopy (Erma, Japan) to ensure that the cannula was properly positioned. Figure 2 depicts the position of the cannula in the VTA.

Statistical analysis of data

The conditioning time score or distance score was calculated as the time spent or distance traveled in a morphine-paired compartment on the fifth day minus the time spent or distance traveled in the morphine-paired compartment on the first day in all groups. In addition, the behavioral reinstatement assessment was defined as the morphine-paired compartment time spent or distance traveled on the ninth day minus the morphine-paired compartment time spent or distance traveled on the first day. All data were presented as mean ± SEM.

The data were analyzed using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) post-hoc analysis. A paired *t*-test was used to determine group differences between conditioning and reinstatement-operated rats based on difference scores for the drug-paired chamber. *P*-values of less than 0.05 were considered statistically significant. The statistical analysis was performed using SPSS 23 analytical software for Windows (SPSS Inc., Chicago, IL, USA). Also, all graphs were plotted using Excel 2016.

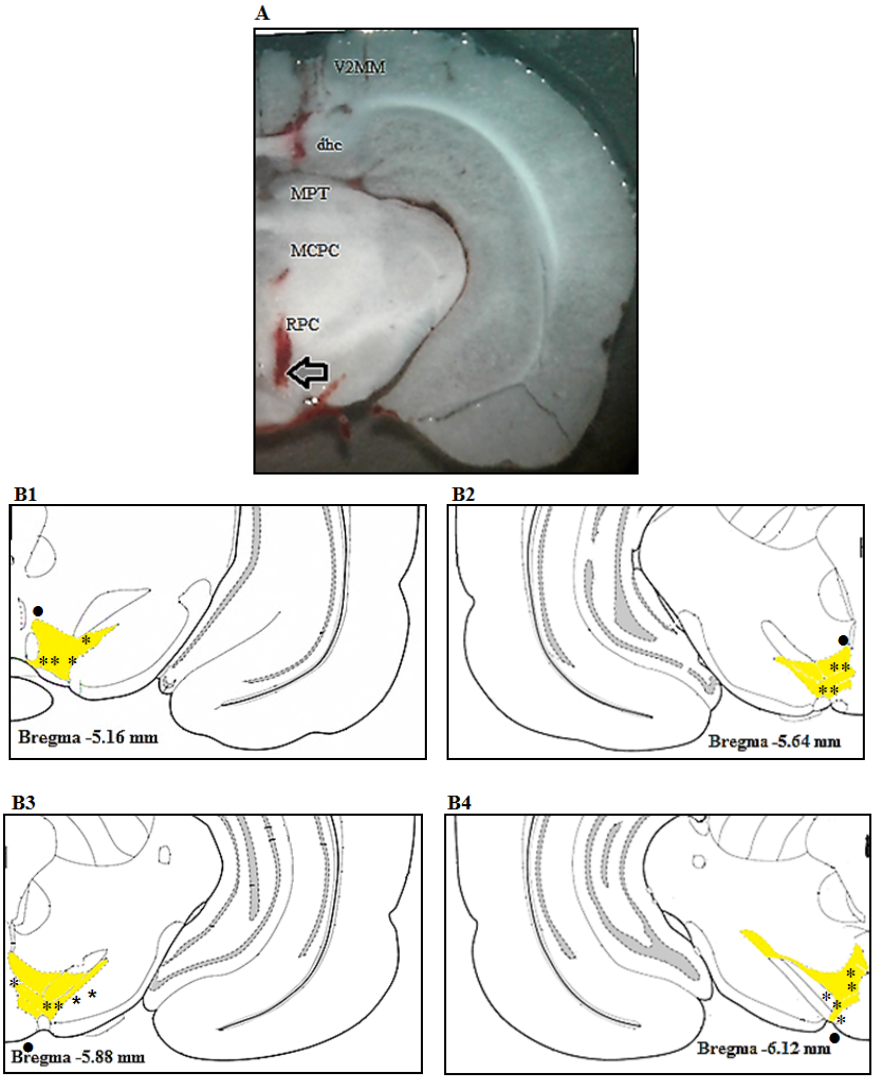


Fig. 2. Confirmation of the cannula position in the VTA. (A) Photographic illustration of a rat brain section showing the correct position of the cannula in the VTA; (B1-B4) schematic representation of stimulation electrode and cannula positions in the VTA. The schematic diagrams of brain coronal sections including bregma -5.16, -5.64, -5.88, and -6.12 mm planes originated from the stereotaxic atlas of Paxinos and Watson (22) and the shaded area is the tissue section of VTA. * and ⇦ represented the tip of the stimulating electrode. The black points (●) are the inaccurate points. Scale bar, 1 mm. V2MM, Secondary visual cortex, mediomedial area; dhc, dorsal hippocampal commissure; MPT, medial pretectal nucleus; MCPC, the magnocellular nucleus of the posterior commissure; RPC, red nucleus parvocellular part.

RESULTS

Morphine dose-response to a place conditioning paradigm in morphine-treated animals

Reinstatement began four days after place conditioning with the dose of morphine (0.5 mg/kg) in rats that had previously received daily morphine (0.5 or 5 mg/kg, s.c.) for three days. Animals with a history of morphine conditioning exhibited enhanced responses to morphine. The extinguished preference for the morphine-paired context was restored by priming the injection of morphine after forced abstinence. Morphine at the dose of 5 mg/kg increased CPP in the PC and produced the greatest effect (reinstatement, $F(2, 19) = 6.080$). There was no significant difference between PC and reinstatement phases in saline ($t(10) = 0.462$, $P = 0.653$), M 0.5 ($t(4) = 1.912$, $P = 0.128$) and M 5 ($t(5) = -0.721$, $P = 0.502$) groups (Fig. 3).

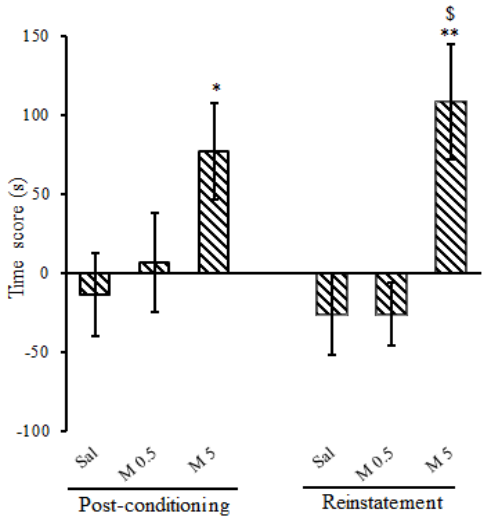


Fig. 3. The effect of various doses of morphine on CPP in both PC and reinstatement phases in experimental groups. The animals received morphine (0.5 or 5 mg/kg, s.c.) during the conditioning phase. After a forced abstinence period, a priming dose of morphine (0.5 mg/kg, s.c.) was administered in the reinstatement phase. Data were represented as the mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate the significant differences in comparison with the Sal group; $^{\$}P < 0.05$ versus the M 0.5 group (n = 5-11). Sal, saline; M 0.5, morphine 0.5 mg/kg; M 5, morphine 5 mg/kg.

Morphine-reinstated CPP effect

As shown in Fig 4A, the administration of morphine 0.5 mg/kg induced no differences among groups in the PC phase ($F (3, 27) = 0.537$, $P = 0.661$) as well as the priming injection of morphine (0.5 mg/kg) showed no reinstatement in these groups ($F (3, 27) = 0.979$, $P = 0.417$) (Fig. 4A).

Fig. 4B illustrated significant differences among groups in the phase of PC ($F (3, 24) = 3.891$). The administration of morphine 5 mg/kg increased the time score in both M 5 and M 5 + sham-operated groups in the PC phase when compared with the Sal group (Fig 4B). Consequently, tissue lesions induced by implanting electrodes into the VTA had no impact on the reinforcing effects of morphine or subsequent drug seeking (Fig. 4B). After four days of forced abstinence, a priming injection of morphine (0.5 mg/kg) reinstated the morphine preference ($F (3, 24) = 5.140$). The administration of saline failed to induce place preference in intact and sham-operated groups (Fig. 4B).

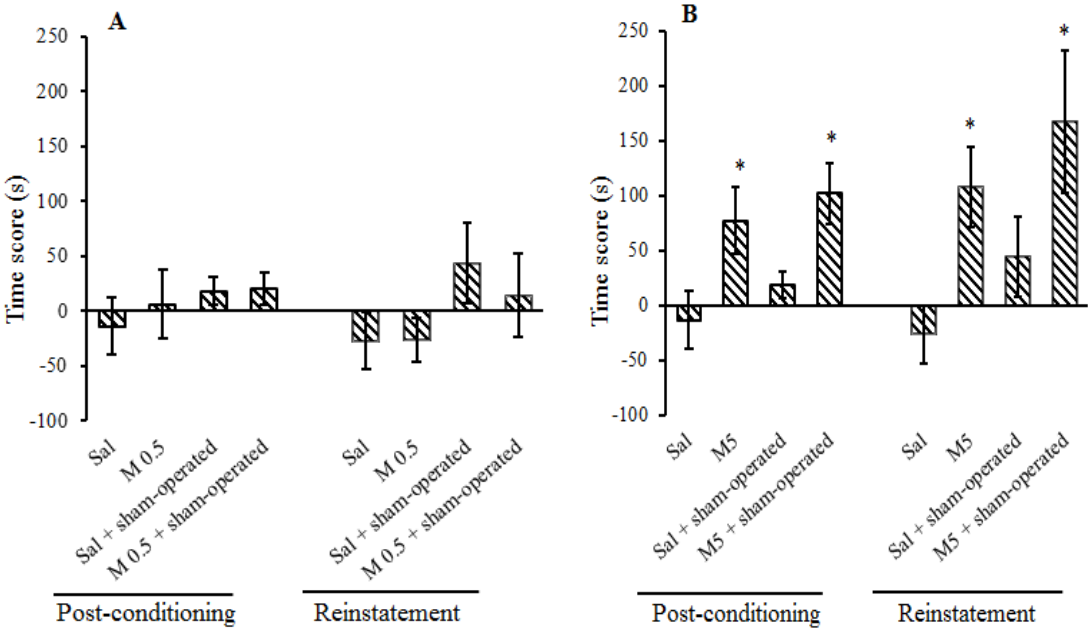


Fig. 4. The comparison of time score in morphine-induced CPP expression in the experimental groups. The animals received morphine (0.5 or 5 mg/kg, s.c.) during the conditioning phase. After a forced abstinence period, a priming dose of morphine (0.5 mg/kg, s.c.) was administered in the reinstatement phase. (A**)** Comparison of time score in the groups conditioned by morphine at the dose of 0.5 mg/kg in both post-conditioning and reinstatement phases; (B) Comparison of time score in the groups conditioned by morphine at the dose of 5 mg/kg in both PC and reinstatement phases. Data were represented as mean ± SEM. $^{*}P < 0.05$ indicates the significant differences in comparison to the Sal group (n = 5 - 11). Sal, saline; M 0.5, morphine 0.5 mg/kg; M 5, morphine 5 mg/kg.

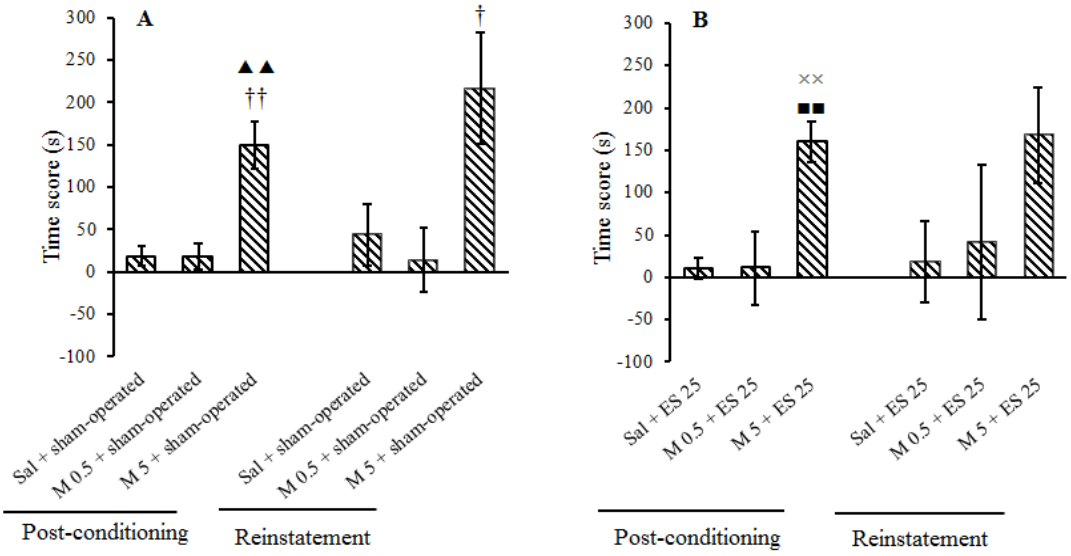
Effects of intra-VTA DBS and lidocaine microinjection on morphine-induced CPP expression in morphine-conditioned rats

Rats received either morphine (0.5 or 5 mg/kg/day, s.c.) or saline (1 ml/kg, s.c.) as control. To investigate the effect of DBS on the expression and subsequent reinstatement of morphine-induced place preference 5 min before testing, electrical stimulation (25 and 150 µA/rat) was applied into the VTA and reinstatement with an ineffective dose of morphine (0.5 mg/kg, s.c.) was performed. The findings are illustrated in Fig. 5.

The results showed that morphine injection (5 mg/kg) for three days in the M 5 + sham-operated group produced a significant increment in CPP scores in the PC for the morphine-paired chamber (F (2,17) = 5.932) in comparison to Sal + sham-operated and M 0.5 + sham-operated groups Fig. 5A. In addition, a high reinstatement score was observed in M 5 + sham-operated group compared to M 0.5 + sham-operated group (Fig. 5A).

To examine if the DBS of VTA could prevent morphine reinforcement and affect withdrawal and subsequent reinstatement, rats received DBS with high (HI) or low intensity (LI) (25 or 150 µA) into the VTA. Then, rats were given a morphine priming dose (0.5 mg/kg) and assessed for reinstatement after 15 min.

The current findings showed that there were significant and insignificant differences in PC (F (2, 12) = 8.492) and reinstatement (F (2,12) = 1.407, P = 0.282) phases among groups, respectively (Fig. 5B). The low intensity of DBS blocked morphine-induced CPP in the reinstatement phase in M 0.5 + ES 25 group, though the M 5 + ES 25 group showed a reinstatement with a priming dose of morphine, insignificantly (Fig. 5B). The findings showed that the administration of morphine at the dose of 5 mg/kg increased morphine-induced CPP in PC in M 5 + ES 150 group compared to Sal + ES 150 group, significantly (F (2,12) = 3.501, P = 0.063). In the reinstatement phase, there were no significant differences in CPP among groups (F (2,12) = 1.593, P = 0.243) (Fig. 5C). Thus, DBS did not successfully inhibit morphine reward after reinforcement, and priming dose of morphine slightly increased the time score in the M 5 + ES 150 group, insignificantly. Paired t-test found no significant changes in reinstatement scores versus PC test scores (Fig. 5C).



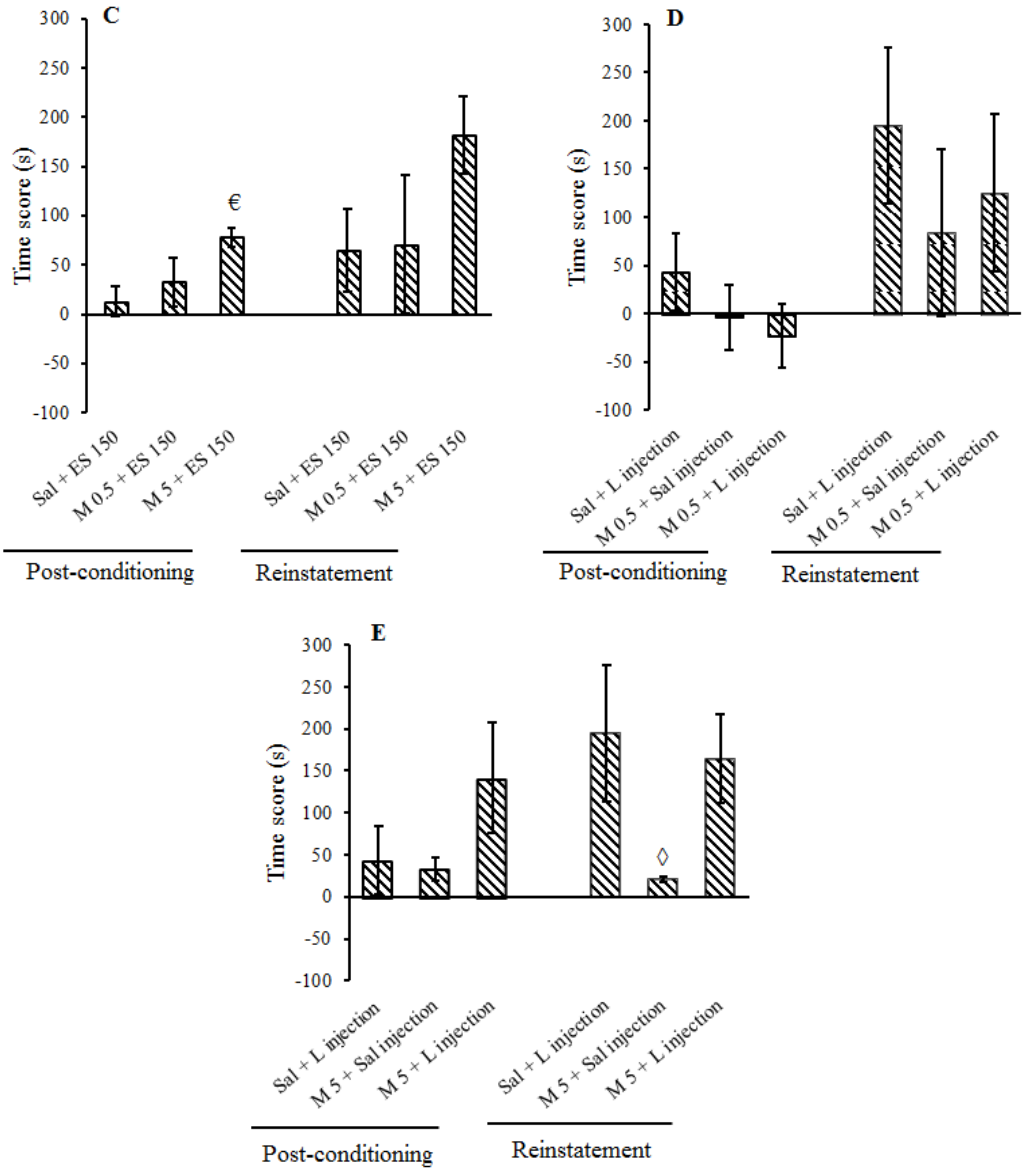


Fig. 5. The comparison of time score in morphine-induced CPP expression in morphine-conditioned rats in the experimental groups (n = 5-11). The animals received morphine (0.5 or 5 mg/kg, s.c.) during conditioning phase. After a forced abstinence period, a priming dose of morphine (0.5 mg/kg, s.c.) was administered in reinstatement phase. In addition, the animals received electrical stimulation or lidocaine injection in reinstatement phase before receiving the priming dose of morphine. (A) Comparison of time score in sham-operated groups conditioned by morphine in both PC and reinstatement phases; (B) the effect of electrical stimulation with the current intensity of 25 µA on time score in groups conditioned by morphine in both PC and reinstatement phases; (C) the effect of electrical stimulation with the current intensity of 150 µA on time score in groups conditioned by morphine in both PC and reinstatement phases; (D) the effect of lidocaine-induced reversible inactivation of the VTA on the expression of morphine-induced CPP in rats receiving morphine 0.5 of mg/kg; (E) the effect of lidocaine-induced reversible inactivation of the VTA on the expression of morphine-induced CPP in rats receiving morphine of 5 mg/kg. The data were represented as mean ± SEM.$^{▲▲}P < 0.01$ indicates a significant difference compared to the Sal + sham-operated group; $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ versus the M 0.5 + sham-operated groups; $^{■■}P < 0.01$ versus the M 0.5 + ES 25 group; $^{××}P < 0.01$ versus the Sal + ES 25 group; $^{€}P < 0.05$ versus the Sal + ES 150 group; $^{◊}P < 0.05$ versus the Sal + L injection group. ES, electrical stimulation; L, lidocaine; M 0.5, morphine 0.5 mg/kg; M 5, morphine 5 mg/kg; Sal, saline.

There were no significant differences in the time spent in the morphine-paired chamber in the PC test among groups receiving morphine 0.5 mg/kg (F (2,13) = 0.899, *P* = 0.431) (Fig. 5D). In addition, these observations were seen in reinstatement phase among groups (F (2,13) = 0.429, *P* = 0.660) (Fig. 5D). Moreover, the administration of effective dose of morphine (5 mg/kg) revealed no significant changes in the expression of PC phase (F (2,12) = 1.717, *P* = 0.221) (Fig. 5E). M 5 + Sal injection group altered the time spent in saline-or morphine-paired chamber in the reinstatement test. The injection of saline decreased the expression of CPP in the M 5 + Sal injection group (Fig. 5E). Paired *t*-test analysis found no significant decrease in time scores between PC and reinstatement tests in the M 5 + L injection group (t (4) = -0.275, *P* = 0.797; Fig. 5E).

Effects of intra-VTA DBS and lidocaine microinjection on locomotor activity during expression of morphine-induced CPP in morphine-conditioned rats

To rule out any non-specific effects of DBS, which could confound the measures of morphine reward, the impact of DBS also was measured on locomotor activity by analyzing the score of total distance traveled during CPP tests. Since LI-DBS did not affect morphine preference, we focused mainly on DBS with high intensity (HI). Locomotor activity was evaluated by quantifying the score of traveled distance in the CPP compartments using ANY-maze software (Fig. 6A).

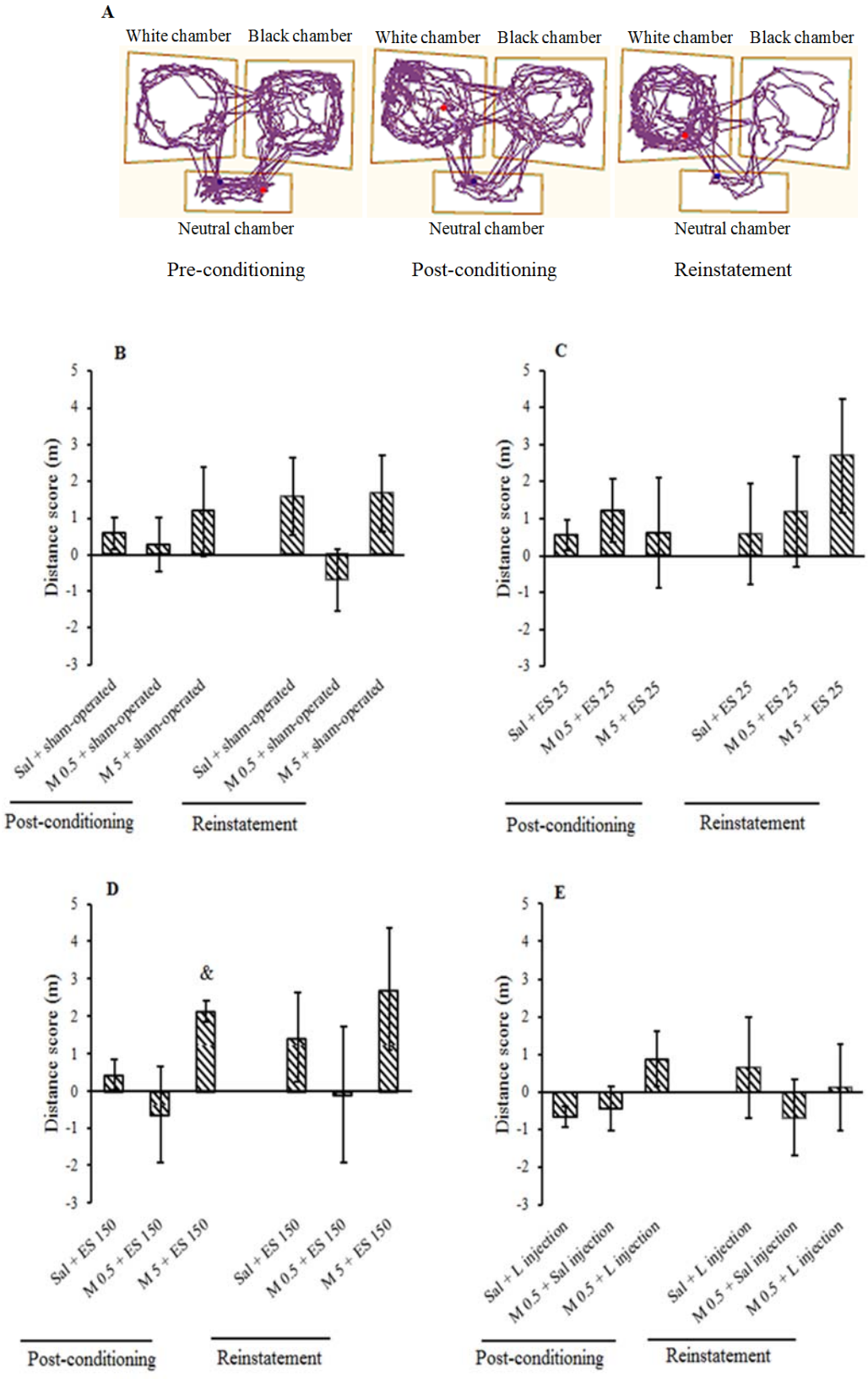
The analysis of one-way ANOVA showed no significant change in the score of distance traveled among the three operated groups in both phases of PC (F (2,17) = 0.300, *P* = 0.745) and reinstatement (F (2,17) = 2.129, *P* = 0.150) (Fig. 6B). In addition, a paired *t*-test analysis revealed no significant difference in the score of distance traveled in the CPP compartments between the phases of PC and the reinstatement in sham-operated animals (t(19) = 0.13, *P* = 0.990) (Fig. 6B).

There were no significant alterations in the activity of locomotion in PC (F(2,12) = 0.131, *P* = 0.878) and reinstatement: (F(2,12) = 0.551, *P* = 0.590) phases (Fig. 6C). The analysis of paired *t*-test revealed no significant changes in the scores of distance traveled between PC and reinstatement phases in all groups (Fig. 6C).

There was a significant preference change during PC in M 5 + ES 150 group versus M 0.5 + ES 150 group (Fig. 6D). As expected, there was no significant change in the score of distance traveled for the CPP paradigm on the expression phase during the reinstatement test (F (2, 12) = 0.810, *P* = 0.468) (Fig. 6D). Paired *t*-test revealed that the priming dose of morphine with 150 µA DBS was not associated with the score changes of distance traveled during reinstatement versus PC in any groups (Fig. 6D).

The current findings revealed that the reversible inactivation of the VTA in rats receiving morphine with the dose of 0.5 mg/kg did not affect their locomotor activity scores (Fig. 6E). As shown in Fig. 6E, there were not any significant changes in distance score among the groups in both phases of PC (F (2, 13) = 2.091, *P* = 0.163) and reinstatement (F (2, 13) = 0.301, *P* = 0.745).”The paired t-test revealed that the injection of lidocaine into VTA failed to show a significant difference between PC and reinstatement phases in each group (t (5) = 0.736, *P* = 0.495) (Fig. 6E).

The administration of morphine at the dose of 5 mg/kg during the conditioning phase could not change distance score in PC phase(F (2, 12) = 0.675, *P* = 0.528) (Fig. 6F). Moreover, it was not observed a significant change in the scores of traveled distance among the groups in the reinstatement phase (F (2, 12) = 0.651, *P* = 0.539) (Fig. 6F). The paired *t*-test revealed no significant difference between the two phases of PC and reinstatement in each group (t (4) = 1.158, *P* = 0.311) (Fig. 6F). Therefore, reinstatement with a priming dose of morphine did not affect locomotor activity.


A
White chamber
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B
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M 0.5 + sham-operated
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Post-conditioning
Reinstatement
C
Sal + ES 25
M 0.5 + ES 25
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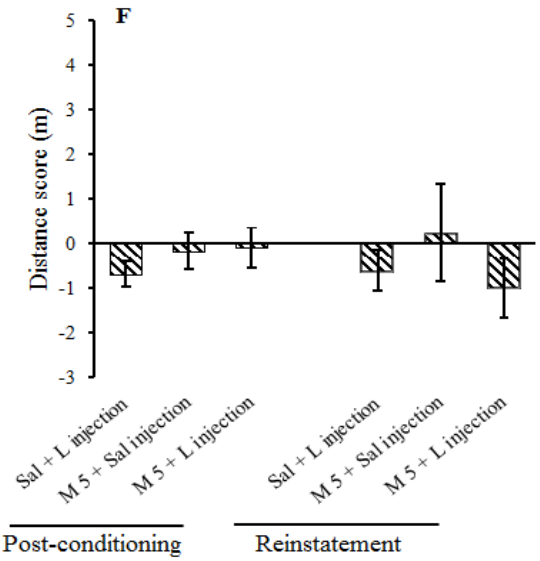


Fig. 6. The comparison of distance score in morphine-induced CPP expression in morphine-conditioned rats in the experimental groups (n = 5-11). The animals received morphine (0.5 or 5 mg/kg, s.c.) during the conditioning phase. After a forced abstinence period, a priming dose of morphine (0.5 mg/kg, s.c.) was administered in the reinstatement phase. In addition, the animals received electrical stimulation or lidocaine injection in the reinstatement phase before receiving the priming dose of morphine. (A) Track plots showing the traveled distance in the CPP compartments. Blue and red spots present start and end points in each track, respectively; (B) the distance scores in the expression of morphine-induced CPP in sham-operated groups; (C) the effect of electrical stimulation with the intensity of 25 μA on distance scores in the expression of morphine-induced CPP; (D) the effect of electrical stimulation with the intensity of 150 μA on distance scores in the expression of morphine-induced CPP; (E) the effect of the reversible inactivation of VTA by the administration of lidocaine on distance scores in the expression of morphine-induced CPP in rats conditioned by morphine of 0.5 mg/kg; (F) the effect of the reversible inactivation of VTA by the administration of lidocaine on distance scores in the expression of morphine-induced CPP in rats conditioned by morphine of 5 mg/kg. The data were represented as mean ± SEM. $^{\&}P < 0.05$ indicates a significant difference in comparison to the M 0.5 + ES 150 group. ES, Electrical stimulation; L, lidocaine; M 0.5, morphine 0.5 mg/kg; M 5, morphine 5 mg/kg; Sal, saline, CPP, conditioned place preference.

Intra-VTA DBS and microinjection of lidocaine on the incubation of craving in the expression of morphine-induced CPP in morphine-conditioned rats.

Rats were further examined to determine if high and low DBS caused non-specific effects. The incubation of craving behavior was measured by counting and comparing compartments entering the CPP test. Preconditioning (day 1), PC (day 5), and reinstatement (day 9) were compared using a paired *t*-test.

The one-way ANOVA followed by the post hoc test showed a significant difference among groups in compartment entering the PC phase of CPP (F (2, 17) = 5.290) (Fig. 7A). The animals receiving morphine at the dose of 0.5 mg/kg moved fewer times between compartments in the PC phase of CPP compared with the Sal + sham-operated group, significantly. On day 9, the priming dose of morphine (0.5 mg/kg, s.c.) failed to increase entry counts, event entry counts were decreased in comparison to the Sal + sham-operated group, significantly (F (2, 17) = 7.446). The comparison of phases of CPP in each group demonstrated that compartment entry increased in both PC and reinstatement phases compared to the preconditioning phase in the Sal + sham-operated group, significantly (Fig. 7A).

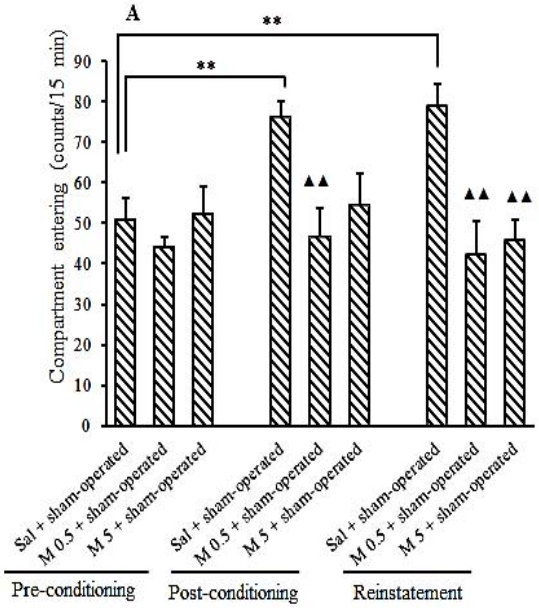
Compartment entering had not any significant differences among groups in both phases of preconditioning ($F(2,12) = 0.354$, $P = 0.709$) and PC ($F(2,12) = 0.221$, $P = 0.805$) (Fig. 7B). Also, there were not any significant differences among groups in the reinstatement

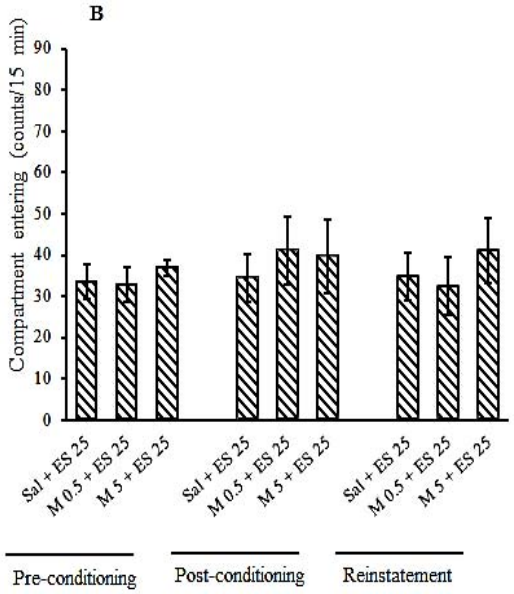
phase (F(2,12) = 0.417, *P* = 0.668) (Fig. 7B). The significant changes were not observed among all phases in each group (Fig. 7B).

The groups shown in Fig. 7C failed to make significant compartment entering changes in both pre-conditioning (F(2,12) = 0.718, *P* = 0.507) and reinstatement (F(2,12) = 0.693, *P* = 0.519) (Fig. 7C). In contrast, the administration of morphine at the dose of 5 mg/kg could enhance compartment entering than other groups in PC phase (F(2,12) = 6.206) (Fig. 7C). The significant changes were not observed in compartment entry in reinstatement test versus preconditioning and PC tests, as revealed by paired *t*-test (Fig.7C).

As shown in Fig. 7D, there was no significant difference in the phase of preconditioning among groups. The administration of morphine at the dose of 0.5 mg/kg increased compartment entering in both M 0.5 + Sal injection and M 0.5 + L injection groups than Sal + L injection group in the PC phase (F (2, 13) = 8.043) (Fig. 7D). Also, the administration of priming morphine dose significantly increased the number of entering compartments in the reinstatement phase in both M 0.5 + Sal injection and M 0.5 + L injection groups than Sal + L injection group (F (2, 13) = 5.382) (Fig. 7D). The maximum response was observed in Sal + L injection group, where compartment entering number significantly decreased in reinstatement phase versus preconditioning phase (t(4) = 11.554) (Fig. 7D).

In the PC test, there was no significant difference in compartment entering among groups (Fig. 7E). Whereas, this parameter changed in the reinstatement phase among groups, significantly (F (2, 12) = 7.959) (Fig. 7E). Also, the results showed that Sal + L injection group had significant differences in compartment entering among three phases of CPP. Although other groups did not exhibit such observations (Fig. 7E).





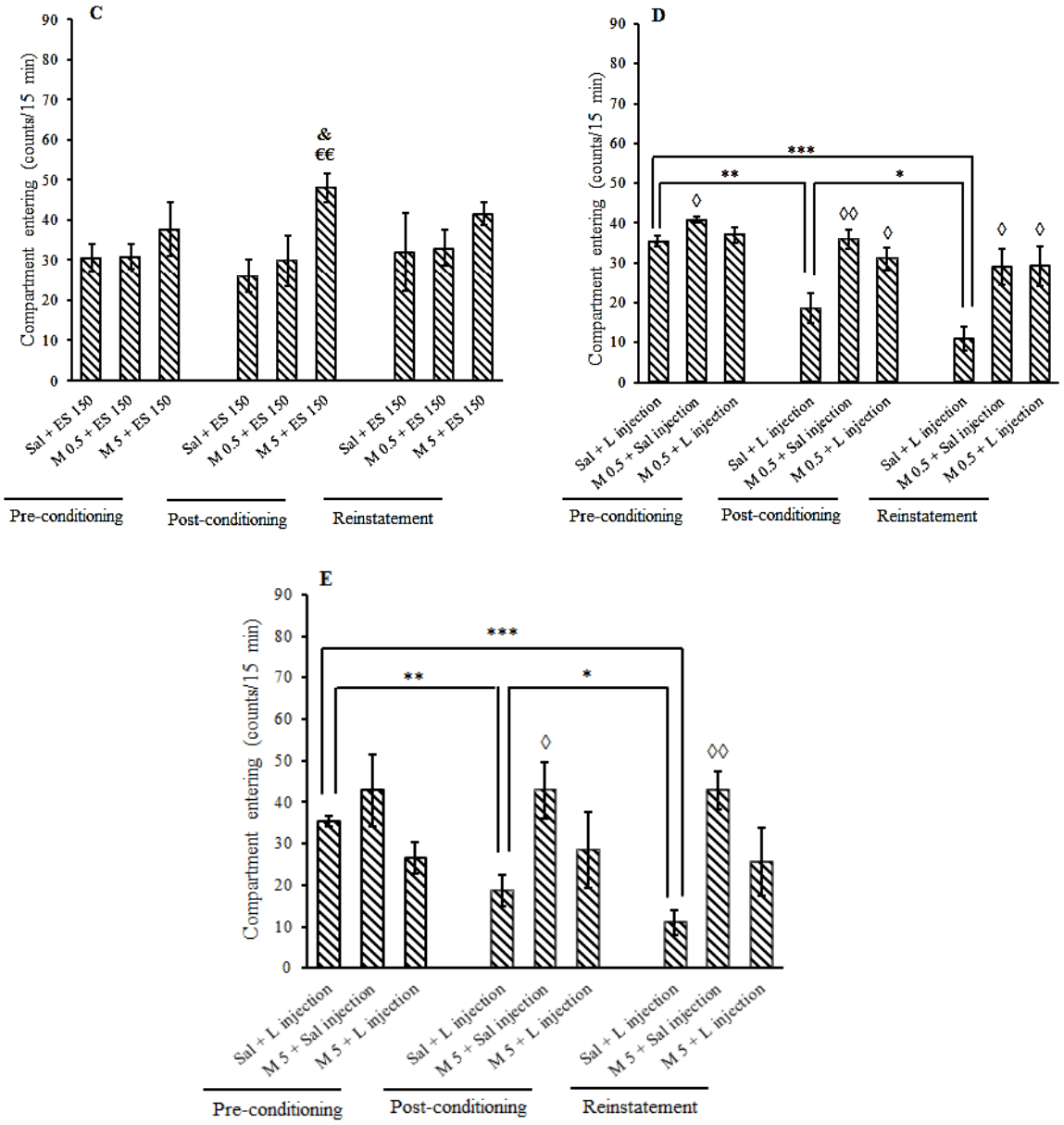


Fig. 7. The comparison of compartment entering numbers in experimental groups conditioned by morphine during CPP (n = 5 - 11). The animals received morphine (0.5 or 5 mg/kg, s.c.) during the conditioning phase. After a forced abstinence period, a priming dose of morphine (0.5 mg/kg, s.c.) was administered in the reinstatement phase. In addition, the animals received electrical stimulation or lidocaine injection in the reinstatement phase before receiving the priming dose of morphine. (A) The number of compartments entering sham-operated groups conditioned by morphine; (B) the effect of electrical stimulation with the intensity of 25 µA on the expression of morphine-induced CPP in morphine-conditioned rats; (C) the effect of electrical stimulation with the intensity of 150 µA on the expression of morphine-induced CPP in morphine-conditioned rats; (D) reversible inactivation of the VTA by the administration of lidocaine on the expression of morphine-induced CPP in rats conditioned by morphine of 0.5 mg/kg; (E) reversible inactivation of the VTA by the administration of lidocaine on the expression of morphine-induced CPP in rats conditioned by morphine of 5 mg/kg. The data were represented by the mean ± SEM. $^{▲▲}P < 0.01$ indicates a significant difference in comparison with the Sal + sham-operated group; $^{€€}P < 0.01$ versus the Sal + ES 150 group; $^{\&}P < 0.05$ versus the M 0.5 + ES 150 group; $^{◊}P < 0.05$ and $^{◊◊}P < 0.01$ indicate significant differences in comparison to the Sal + L injection group; ES, electrical stimulation; L, lidocaine; M 0.5, morphine 0.5 mg/kg; M 5, morphine 5 mg/kg; Sal, Saline; VTA, ventral tegmental area; CPP, conditioned place preference; DBS, deep brain stimulation.

DISCUSSION

There is limited information regarding the effects of DBS in VTA on morphine-induced place conditioning in morphine-sensitized rats. We explored the effects of DBS on the VTA activity within the mesocorticolimbic system to better understand the mechanism behind the use of DBS as a viable therapy for morphine-induced rats. Overall, the present study investigated the effects of HI or LI DBS in the VTA on the expression of morphine-induced place conditioning in morphine-sensitized rats. CPP test established a successful state of morphine-conditioned preference in rats treated with systemic morphine administration (Figs. 3 and 4B). The study found that the repeated injections of morphine increased its rewarding properties and induced sensitization in the animals. The animals showed less response to a low dose of morphine (0.5 mg/kg), which did not induce place conditioning in morphine-naive animals (Fig. 3). Additionally, the administration of morphine did not inhibit the expression of morphine-induced CPP in morphine-conditioned rats (Fig. 4B).

The study also investigated the potential of DBS in the VTA to reduce the development of morphine reinforcement as well as forced abstinence and the reinstatement of morphine-seeking behavior in morphine-sensitized rats.

Drug addiction is a chronically relapsing disorder that is characterized by (1) the compulsion to seek out and use the drug; (2) the loss of control in limiting intake; and (3) the emergence of a negative emotional state (such as dysphoria, anxiety, or irritability) reflecting a motivational withdrawal syndrome when access to the drug is restricted (35). According to the incentive-sensitization theory, the excessive amplification of psychological "wanting," particularly when sparked by cues, rather than necessarily an amplification of "liking," constitutes the core of drug addiction (36). At a glance, the morphine-sensitized animals are characterized by an increase in Mu opioid receptor, net [35S] GTP gamma S binding, and basal cAMP levels (37), D1 DA receptor (38), orexin receptors (39), and the response of systems. These functional changes have demonstrated that the increased responsiveness to an ineffective dose of morphine induces an increase in time spent by rats in a morphine-paired compartment, confirming that sensitization to CPP has been developed (32,40). A similar mechanism(s) may have been involved in the morphine-sensitized animals' responses to the low doses of morphine in our experiments. Furthermore, our findings (Figs. 3 and 4B) are consistent with previous research demonstrating that the reinstatement of CPP was induced by the low doses of morphine in rats that had previously been conditioned to morphine (32).

Although many studies have focused on the issues surrounding morphine reinstatement, the nature of morphine reinstatement, as well as the neurotransmitters and neural sites involved in this phenomenon, is still unknown. The results suggest that DBS, particularly in the VTA, may play a key role in reinstating morphine-induced CPP in morphine-conditioned rats.

The priming dose was effective in reinstatement of the induction of the expression suppressed by intra-VTA DBS in the morphine (5 mg/kg)-induced CPP of morphine-conditioned rats (Fig. 5B and C).

The possible mechanisms involved in these observations are suggested as follows: the electrical stimulation of the VTA is dependent on DA D1-like receptor activation (28). The DBS of intra-VTA activates the dopaminergic pathway and increases DA secretion from VTA cells (27). Peripheral electrical stimulation at 2 and 100 Hz, given for 30 min per day for three days, inhibited both morphine-induced CPP expression and the reinstatement of extinguished CPP (41), and induced DA release into the NAc, a key region of the mesolimbic dopaminergic system involved in reward processing. Previous studies have demonstrated that the electrical stimulation of the VTA increases DA levels in multiple brain regions, including mPFC sub-regions (28). It is now clear that canonical blood oxygen level-dependent responses in the reward system represent mainly the activity of non-dopaminergic neurons. Thus, the minor effects of projecting dopaminergic neurons are concealed by non-dopaminergic activity (42).

Moreover, the VTA is one of the primary sites in the brain where addictive drugs like

cocaine (43) and morphine (44) act. Morphine sensitization is associated with increased DA release and changes in the sensitivity of dopaminergic D1 receptors in mesolimbic structures such as the striatum, NAc, VTA, hippocampus, and prefrontal cortex. The pharmacological blockade of D1 receptors impairs the expression of sensitization, because antagonists of N-Methyl-D-aspartate and AMPA receptors inhibit the acquisition, but not the expression of behavioral sensitization, and the development of behavioral sensitization is associated with the glutamatergic system and VTA (6). DA neurons of VTA are innervated by norepinephrine (NE) neurons coming from the LC, which in turn affects VTA DA neuronal activity. The factors such as D2 receptors, DA transporters (DAT), and α_1 -adrenergic receptors included in the VTA participated in the regulation of electrically induced DA in the NAc, but neither D2 receptors nor DAT involved in the regulation of NE release in the VTA. The release of NE is regulated by both α_2 -adrenergic receptors and NE transporters (45). By controlling the creation of new required proteins for this process through the D1/D5 dopaminergic receptors of the hippocampus, the VTA regulates the consolidation of memories. Furthermore, the LC may function as the second component with a similar role, acting both independently and in conjunction with the VTA through the beta-adrenergic receptors in the hippocampus (46). Based on the mentioned studies, different neurotransmitters play an important role in the expression of the morphine-induced reinstatement of CPP.

The study found that high electrical stimulation increased the time spent by rats in the non-preferred compartment (Fig. 5C), while lidocaine reduced it (Fig. 5E). These results are in agreement with the results of other studies performed the expression of morphine-induced CPP, but not significantly (10). The study also found that the ineffective morphine dose (0.5 mg/kg) was less important in the expression of sensitization to CPP in morphine-conditioned rats, because the dose did not induce any significant effects on the conditioning of rats, but its role should not be completely excluded. The high dose of morphine resulted in a high score of conditioning, and a lower score of reinstatement, which tended to a statistical significance. However, there is no evidence in the literature to support the effects of electrical stimulation on morphine reinstatement expression. Interestingly, the blockade of VTA by lidocaine did not induce any effects on the expression of reinstatement in morphine-conditioned rats, which is possibly associated with the interaction of receptors. The study used the effective and infective doses of morphine, and the involvement of lidocaine appears to be insignificant for the obtained results. However, the lack of effect of morphine expression may be explained by the interaction between inactivated VTA to make the morphine priming dose useless.

The participation of the dopaminergic system in behavioral reinstatement has already been described in our previous paper (30). Then, we demonstrated that electrical stimulation with both low and high intensities was able to inhibit the acquisition of morphine-seeking behavior in rats, observed as the reinstatement. Thus, this paper presented the beneficial role of electrical stimulation in various tests reflecting the relapse of drug use (23). Because the effect of electrical stimulation was examined in the expression of reinstatement to CPP, we have greatly extended this information in rats. This test revealed the reinstatement of the rewarding effect of morphine. In this context, the participation of electrical stimulation was examined for the first time. We comprehensively showed the important role of electrical stimulation in various aspects of morphine reinstatement.

We also found that the HI DBS of VTA did not significantly increase locomotor activity (Fig. 6D) or compartment entry (Fig. 7C), which may be considered as the incubation of craving behavior during CPP tests in rats. We also observed that the LI-DBS of the VTA did not affect the measures of morphine reinforcement. For the first time, this study investigated the effect of electrical stimulation on locomotor activity in morphine-induced CPP. However, as shown in Fig. 6C, LI DBS causes an insignificant increase in the traveled distance reinstatement. This observation suggests that the effects of DBS and lidocaine

on morphine reward were not likely due to altered locomotion but rather a direct modulation of VTA neurotransmission.

Overall, the present study provides valuable insights into the potential role of DBS in the VTA in morphine reinstatement in morphine-sensitized rats. However, further research is necessary to fully understand the effects of DBS on morphine addiction and the mechanisms involved in morphine reinstatement for developing effective DBS therapies in morphine addiction in humans.

CONCLUSION

The study used different doses of morphine and electrical stimulation amplitudes to observe differences and determine the optimum parameters for future studies. The results of this study could provide insights into the potential use of DBS and lidocaine for the treatment of addiction and the prevention of relapse. Overall, several major highlights could be drawn from the findings of the present study, based on the role of the VTA in learning and memory. Firstly, unilateral intra-VTA HI DBS may not prevent the attenuation of morphine-induced place conditioning after forced abstinence in morphine-treated rats without blocking its reinstatement induced by morphine priming. This finding may underpin the mechanisms of VTA stimulation in alleviating drug addiction. Secondly, HI DBS of VTA is more effective in CPP expression than LI DBS. Thirdly, the intra-VTA microinjection of lidocaine bilaterally plays different roles in suppressing CPP expression as two morphine doses. Finally, HI DBS was not associated with locomotor activity. Based on previous work (23) and the current study, it is proposed that DBS-based manipulation of the VTA activity could be a potential therapeutic target for future research toward the potential intervention of DBS-based treatment in the intractable disorders of addictive substances. These findings warrant further studies to assess their translatability to clinical use.

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Conflict of interest statement

All authors declared no conflict of interest in this study.

Authors' contributions

M. Ghobadi Pour and H. Alaei were involved in the conception, design, methodology, and project administration. M. Ghobadi Pour participated in material preparation, data collection and analysis, data curation, investigation, software works, visualization, and first draft writing. H. Alaei was involved in funding resources, supervision of the study, validation, review, and the editing of manuscript. The final version of the manuscript was approved by all authors.

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