

The effect of microinjection of CART 55-102 into the nucleus accumbens shell on morphine-induced conditioned place preference in rats: Involvement of the NMDA receptor

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Abstract

Background

The addictive properties of opioids may be mediated to some extent by cocaine- and amphetamine-regulated transcript (CART) in the reward pathway. There are also some claims regarding the interaction of CART and glutamate system. Drug-paired learning and memory may induce conditioned place preference (CPP) or conditioned place aversion (CPA). Here, we have evaluated whether intra-nucleus accumbens (NAc) shell infusions of CART induces CPP or CPA and affect morphine reward. In addition, we have measured the expression of the NR1 subunit of the N-methyl-D-aspartate (NMDA) glutamate receptor in various parts of the reward pathway (NAc, prefrontal cortex (PFC), and hippocampus) after conditioning tests. Bilateral cannulas were implanted in the rats NAc shell and then the animals were exposed to place conditioning. Animals were place-conditioned with several doses of subcutaneous (s.c.) morphine prior to the intra-NAc shell infusion of artificial cerebral spinal fluid (aCSF). Immunohistochemistry (IHC) data showed a dose-dependent increase in the expression of the NR1 subunit in all examined parts. Then, rats were conditioned with intra-NAc shell infusion of different doses of CART. CPP and CPA were induced with 2.5 and 5 µg/side, respectively.

Results

IHC showed an elevated level of NR1 with 2.5 µg/side and a decrease with 5 µg/side in all areas. Administration of a sub-rewarding dose of CART (1.25 µg/side) prior to the injection of a sub-rewarding dose of morphine (2.5 mg/kg) induced CPP and IHC analysis showed an increased amount of NR1 in all examined tissues. However, infusion of an aversive dose of CART (5 µg/side) prior to the injection of a rewarding dose of morphine (5 mg/kg) produced neither CPP nor CPA and IHC data showed a significant decrease in the amount of NR1 subunit in the NAc and hippocampus.

Conclusions

It seems that the rewarding or aversive effects of intra-NAc shell CART and its facilitating or inhibiting effects on morphine reward are dose-dependent. Furthermore, the NMDA receptor may be closely involved in the affective properties of opioids and CART in the reward pathway.

1. Introduction

1.1. Background

Chronic consumption of drugs of abuse such as opioids could result in adverse neuroadaptations in the brain reward pathway [1]. Nucleus Accumbens (NAc) as a part of the pathway is a significant target of addictive drugs [2] and has different neurotransmitter systems which modulate the reinstatement of drug-seeking behavior [3]. The location of NAc at the interface of the limbic projections from the amygdala, hippocampus, and cingulate cortex results in receiving many fibers from the midbrain dopaminergic

system [4]. Functional imaging studies have revealed that environmental cues which are associated with addictive substances lead to dopamine release in the NAc [5].

Glutamate, the primary excitatory neurotransmitter in the brain, is another critical molecule in the NAc regarding the addiction phenomenon. The role of glutamate in the neuroplasticity of the reward pathway, especially the circuitry from the prefrontal cortex (PFC) to the NAc is undeniable [6]. Long-lasting drug consumption could disrupt the release of glutamate from PFC to NAc and affect the glutamate homeostasis in the NAc, causing a significant upsurge in addictive behaviors [7, 8]. The most critical glutamate receptor that is involved in the mentioned phenomenon is the ionotropic N-methyl-D-aspartate (NMDA) receptor. The receptor is a tetramer composed of different subunit families, including GluN1, GluN2 (GluN2A-D), and GluN3 (GluN3A and B), which could be assembled as GluN1/GluN2 or GluN1/GluN2/GluN3 [9]. A noticeable interaction has been reported between the mu-opioid receptor and the NMDA glutamate receptor which has a critical role in the processing of opioid-induced neuroplasticity [10].

Beside numerous neurotransmitters that are involved in drug dependence, relapse, and craving behaviors, cocaine-and amphetamine-regulated transcript (CART) peptide is another molecule that has a close interaction with dopamine. The peptide takes part in several physiological functions such as learning, memory [11], drug reward, reinforcement, addiction [12], etc. Based on a growing body of evidence, it is believed that CART is involved in the action of psychostimulant and opioid drugs [13–15]. CART peptide in the NAc shell acts downstream to dopamine and may work as a mediator in morphine-induced reward and reinforcement [16]. Recently, our laboratory reported that the levels of CART mRNA and the peptide is considerably up-regulated in the NAc when rats become addicted to the escalating doses of morphine. Our study showed that CART peptide in the NAc has a crucial role in morphine addiction [13] and morphine-induced sensitization [17]. Elevation of the endogenous CART in the NAc shell following training rats in the Morris water maze demonstrates that CART is involved in the formation of spatial learning and memory [18].

1.2. Objectives

Various brain regions like PFC, striatum, hippocampus, amygdala, and NAc are involved in drug-induced conditioned place preference (CPP). In the present study, we have studied the possible effects of intra-NAc shell infusion of CART on induction of CPP or conditioned place aversion (CPA) and the likely influence of this peptide on morphine reinforcement. NAc was selected among brain reward areas because of its massive natural amount of CART mRNA and peptide as well as the strong sensitization of its endogenous CART to morphine administration [13]. Furthermore, some evidence exists that the glutamatergic system may be involved in the rewarding effects of CART [19]. To assess the involvement of the glutamatergic system in CART-induced CPP or CPA, we have studied the expression of NMDA receptor in the NAc, PFC, and hippocampus using antibodies against its NR1 subunit in an immunohistochemistry protocol.

2. Methods

2.1. Animals

150 Adult male albino Wistar rats (240–270 g, aged 12–14 week) were purchased from Iran University of Medical Sciences (Tehran, Iran). Animals were housed four per Plexiglas cages (40 × 30 × 25 cm) with food and water freely available *ad libitum*, except for the periods of behavioral testing, under an artificial 12:12 h light/dark schedule (light on at 07:00 a.m.), humidity 40–70%, and constant temperature (21 ± 2 °C). The rats were first handled and adapted to the group cages for two weeks before the beginning of an experiment. Each animal was used only once. All of the animals used in this study were maintained, and all of the procedures of the study were done according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 85–23, revised 1985) and also in accordance with the Research and Ethics Committee of Tehran University of Medical Sciences, Iran, TUMS Institutional Review Board (IRB), the core body responsible for this approval, which runs under the supervision of the Vice-Chancellor for Research and acts completely in accordance with Declaration of Helsinki and other recognized statements.

2.2. Drugs

The following drugs and chemicals were used in this study: Morphine Sulfate (Temad, Tehran, Iran), Ketamine (Rotexmed-ICA, Trittau, Germany), Xylazine (Alfasan, Woerden, Holland), Rat CART peptide 55–102 (Sigma-Aldrich, United Kingdom), Anti-NMDAR1 antibody-Neuronal Marker ab17345 (Abcam, United Kingdom), and Goat Anti-Rabbit IgG H&L (HRP; Abcam, United Kingdom). Morphine Sulfate was dissolved in 0.9% sterile saline and Rat CART peptide 55–102 was dissolved in artificial cerebral spinal fluid (aCSF; Harvard Apparatus, Holliston, MA, USA).

2.3. Primary and Secondary Outcomes

The main primary experimental outcome was assessing the probable ability of CART 55–102 peptide to improve or impair learning and memory processes via NAc shell (injected into the NAc shell), and whether this peptide microinjection in this area has any influence on morphine-induced conditioning, as assessed by the difference between the time spent in the drug-associated compartment and the time spent in the saline-paired side (or any control substance) on the testing day. The secondary outcomes were assessing the possible involvement of NMDA glutamate receptor in CART-modulated place preference as well as in morphine-CART interactions-induced learning and memory during CPP procedure as measured by the changes in NR1 subunit expression within the key structures of the brain mesocorticolimbic reward circuits (NAc, PFC and Hippocampus) by applying immunohistochemistry.

2.4. Surgical and infusion procedures

Stereotaxic surgical procedures were done in all rats before entering the CPP apparatus. The animals were anesthetized with intraperitoneal (i.p.) injections of ketamine (60 mg/kg) / xylazine (15 mg/kg) delivered in 1 min and placed in the stereotaxic apparatus (Stoelting Instruments, USA) when we became

sure that they were completely anesthetized, using a gentle toe pinch withdraw reflex. To prevent corneal drying and trauma during anesthesia, ophthalmic ointment was applied. A sagittal midline incision was made in the scalp, and the skull was cleaned. By using the Burr hole, the skull over the NAc shell was drilled bilaterally according to the stereotaxic coordinates: +0.7 mm anterior, ± 1.0 mm bilateral to the midline and 6.5 mm ventral to bregma as can be found in the stereotaxic atlas described by Paxinos and Watson [20] (Fig. 1). Two 27-gauge guide cannulas (made of stainless-steel tubing) were secured by acrylic dental cement and anchored to stainless steel screws fixed to the skull. Cannulas were implanted 1 mm above the NAc shell. A single dose of penicillin (50 mg/kg, intramuscular) was injected and local antibiotic treatment (bacitracin ointment) was administered following surgery. During and after surgery, rats were placed on a heating pad to maintain body temperature at 37 ± 0.5 °C. A bolus of Lactated Ringers of the 0.9% saline is given at the end of surgery (5 ml, s.c.) to prevent dehydration. Buprenorphine (0.2 mg/kg, s.c.) was also administered twice daily on the operation day and also on the day after the surgery and then, on an as-needed basis, if it appears that rats had pain. After stereotaxic surgery, animals are individually housed with food and water accessible ad libitum. To prevent clogging, the stainless-steel stylets (30 gauge) were placed in the guide cannulas until the animals received the intra-NAc shell injection. The animals were allowed to recover for 10 days before place conditioning. After the recovery period, rats with normal locomotor activity and weight gain were allowed to enter the next experiments. For drug infusion, the stylets were withdrawn and replaced by the injection units (27-gauge stainless steel tubing), ending 1 mm below the tip of the guides. Each injection unit was connected by polyethylene tubing to a 1- μ l Hamilton syringe. Each intra-NAc shell injection was done with 0.5 μ l solutions over one minute. The cannulas were left in place for an additional one minute to allow diffusion, and then the stylets were re-inserted into the guide cannulas. During the infusion procedure, the experimenter loosely held the animal. The sham groups received the same volume of aCSF instead of drug solutions. The body weight was controlled and recorded every three days and finally 5 rats were excluded from the rest of the study due to not gaining enough weight during 10 days of the recovery period.

2.5. Place conditioning apparatus

Place conditioning studies were performed in a three-compartment apparatus. The two larger, outer compartments (A and B; 25 × 30 × 32 cm) were separated by a central compartment (C; 10 × 25 × 32 cm) and differed in both visual and tactile cues. One outer compartment had white vertical lines on the walls, and the other one had white horizontal lines. The floors of the two compartments had different textures. The central compartment had white walls and a Plexiglas floor and was designed to allow animals to move freely between the two outer compartments unless it was barred by two white partitions, which restricted movement between compartments during conditioning sessions. Conditioning and test sessions were done under dim illumination and white noise presence. During the procedure, the walls were thoroughly washed, and floors were replaced between each session.

2.5.1. Place conditioning

The CPP paradigm took place in five consecutive days using an unbiased procedure. The experiment consisted of three distinct phases according to the method described by De Fonseca et al. (1995): preconditioning, conditioning, and testing [21]. All experiments were performed under neon ceiling light (58 W, 1 m distance) during the day (7:00–19:00 h).

2.5.1.1. Preconditioning

On the first day, each rat was placed separately into the apparatus for 45 min with free access to all compartments (A, B and C), and the amount of time spent in each compartment was measured. The position of the animal was defined by the position of its front paws. Rats who did not show a significant innate preference for either of the conditioning compartments were selected. After testing all the animals, we finally excluded 25 rats who showed significant preference for one compartment of the CPP apparatus and 120 male rats remained for our experimental groups. Each group consisted of nine animals which completely performed on a randomized basis. All assigned rats were conducted through CPP paradigm.

2.5.1.2. Conditioning

Place conditioning phase (in three continuous days) was done after preconditioning. This phase consisted of six 30-min sessions (three saline- and three drug-paired periods). Conditioning phase started the day after pre-conditioning phase. These periods were conducted twice a day (days 2–4) with a 6-h interval. On each of these days, separate groups of animals received one conditioning session with drug and one with saline or aCSF. We administered morphine or saline subcutaneously (s.c.) based on previous studies showing the effectiveness of s.c. rout compared to the i.p. one in the induction of CPP without changes in locomotor activity [22]. Animals were injected with drugs and immediately confined to one compartment of the apparatus for 30 min by closing the removable door. Six h later, following the administration of saline or aCSF, the animals were restricted to the other compartment. The order of drug presentation was counterbalanced on either day. This schedule was performed to avoid circadian variability (morning/evening) [21].

2.5.1.3. Testing

On day 5, each animal was allowed to have free access to all compartments of the apparatus for 15 min without any injection. The time spent in the drug-paired compartment was recorded for each animal. Place preference for a drug was defined if the time spent in the drug-paired side was greater than the time spent in saline (or any control substance)-paired side. In contrast, conditioned place aversion was recorded if the time spent in the drug-paired side was less than the time spent in saline (or any control substance)-paired side [23].

2.6. Locomotor testing

Locomotor activity was measured during the testing phase. For this purpose, the floors of the two conditioning compartments were divided into four equal-sized areas. Locomotor activity was evaluated

as the number of line crossings for 15 min.

2.7. Experimental design

2.7.1. Experiment 1: Dose-response curve for morphine-induced place preference

All randomized rats (overall n = 55, each group n = 9) which classified in this group were used. This experiment was designed to plot a dose-response curve for morphine place conditioning. Different doses of morphine (0.5, 2.5, 5, 7.5, and 10 mg/kg; s.c.) were tested for their ability to induce place conditioning. One group of animals received only saline (1 ml/kg) and five groups were injected with saline (1 ml/kg) or morphine (s.c.) on alternate sessions for three days as it was explained above. Also, all the rats were infused with aCSF (0.5 µl/side; intra-NAc shell) before receiving s.c. injection. Locomotor activity was also assessed in the testing phase.

2.7.2. Experiment 2: Dose-response curve for CART peptide-induced place preference or place aversion

All randomized rats (overall n = 45, each group n = 9) which classified in this group were used. The ability of CART 55–102 peptide to induce CPP or CPA was evaluated by the administration of aCSF (0.5 µl/side) or four doses of the peptide (0.625, 1.25, 2.5, and 5 µg/side in the volume of 0.5 µl/side, intra-NAc shell) on alternative sessions in three conditioning days and one group of animals received only aCSF (0.5 µl/side) as the control group. It should be mentioned that all of the animals received s.c. injections of saline after administration of aCSF or CART. Locomotor activity was also calculated on the testing day.

2.7.3. Experiment 3: administration of a dose of CART that did not produce CPP or CPA plus a dose of morphine that did not produce CPP or CPA

All randomized rats which classified in this group were used (n = 10). In the third experiment, the animals received the maximum dose of CART 55–102 (intra-NAc shell) that induced neither CPP nor CPA (1.25 µg/side) before injection of the highest dose of morphine that also did not produce either CPP or CPA (2.5 mg/kg; s.c.) in three days of the conditioning phase. For alternative sessions, we administered aCSF (0.5 µl/side) plus saline (1 ml/kg) and placed the rats in the other compartment. Locomotor activity was evaluated during the testing day.

2.7.4. Experiment 4: Intra-NAc shell administration of a dose of CART 55–102 that produced CPA plus systemic administration of a dose of morphine that produced CPP

All randomized rats which classified in this group were used (n = 10). The animals received the highest dose of CART 55–102 that produced the CPA (5 µg/side; intra-NAc shell) before injection of the lowest dose of morphine that produced CPP (5 mg/kg; s.c.) in three days of the conditioning phase. Alternative sessions were designed similar to the experiment 3. Locomotor activity was also assessed in the testing phase.

2.8. Histology

Immediately after the CPP test, the animals were deeply anesthetized by i.p. injection of sodium pentobarbital (75 mg/kg) and after around 5 minutes, when the rats became completely anesthetized and not reacted to toe pinch, they were perfused transcardially with phosphate-buffered saline solution (PBS) (pH 7.4), followed by 10% paraformaldehyde (PFA). After decapitation, brains were removed, and after blocking procedures, they were cut by microtome device. The brains that the cannulas were correctly placed in the NAc shells were selected (n = 104). Data acquired from the animals that cannulas were located outside of the NAc shell were not used in both behavioral and immunohistochemistry analysis.

2.9. Immunohistochemistry (IHC)

After taking out the brain from the skull, to preserve tissue morphology and retain the antigenicity of the target molecules, the tissues were fixed by vascular perfusion of 500 ml PBS, followed by 500 ml PFA (10%, v/v) fixative solution. Then, the brains were fixed by immersion in 4% PFA for 24 h and then 48 h in 30% sucrose (in falcon tubes) until the brain sank. When the brain tissues became fully dehydrated, they were put in the special block. Subsequently, the brains were embedded in optimum cutting temperature cryostat-embedding compound (Tissue-Tek, Torrance, CA) and the base mold containing the tissue block was placed into the liquid nitrogen until the entire tissue block was submerged into the liquid, and then the frozen tissue block was stored at -80 °C. Ten µm thick tissue sections were cut using a cryotome cryostat (Leica Microsystems, Wetzlar, Germany) while the cryostat temperature was kept between 25 and 30 °C. The selected brain areas were NAc, hippocampus, and PFC. These tissue sections were placed on glass slides suitable for IHC (poly-L-lysine-coated). After drying the sections overnight at room temperature, they were stored in a sealed slide box at -80 °C for later use. To fix the tissue sections, they were immersed in pre-cooled acetone (-20 °C) for 10 min. After that, to evaporate the acetone from tissue sections, they were put for more than 20 min at room temperature. Then the slides were rinsed two times with 300 ml PBS at a neutral pH. Because NR1 staining is visible on the cell membrane as well as in the cytoplasm [24], we incubated the samples for 10 min with PBS solution containing 0.25% Triton X-100 to improve the antibody penetration. To block the endogenous peroxidase activity, the slides were incubated in 0.3% H₂O₂ solution in PBS at room temperature (for 10 min). After incubation, the slides were rinsed with 300 ml PBS two times. Then, more than 100 µl 10% BSA in PBS was added as a blocking buffer onto the slides, and they were incubated in a humidified chamber at room temperature for one h. After raising off the blocking buffer, we applied more than 100 µl of an appropriately diluted primary antibody in 0.5% BSA to the sections on the slides and incubated them in a humidified chamber at 4 °C overnight. Then, the slides were rinsed with 300 ml PBS two times. After that, by applying 100 µl fresh DAB substrate solution to the sections, the color of the antibody staining was revealed in about 5 min. Then, the slides

were washed with 300 ml PBS two times and counterstained by immersion in Hematoxylin for 1 min. The slides were then rinsed in running tap water for about 5 min. Afterward, the tissue slides were dehydrated through 4 steps (5 min each) of alcohol exposure (95%, 95%, 100%, and 100%). The tissue slides were then cleared in three changes of xylene and cover-slipped by the mounting solution. At last, the color of the antibody staining in the tissue sections was observed under the microscope.

2.10. Cell counting

Sections were imaged sequentially under light microscopy (Nikon Corporation, Japan) with an attached image capture analysis system. Individual cells were manually counted along the bilateral PFC, hippocampus and NAc regions of each animal at 40 × magnification. Quantification was performed using the Image J software. Five different images of the areas were quantified per side per animal and the average was used for further analysis. The results are expressed as the number of immunopositive cells per square millimeter.

2.11. Statistical analysis

In all the experiments, CPP and CPA scores were conveyed as the difference between the time spent in the drug-associated compartment and the time spent in the saline-paired side (or any control substance) on the testing day. Locomotor activity was reported as the number of crossed lines inside the two main conditioning compartments during the testing phase. Data were expressed as mean ± SEM (n = 8; because 16 animals were excluded due to incorrect site of cannula, the approximate number of animals in each group that their data used for statistical analysis was eight) and analyzed by one-way repeated measures analysis of variance (ANOVA). Following a significant F-value, post-hoc testing (Tukey's test) was performed for assessing specific group comparisons. Data comparisons in the two groups were performed by Student's t-test. The level of statistical significance was set at $P < 0.05$. All data were analyzed using SPSS 22.0 Software for Windows and were plotted by GraphPad Prism 6. Statistical analysis of IHC data was done by ANOVA test with Bonferroni post hoc correction for multiple t-tests (Prism software, GraphPad, CA).

3. Results

3.1. Histology

Figure 1 illustrates the site for drug injection in the NAc shell and Fig. 2 is the schematics to show the regions of analysis in NAc, PFC, and hippocampus and with some of the well-known neurotransmitter pathways established between these key structures of the reward system. The histological results were plotted on representative sections taken from the rat brain atlas of Paxinos and Watson [20]. Data from the animals with injection sites located outside the NAc shell were not used in either behavioral or IHC analysis.

3.2. Behavioral analysis

3.2.1. Experiment 1: The place conditioning and locomotor effects of s.c. morphine administration

Our data showed that morphine induced CPP dose-dependently (Fig. 3a, left panel). One-way ANOVA revealed that morphine induced a noteworthy dose-related place preference [$F(5, 42) = 49.09, P < 0.001$]. Post-hoc analysis showed statistically significant conditioning at doses of 5, 7.5 and 10 mg/kg. In the locomotor activity graph (Fig. 3b, left panel), one-way ANOVA analysis showed a significant effect on the testing day [$F(5, 42) = 2.534, p < 0.05$]. Post-hoc analysis indicated that only the highest dose of morphine had a significant effect. Therefore, the maximum CPP effect of morphine without notable changes in locomotion was seen with 7.5 mg/kg.

3.2.2. Experiment 2: The place conditioning and locomotor effects of intra-NAc shell administration of CART 55–102 peptide

One-way ANOVA analysis demonstrated that CART induced place preference or aversion dose-dependently [$F(4, 35) = 70.64, P < 0.01$] (Fig. 3a, middle panel). The animals that received intra-NAc shell infusion of aCSF (0.5 μ l/side) or CART 55–102 (0.625 and 1.25 μ g/side), showed no differences in the time spent in either compartment during the CPP test. However, rats that received 2.5 μ g/side concentration of CART presented significant place conditioning ($p < 0.01$). In contrast, animals that were injected with 5 μ g/side CART 55–102, spent more time in the aCSF-paired compartment than the CART-paired compartment during the test ($p < 0.01$). These injections did not alter the locomotion activity [$F(4, 35) = 4.652, P > 0.05$] (Fig. 3b, middle panel).

3.2.3. Experiment 3: The effect of a sub-threshold dose of CART 55–102 on conditioning response induced by the sub-threshold dose of morphine

Independent t-test analysis indicated that animals who received an ineffective dose of CART (1.25 μ g/side, intra-NAc shell) plus an ineffective dose of morphine (2.5 mg/kg, s.c.) spent more time in CART + morphine-paired compartment compared to the aCSF + morphine-paired or CART + saline-paired compartment during the place conditioning test and showed significant CPP ($p < 0.01$; Fig. 3a, right panel). No significant changes in locomotor activity were found during the testing day ($P > 0.05$; Fig. 3b, right panel).

3.2.4. Experiment 4: The effect of CART-induced CPA on morphine-induced CPP

To determine the effects of CART on morphine reward, rats received intra-NAc shell infusion of a dose of CART 55–102 that produced the CPA (5 µg/side) before the injection of a dose of morphine that produced CPP (5 mg/kg; s.c.). Based on the results of previous experiments, 5 mg/kg was the minimum dose of morphine that produced CPP. Independent t-test analysis showed that the effective CPA inducer dose of CART (5 µg/side) abolished the CPP of 5 mg/kg morphine ($P < 0.01$; Fig. 3a, right panel), while did not alter the locomotion ($P > 0.05$; Fig. 3b, right panel).

3.3. IHC analysis in the rat brains

3.3.1. IHC data of NR1 protein in the NAc

IHC analysis of brain tissues extracted from control rats revealed that the NR1 subunit peptide is expressed in all examined areas of this study (hippocampus, NAc, and PFC). In the NAc, one-way ANOVA showed that morphine CPP could induce a dose-dependent increase in the expression of the NR1 subunit [$F(5, 24) = 49.05, P < 0.001$] (Fig. 4a, left panel). The NR1 expression level was significantly different from the saline group when morphine was administered with doses of 5 ($P < 0.01$), 7.5 and 10 mg/kg ($P < 0.001$). One-way ANOVA analysis revealed that the level of NR1 was changed after intra-NAc shell infusion of four different doses of CART 55–102 in place conditioning test [$F(4, 20) = 18.29, P < 0.001$] (Fig. 4a, middle panel). Although the NR1 content was increased significantly after administration of 2.5 µg/side CART ($P < 0.01$), 5 µg/side infusion of the drug induced a noteworthy decrease in NR1 expression ($P < 0.01$). Infusion of a dose of CART that produced neither CPP nor CPA prior to the injection of an ineffective dose of morphine caused an increase in the quantity of NR1 in the NAc in comparison to the level of this peptide after administration of only a sub-threshold dose of morphine ($P < 0.01$) (Fig. 4a, right panel). The IHC assessment of NAc samples in rats who received intra-NAc shell infusion of a dose of CART that produced CPA prior to the injection of the lowest dose of morphine that produced CPP, indicated a significant decrease in the NR1 subunit level compared to the morphine group (5 mg/kg; s.c.) ($P < 0.01$) (Fig. 4a, right panel). Some selected images from each panel have been demonstrated in Fig. 4b.

3.3.2. IHC data of NR1 protein in the PFC

One-way ANOVA demonstrated that morphine induced a dose-dependent upsurge in the expression of the NR1 peptide in the PFC [$F(5, 24) = 39.98, P < 0.001$] (Fig. 5a, left panel). The NR1 content was significantly elevated with the doses of 5 ($P < 0.01$), 7.5, and 10 mg/kg ($P < 0.001$). Analysis by one-way ANOVA showed that CART 55–102 induced a significant dose-dependent change in the NR1 content [$F(4, 20) = 46.78, P < 0.001$] (Fig. 5a, middle panel). The amount of NR1 was increased significantly with a dose of 2.5 µg/side ($P < 0.01$) and was decreased with a dose of 5 µg/side ($P < 0.01$). Co-administration of sub-threshold doses of CART and morphine together caused an elevation in the amount of NR1 in the PFC in

comparison to the 2.5 mg/kg morphine group ($P < 0.01$) (Fig. 5a, right panel). Administration of a dose of CART that produced CPA before the injection of the lowest dose of morphine which produced CPP had no significant effect on the NR1 level in the PFC in comparison to 5 mg/kg morphine group (Fig. 5a, right panel). Some images from each panel have been shown in Fig. 5b.

3.3.3. IHC data of NR1 protein in the hippocampus

One-way ANOVA showed that morphine induced a dose-dependent increase in the NR1 subunit expression in the hippocampus [$F(5, 24) = 64.75, P < 0.001$] (Fig. 6a, left panel). In comparison to control animals, the amount of NR1 in the hippocampus was increased significantly with the doses of 2.5 ($P < 0.05$), 5 ($P < 0.01$), 7.5, and 10 mg/kg ($P < 0.001$) of morphine. One-way ANOVA also showed an obvious change in the NR1 content in the hippocampus by intra-NAc shell infusion of four different doses of CART 55–102 [$F(4, 20) = 36.20, P < 0.001$] (Fig. 6a, middle panel). Although the amount of this peptide was elevated by the dose of 1.25 and 2.5 $\mu\text{g}/\text{side}$ of CART ($P < 0.05$ and $P < 0.001$, respectively), it was decreased significantly with 5 $\mu\text{g}/\text{side}$ ($P < 0.001$). Administration of CART with a dose that produced neither CPP nor CPA before injection of a sub-threshold dose of morphine resulted in a significant increase in NR1 content ($P < 0.01$) (Fig. 6a, right panel). As it is shown in the right panel of Fig. 6a, infusion of a dose of CART that induced CPA before the injection of a dose of morphine that induced CPA decreased the content of hippocampal NR1 subunit ($P < 0.01$). Some selected images of each panel can be found in Fig. 6b.

4. Discussion

Although this study was designed completely in accordance with the 3Rs principles (replacement, refinement and reduction) of the use of animals in research, replacing rats were not possible with nor lower species, neither alternative laboratory techniques. As this study had been planned to evaluate the probable effects of CART peptide on learning and memory processes in the brain, there was no suitable alternative way to gain the same quality of results, and rationalize the benefits of proposed research compared with the costs to the animals. Attributable to complex neuroanatomical organization of the prefrontal cortex and the topographic ordering of cortical and subcortical connections, our current knowledge of reward pathway, primary and secondary reinforcement and the effects of reinforcement on behavioral and neurobiological bases of reward-induced learning and memory formation as well as place preference or aversion all arise significantly from animal research and thus using in vivo methods are still the best available method for gaining meaningful and detailed data about complex cortical and subcortical networks of the reward, learning and memory pathways.

As the second R in the 3Rs principle is Reduction, we tried to use methods which minimize the number of animals used per experiment by calculating the sample size by power analysis. Based on our laboratory previous findings of morphine-induced CPP as well as pilot studies of CART-induced place preference or aversion and also by having enough knowledge and information about effect size, standard deviation, type 1 error, power of a study, statistical tests, expected attrition or death of animals we applied G Power

program to calculate the sample size, the exact number of animals which not only led to missing of any significant difference, but also resulted in any prevention from unnecessary wastage of animal and resources.

The third R is Refinement methods which about minimizing the pain, suffering, distress or lasting harm that may be experienced by research animals, and improving their welfare. In our study, refinement were applied to all aspects of animal use, from their housing and husbandry to the scientific procedures performed on them; including ensuring the animals are provided with housing and environmental enrichment that allows the expression of species-specific behaviors, using appropriate anesthesia and analgesic regimes for pain relief, and training animals to voluntarily co-operate with procedures to have greater control over the procedure in order to reduce any distress.

An enormous bulk of experiments has shown the main role of CART peptide in the action of psychostimulants and alcohol. In addition to these studies, some recent experiments have reported the association between the CART peptide and opiate compounds. Some examples may be the increase of the anti-nociceptive effect of morphine by CART [14] or the action of CART (85–102) in attenuating the expression of chronic morphine sensitization [15]. Upadhyaya and colleagues have suggested that in the framework of NAc shell, CART 55–102 serves as the final output of the endogenous opioid-mesolimbic-dopamine system [16]. Our previous studies demonstrated that not only the content of CART 55–102 in some reward areas in the rat brain is altered by acute and chronic morphine dependence and abstinence syndrome [13], but also it changes in the CSF and plasma [25]. Still, relapse is the most important problem of opiate addiction, which is related to drug-induced learning and memory [26]. In the current study, we evaluated the probable interaction between the CART peptide and morphine in producing drug-related learning and memory. Then, we tried to find out whether the NMDA receptor content in the reward system would change in response to the administration of morphine, CART, and their combination. Place conditioning studies have verified that large amounts of neuronal circuits, neurotransmitters, neuromodulators, receptors, and discrete brain sites are involved in the brain reward mechanisms [27]. Mesocorticolimbic system, which initiates from the ventral tegmental area (VTA) and projects to the NAc, limbic system, and many cortical areas, is the most important pathway in the reward process [28]. Our data showed that morphine administration to rats could induce CPP dose-dependently. Numerous previous studies have reported such results. The morphine dose selection in our study was based on what Rezayof and colleagues have reported in 2002 [29]. Since many years ago, numerous studies have been done to clarify the neurotransmitters and neuroanatomical sites, which are involved in producing CPP or CPA. Now it is clear that opioid neuronal system plays an important role in CPP learning and memory [30]. Also, the key role of the NMDA receptor in modulating reward memory reconsolidation, including drug-induced reward and memory has been reported previously [31, 32]. Our IHC results showed that by increasing the dose of morphine, the number of NR1 subunits of the NMDA receptor in all the examined parts of the reward pathway was increased. Considering the essential role of the NAc as a key structure in motivation and memory, Ahmadi and colleagues have demonstrated that systemic administration of morphine may induce passive avoidance memory through NMDA function in the NAc

[33]. In another study, it was established that NMDA receptors in the NAc are involved in the reconsolidation of aversive and positive morphine-associated memories [34–36].

Our data also indicated that the intra-NAc shell infusion of the 2.5 µg CART produced CPP, while the higher concentration of the drug (5 µg/side) induced CPA. To the best of our knowledge, there is no previous study similar to our work. There is only one study that has evaluated the ability of intra-basolateral amygdala (BLA) infusion of CART 55–102 (with different doses of 1, 2, or 4 µg/side) to induce conditioned place preference or aversion. The results of the mentioned study are so impressive and are similar to our data. Intra-BLA infusion of aCSF or CART (1 µg/side) has produced neither CPP nor CPA, while 2 µg/side of the drug has induced CPP and 4 µg/side has made CPA [37]. Other studies have reported that intra-VTA administration of CART 55–102 could raise locomotor activity and induce CPP [38]. Also, injection of CART 55–102 into the NAc could decrease the locomotor activity induced by the systemic infusion of cocaine and D-amphetamine. However, it has no effect on locomotor activity when it is administered alone [39]. Also, in CART null mice, the effect of D-amphetamine in producing CPP was attenuated. The authors proposed a modulatory role for CART peptide in locomotor activity and other affective behaviors related to the D-amphetamine administration [40]. Our results showed that different doses of CART 55–102 (infused in the NAc shell) have rewarding or aversive effects. Furthermore, our findings also revealed that administration of CART 55–102 into the NAc shell had no significant effect on locomotor activity. Previous studies have established that the NAc shell does not play a significant role in locomotor activity [41]. Previous studies have reported that not only CART mRNA and peptides are found in several distinct brain nuclei including the NAc, a site with a robust dopamine input, but also dopamine receptors are found on CART neurons [42]. Considerable evidence exists showing that tyrosine hydroxylase-positive nerve terminals synapse on CART peptide-containing neurons in the NAc [16, 43]. It seems that CART, released from the axonal terminals in the framework of NAc-shell, may be the final output of the endogenous mesolimbic-dopamine circuitry that processes reward [16]. Injection of CART 55–102 peptide into the ventricles leads to the increased turnover of Dopamine [44]. The existing data propose that in the NAc, CART 55–102 peptide functions as a homeostatic regulator of dopamine-mediated activity [45]. In other words, as dopamine signaling or its activity in the NAc becomes high, CART peptide is released to oppose the actions of dopamine by acting like an inhibitory neurotransmitter. On the other hand, when dopamine activity is low, CART peptide should be a less inhibitory or even an excitatory molecule. Several studies have reported that the exogenously injected CART peptide may simulate the effects of endogenously released peptide. Job has suggested that the effects of CART peptide are fully dependent on the conditions it acts in it. He has claimed that under certain settings, intra-NAc CART peptide may have no effect or enhance/facilitate some dopamine-induced behavioral effects [46]. Our results were highly compatible with this suggestion. Moreover, it seems that a reciprocal interaction between dopamine and CART peptide exists. We hypothesize that while the level of CART peptide is increased by exogenous injection of the peptide into the NAc-shell, the dopamine level is raised and CPP is induced, but this effect completely depends on the dose of the injected CART peptide. Exogenous CART in high doses not only impairs the acquisition of CPP, but also results in driving aversion. Therefore, it seems that without a comprehensive systematic understanding of the CART

peptide effects, it is challenging to guess the influence of CART peptide in low dose or high dose in any given brain structure and further studies are needed to fully realize how CART peptide actually acts.

Various studies have shown the association between the CART peptide and NMDA. One study has demonstrated that CART 55–102 could increase the levels of phosphoserine 896 and 897 on the NR1 subunit of the NMDA receptor through protein kinase A and C (leading to the elevation of NMDA function) [47]. Also, it was revealed that the NMDA-mediated nociception in the spine is potentiated by CART administration via protein kinase A and C signaling pathways [48]. CART may be able to stimulate the sympathetic neural axis both directly and indirectly by potentiating the glutamate action on NMDA receptor [49]. Besides, it has been reported that CART may facilitate NMDA-mediated currents in the central amygdala [50]. All these studies show the possibility of the existence of an interaction between CART peptide and NMDA glutamate receptor. Based on these studies and the close impact of NMDA on memory induction, we can propose the probability of CART role in the induction of CPP and CPA dose-dependently.

Our data also showed that the intra-NAc shell infusion of a dose of CART that produced neither CPP nor CPA plus an ineffective dose of morphine induced CPP. It is supposed that the CART peptide could facilitate morphine reward through the NAc shell. Endogenous CART in the framework of reward anatomical regions may be an essential element for the opioid-mesolimbic-dopamine structures and the action of drugs of abuse. CART peptide secreting neurons have been discovered in the NAc. However, the exact cellular mechanisms of CART to modify drug-induced reward are not fully understood [13, 16, 51].

To our knowledge, this is the first study evaluating the possible interaction between opioid and CART in the NAc shell framework and evaluation of the NMDA involvement in reward or aversion-induced learning and memory caused by opioid and CART interaction. In our previous studies, we tried to show the homeostatic role of endogenous CART circuitry as a mediator in the action of morphine [13]. In the brain reward system, activation of mu opioid receptors changes the level of dopamine formation, release, and diffusion in the synaptic space. In the NAc shell, administration of a mu opioid receptor agonist elevates drug consumption, seeking behavior [52], and conditioned reinforcement by enhancing the drug motivational properties [53]. According to Upadhyaya study, it seems that in the NAc shell, CART peptide acts downstream to dopamine and mediates the morphine-induced reward and reinforcement [16]. According to all discussed above, it is proposed that both morphine and CART have rewarding effects and when they are administered together, enforce each other via a final common pathway (dopamine). Data analysis of IHC staining showed a higher quantity of the NR1 subunit in all examined areas. As mentioned above, place preference is a good behavioral test for evaluating drug-induced learning and memory. It is not far from the mind that the level of NMDA in some parts of the reward pathway was elevated because NMDA has a potential role in memory formation [54].

In order to discuss whether increased NR1 synthesis is indicative of increased NR1 functional activity or not, we have to say that most NMDA receptors in the brain are heteromeric complexes with NR1 as constructive and NR2 (A–D) as functional subunits which increase the NMDA receptor-mediated current.

The NR1 subunit serves as an important subunit critical for ion selectivity and agonist binding of the NMDA receptors [55]. In our study, while morphine treatment induced dose-dependent CPP, the amount of NR1 subunit increased in several regions of the reward pathway. Moreover, when intra-NAc shell microinjection of 2.5 µl/side CART produced CPP, the level of NR1 immunopositive neurons increased in all regions of study. It was also observed that place-aversion learning with 5 µl/side intra-NAc shell infusion of CART peptide was accompanied by a significant decrease in NR1 subunit immunoreactivity in the reward pathway. Thus, according to the crucial role of NMDA receptors in the expression of drug-induced CPP or CPA, developing several forms of learning and memory, and the important structural role and pharmacological characteristics of NR1 subunit protein in the NMDA receptor building and function, it seems that increased NR1 synthesis can be indicative of increased NR1 functional activity. Confirmation of this hypothesis should be assessed by further studies in future.

Another question that may come to mind is whether there is precedent for learning processes to be accompanied by increased NR1 synthesis. There are a number of studies demonstrating that several forms of learning and memory are accompanied by increased NR1 synthesis. Cui and colleagues showed that transient NR1 knockout at the time of learning impaired consolidation and storage of nondeclarative taste memory [56]. It was also demonstrated by Kalev-Zylinska and colleagues that knocking down of the NR1 subunit causes learning impairment, and NR1 overexpression increases fear learning [57]. Furthermore, using striatal NR1-knockout mice, it was shown that striatal-specific deletion of NR1 subunit of NMDA receptors disrupts the development of start/stop activity and impairs sequence learning [58]. Using RNA interference in the honeybee brain during and shortly after appetitive learning, it was indicated that acute disruption of the NMDA receptor subunit NR1 selectively impairs learning as well as mid-term and early long-term memory formation [59]. Moreover, a study in 2009 found that in the Morris water maze test, where identifying the location of an escape platform depends on visual cue-dependent learning, NR1DATCre mice showed a significant longer latency to locate the platform [60]. Furthermore, Ping Li and co-workers found that high-intensity ultrasound irradiation could decrease learning and memory abilities by reducing the expression of NR1 and NR2B in the hippocampal regions and damaging the structure of synapses. In contrast, low-intensity ultrasound irradiation can enhance the learning and memory abilities of the offspring rats by increasing the expression of NR1 and NR2B in the hippocampal regions [61]. In addition, it was found in clinic that a patient with Multiple Sclerosis who developed severe cognitive impairments like learning and memory problems had intrathecal antibodies against the NR1 subunit of the NMDA receptor [62].

Our data also showed that CART-induced CPA could abolish the morphine CPP. It seems that CART 55–102 with aversive dose acts on NAc shell circuitry to modify the morphine reward and reinforcement (related to drug induced-learning and memory). In agreement with our data, Rademacher and colleagues have shown that intra-basolateral amygdala administration of an aversive dose of CART 55–102 before the systemic administration of a rewarding dose of amphetamine produced neither CPP nor CPA. They proposed that CART in an aversive dose acts on basolateral amygdala circuitry to block amphetamine reward [37]. Brain-imaging studies have identified NAc and the amygdala as putative neuroanatomical regions involved in drug conditioning [63]. The amygdala-accumbens pathway modulates drug-induced

learning and memory through several neurotransmitters in this circuitry like dopamine [64]. Based on the results of these studies, it was predictable that we get such results because amphetamine and morphine act via dopaminergic pathway and CART acts downstream to dopamine.

In our study, although the levels of the NR1 subunit in the NAc and hippocampus were reduced meaningfully in comparison to aCSF + morphine group (5 mg/kg), no significant changes were observed in the PFC. We have previously reported that the PFC has the lowest amount of CART peptide compared to the NAc, striatum, and hippocampus. Furthermore, it was much less influenced than the other sites by acute and chronic morphine administration and also acute naloxone injection to addicted rats [13]. This may be a hypothesis for the lack of an apparent change in NR1 expression in the PFC in comparison to two other sites. Although we did not find direct evidences in the literature in support of our hypothesis, it has been claimed that exogenously injected CART peptide is able to selectively increase NMDA receptor-mediated glutamatergic neurotransmission [65]. Altogether, it can be proposed that the NMDA receptor may interact with the CART peptide in potentiating or inhibiting morphine-induced reward, reinforcement and place conditioning. Further studies are needed in this regard to evaluate the effect of CART peptide administration on NR1 subunit expression in NMDA receptor.

5. Conclusions

This is the first study aimed at evaluation of the possible interaction between CART 55–102, opioid and NMDA glutamate system in the framework of the NAc. According to the ability of CART in producing both CPP and CPA (dose-dependently) and also the critical role of the CART signaling system in modulation of the rewarding effects of psychostimulants, alcohol, and opioids, it is not far from mind that in the current study, intra-NAc shell administration of CART 55–102 facilitates, attenuates, or blocks morphine-induced reward and reinforcement. NMDA receptors are found in the critical sites of the reward pathway. Based on our results, it appears that these receptors are involved in producing aversive conditioning memory in addition to preference conditioning. Our results suggest a probable close and accurate association between opioid, CART and NMDA systems in the framework of NAc or generally reward circuitry. Since the drug-associated stimuli evoke the craving in humans, CART peptide and NMDA receptors could be a promising target for developing therapeutic agents to diminish drug-induced learning and memory and finally prevent relapse in opiate addicts. Further studies focused on the exact molecular mechanisms underlying opiate, CART and glutamate interaction are required.

Abbreviations

CART: cocaine- and amphetamine-regulated transcript

CPP: conditioned place preference

CPA: conditioned place aversion

NAc: nucleus accumbens

NMDA: N-methyl-D-aspartate

PFC: prefrontal cortex

s.c.: subcutaneous

i.p.: intraperitoneal

aCSF: artificial cerebral spinal fluid

IHC: Immunohistochemistry

VTA: ventral tegmental area

ANOVA: analysis of variance

Declarations

Ethics approval and consent to participate

All experimental protocols of this study were approved by the local Research and Ethics Committee of Tehran University of Medical Sciences (Tehran, Iran).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

Conceptualization of the study was done by AB, NV, and MRZ. Ethical approval was obtained by MN and MRZ. All data were collected and analyzed by AB and NS. The manuscript draft was written by AB and all authors helped to revise the manuscript. All authors read and approved the final manuscript.

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References

1. Christie MJ. Cellular neuroadaptations to chronic opioids: tolerance, withdrawal and addiction. *British journal of pharmacology*. 2008;154(2): 384-96. <https://doi.org/10.1038/bjp.2008.100>
2. Hearing MC, Jedynak J, Ebner SR, Ingebretson A, Asp AJ, Fischer RA, et al. Reversal of morphine-induced cell-type-specific synaptic plasticity in the nucleus accumbens shell blocks reinstatement. *Proceedings of the National Academy of Sciences of the United States of America*. 2016;113(3): 757-62. <https://doi.org/10.1073/pnas.1519248113>
3. Pecina S, Berridge KC. Dopamine or opioid stimulation of nucleus accumbens similarly amplify cue-triggered 'wanting' for reward: entire core and medial shell mapped as substrates for PIT enhancement. *The European journal of neuroscience*. 2013;37(9):1529-40. <https://doi.org/10.1111/ejn.12174>
4. Volkow ND, Wang GJ, Tomasi D, Baler RD. The addictive dimensionality of obesity. *Biological psychiatry*. 2013;73(9):811-8. <https://doi.org/10.1016/j.biopsych.2012.12.020>
5. Carlson NR. *Foundations of behavioral neuroscience*: Pearson Higher Ed; 2013.
6. Quintero GC. Role of nucleus accumbens glutamatergic plasticity in drug addiction. *Neuropsychiatric disease and treatment*. 2013;9:1499-512. <https://doi.org/10.2147/NDT.S45963>
7. Reissner KJ, Kalivas PW. Using glutamate homeostasis as a target for treating addictive disorders. *Behavioural pharmacology*. 2010;21(5-6):514-22. doi: 10.1097/FBP.0b013e32833d41b2
8. LaLumiere RT, Kalivas PW. Glutamate release in the nucleus accumbens core is necessary for heroin seeking. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2008;28(12):3170-7. <https://doi.org/10.1523/JNEUROSCI.5129-07.2008>
9. Kalivas PW, Volkow ND. The neural basis of addiction: a pathology of motivation and choice. *The American journal of psychiatry*. 2005;162(8):1403-13. DOI: 10.1176/appi.ajp.162.8.1403
10. Trujillo KA, Akil H. Inhibition of morphine tolerance and dependence by the NMDA receptor antagonist MK-801. *Science (New York, NY)*. 1991;251(4989):85-7. DOI: 10.1126/science.1824728
11. Bharne AP, Borkar CD, Bodakuntla S, Lahiri M, Subhedar NK, Kokare DM. Pro-cognitive action of CART is mediated via ERK in the hippocampus. *Hippocampus*. 2016;26(10):1313-27. <https://doi.org/10.1002/hipo.22608>

12. Gilon P. Cocaine- and amphetamine-regulated transcript: a novel regulator of energy homeostasis expressed in a subpopulation of pancreatic islet cells. *Diabetologia*. 2016;59(9):1855-9. <https://doi.org/10.1007/s0012>
13. Bakhtazad A, Vousooghi N, Garmabi B, Zarrindast MR. CART peptide and opioid addiction: expression changes in male rat brain. *Neuroscience*. 2016. <https://doi.org/10.1016/j.neuroscience.2016.02.071>
14. Damaj MI, Hunter RG, Martin BR, Kuhar MJ. Intrathecal CART (55-102) enhances the spinal analgesic actions of morphine in mice. *Brain research*. 2004;1024(1-2):146-9. <https://doi.org/10.1016/j.brainres.2004.07.058>
15. Dylag T, Kotlinska J, Rafalski P, Pachuta A, Silberring J. The activity of CART peptide fragments. *Peptides*. 2006;27(8):1926-33. <https://doi.org/10.1016/j.peptides.2005.10.025>
16. Upadhya MA, Nakhate KT, Kokare DM, Singh U, Singru PS, Subhedar NK. CART peptide in the nucleus accumbens shell acts downstream to dopamine and mediates the reward and reinforcement actions of morphine. *Neuropharmacology*. 2012;62(4):1823-33. <https://doi.org/10.1016/j.neuropharm.2011.12.004>
17. Bakhtazad A, Vousooghi N, Garmabi B, Zarrindast MR. Evaluation of the CART peptide expression in morphine sensitization in male rats. *European journal of pharmacology*. 2017;802:52-9. <https://doi.org/10.1016/j.ejphar.2017.02.040>
18. Upadhya MA, Nakhate KT, Kokare DM, Singru PS, Subhedar NK. Cocaine- and amphetamine-regulated transcript peptide increases spatial learning and memory in rats. *Life sciences*. 2011;88(7-8):322-34. <https://doi.org/10.1016/j.lfs.2010.12.008>
19. Choudhary AG, Somalwar AR, Sagarkar S, Rale A, Sakharkar A, Subhedar NK, et al. CART neurons in the lateral hypothalamus communicate with the nucleus accumbens shell via glutamatergic neurons in paraventricular thalamic nucleus to modulate reward behavior. *Brain structure & function*. 2018;223(3):1313-28. <https://doi.org/10.1007/s00429-017-1544-6>
20. Paxinos G, Watson C. *The Rat Brain in stereotaxic coordinates* (6 ed). Academic Press. London, Uk. 2007.
21. Rodriguez De Fonseca F, Rubio P, Martin-Calderon JL, Caine SB, Koob GF, Navarro M. The dopamine receptor agonist 7-OH-DPAT modulates the acquisition and expression of morphine-induced place preference. *European journal of pharmacology*. 1995;274(1-3):47-55. [https://doi.org/10.1016/0014-2999\(94\)00708-F](https://doi.org/10.1016/0014-2999(94)00708-F)
22. Heinrichs SC, Leite-Morris KA, Carey RJ, Kaplan GB. Baclofen enhances extinction of opiate conditioned place preference. *Behavioural brain research*. 2010;207(2):353-9. <https://doi.org/10.1016/j.bbr.2009.10.013>
23. Le Pen G, Duterte-Boucher D, Costentin J. Sensitization to the rewarding effects of the specific dopamine uptake inhibitor GBR12783. *The Journal of pharmacology and experimental therapeutics*. 1998;286(2):688-96.

24. Sans N, Prybylowski K, Petralia RS, Chang K, Wang YX, Racca C, et al. NMDA receptor trafficking through an interaction between PDZ proteins and the exocyst complex. *Nature cell biology*. 2003;5(6):520-30. <https://doi.org/10.1038/ncb990>
25. Bakhtazad A, Vousooghi N, Garmabi B, Zarrindast MR. Evaluation of CART peptide level in rat plasma and CSF: possible role as a biomarker in opioid addiction. *Peptides*. 2016;84:1-6. <https://doi.org/10.1016/j.peptides.2016.06.010>
26. Nestler EJ. Molecular basis of long-term plasticity underlying addiction. *Nature reviews Neuroscience*. 2001;2(2):119-28. <https://doi.org/10.1038/35053570>
27. McBride WJ, Murphy JM, Ikemoto S. Localization of brain reinforcement mechanisms: intracranial self-administration and intracranial place-conditioning studies. *Behavioural brain research*. 1999;101(2):129-52. [https://doi.org/10.1016/S0166-4328\(99\)00022-4](https://doi.org/10.1016/S0166-4328(99)00022-4)
28. MacLaren DA, Wilson DI, Winn P. Selective lesions of the cholinergic neurons within the posterior pedunculopontine do not alter operant learning or nicotine sensitization. *Brain structure & function*. 2016;221(3):1481-97. <https://doi.org/10.1007/s00429-014-0985-4>
29. Rezayof A, Zarrindast MR, Sahraei H, Haeri-Rohani AH. Involvement of dopamine D2 receptors of the central amygdala on the acquisition and expression of morphine-induced place preference in rat. *Pharmacology, biochemistry, and behavior*. 2002;74(1):187-97. [https://doi.org/10.1016/S0091-3057\(02\)00989-9](https://doi.org/10.1016/S0091-3057(02)00989-9)
30. Ukai M, Lin HP. Endomorphins 1 and 2 induce amnesia via selective modulation of dopamine receptors in mice. *European journal of pharmacology*. 2002;446(1-3):97-101. [https://doi.org/10.1016/S0014-2999\(02\)01760-0](https://doi.org/10.1016/S0014-2999(02)01760-0)
31. Brown TE, Lee BR, Sorg BA. The NMDA antagonist MK-801 disrupts reconsolidation of a cocaine-associated memory for conditioned place preference but not for self-administration in rats. *Learning & memory (Cold Spring Harbor, NY)*. 2008;15(12):857-65. DOI: 10.1101/lm.1152808
32. Milton AL, Lee JL, Butler VJ, Gardner R, Everitt BJ. Intra-amygdala and systemic antagonism of NMDA receptors prevents the reconsolidation of drug-associated memory and impairs subsequently both novel and previously acquired drug-seeking behaviors. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2008;28(33):8230-7. <https://doi.org/10.1523/JNEUROSCI.1723-08.2008>
33. Ahmadi S, Zarrindast MR, Haeri-Rohani A, Rezayof A, Nouri M. Nicotine improves morphine-induced impairment of memory: possible involvement of N-methyl-D-aspartate receptors in the nucleus accumbens. *Developmental neurobiology*. 2007;67(8):1118-27. <https://doi.org/10.1002/dneu.20456>
34. Wu Y, Li Y, Gao J, Sui N. Differential effect of NMDA receptor antagonist in the nucleus accumbens on reconsolidation of morphine-related positive and aversive memory in rats. *European journal of pharmacology*. 2012;674(2-3):321-6. <https://doi.org/10.1016/j.ejphar.2011.11.011>
35. Li Y, Ping X, Yu P, Liang J, Shen F, Han, Cui C. Over-expression of the GluN2B subunit in the forebrain facilitates the acquisition of morphine-related positive and aversive memory in rats. *Behavioural Brain Research*. 2016;311:416-424. <https://doi.org/10.1016/j.bbr.2016.05.039>

36. Tomazi L, Mello CF, Schöffner AP, Girardi BA, Frühauf PK, Rubin MA. A Nonrewarding NMDA Receptor Antagonist Impairs the Acquisition, Consolidation, and Expression of Morphine Conditioned Place Preference in Mice. *Molecular Neurobiology*. 2017;54(1):710–721. <https://doi.org/10.1007/s12035-015-9678-0>
37. Rademacher DJ, Sullivan EM, Figge DA. The effects of infusions of CART 55-102 into the basolateral amygdala on amphetamine-induced conditioned place preference in rats. *Psychopharmacology*. 2010;208(3):499-509. <https://doi.org/10.1007/s00213-009-1748-4>
38. Kimmel HL, Gong W, Vechia SD, Hunter RG, Kuhar MJ. Intra-ventral tegmental area injection of rat cocaine and amphetamine-regulated transcript peptide 55-102 induces locomotor activity and promotes conditioned place preference. *The Journal of pharmacology and experimental therapeutics*. 2000;294(2):784-92.
39. Jaworski JN, Kozel MA, Philpot KB, Kuhar MJ. Intra-accumbal injection of CART (cocaine-amphetamine regulated transcript) peptide reduces cocaine-induced locomotor activity. *The Journal of pharmacology and experimental therapeutics*. 2003;307(3):1038-44. <https://doi.org/10.1124/jpet.103.052332>
40. Couceyro PR, Evans C, McKinzie A, Mitchell D, Dube M, Hagshenas L, et al. Cocaine- and amphetamine-regulated transcript (CART) peptides modulate the locomotor and motivational properties of psychostimulants. *The Journal of pharmacology and experimental therapeutics*. 2005;315(3):1091-100. <https://doi.org/10.1124/jpet.105.091678>
41. Koob GF, Swerdlow NR. The functional output of the mesolimbic dopamine system. *Annals of the New York Academy of Sciences*. 1988;537:216-27. <https://psycnet.apa.org/doi/10.1111/j.1749-6632.1988.tb42108.x>
42. Douglass J, McKinzie AA, Couceyro P. PCR differential display identifies a rat brain mRNA that is transcriptionally regulated by cocaine and amphetamine. *Journal of Neuroscience*, 1995;15:2471–2481. <https://doi.org/10.1523/JNEUROSCI.15-03-02471.1995>
43. Smith Y, Kieval J, Couceyro PR, Kuhar MJ. CART peptide-immunoreactive neurons in the nucleus accumbens in monkeys: Ultrastructural analysis, colocalization studies, and synaptic interactions with dopaminergic afferents. *The Journal of Comparative Neurology*, 1999;407(4):491-511. [https://doi.org/10.1002/\(SICI\)1096-9861\(19990517\)407:4%3C491::AID-CNE3%3E3.0.CO;2-0](https://doi.org/10.1002/(SICI)1096-9861(19990517)407:4%3C491::AID-CNE3%3E3.0.CO;2-0)
44. Hubert GW, Jones DC, Moffett MC, Rogge G, Kuhar MJ. CART Peptides as Modulators of Dopamine and Psychostimulants and Interactions with the Mesolimbic Dopaminergic System. *Biochemical Pharmacology*, 2008; 75(1):57–62. <https://doi.org/10.1016/j.bcp.2007.07.028>
45. Rogge G, Jones D, Hubert GW, Lin Y, Kuhar MJ. CART peptides: regulators of body weight, reward and other functions. *Nature Reviews Neuroscience*. 2008; 9:747–758. <https://doi.org/10.1038/nrn2493>
46. JOB MO, Kuhar MJ. CART peptide in the nucleus accumbens regulates psychostimulants: Correlations between psychostimulant and CART peptide effects. *Neuroscience*. 2017; 348:135–142. <https://doi.org/10.1016/j.neuroscience.2017.02.012>

47. Chiu HY, Lin HH, Lai CC. Cocaine- and amphetamine-regulated transcript (CART) peptide activates ERK pathways via NMDA receptors in rat spinal cord dorsal horn in an age-dependent manner. *Regulatory peptides*. 2010;164(2-3):90-6. <https://doi.org/10.1016/j.regpep.2010.05.012>
48. Chiu HY, Lin HH, Lai CC. Potentiation of spinal NMDA-mediated nociception by cocaine- and amphetamine-regulated transcript peptide via PKA and PKC signaling pathways in rats. *Regulatory peptides*. 2009;158(1-3):77-85. <https://doi.org/10.1016/j.regpep.2009.07.012>
49. Dun SL, Brailoiu GC, Yang J, Chang JK, Dun NJ. Cocaine- and amphetamine-regulated transcript peptide and sympatho-adrenal axis. *Peptides*. 2006;27(8):1949-55. <https://doi.org/10.1016/j.peptides.2005.10.027>
50. Salinas A. Central amygdala CART modulates ethanol withdrawal-induced anxiety 2013.
51. Armbruszt S, Abraham H, Figler M, Kozicz T, Hajnal A. Cocaine- and amphetamine-regulated transcript (CART) peptide immunoreactivity in feeding- and reward-related brain areas of young OLETF rats. *Journal of chemical neuroanatomy*. 2013;50-51:75-84. <https://doi.org/10.1016/j.jchemneu.2013.03.003>
52. Contet C, Kieffer BL, Befort K. Mu opioid receptor: a gateway to drug addiction. *Current opinion in neurobiology*. 2004;14(3):370-8. <https://doi.org/10.1016/j.conb.2004.05.005>
53. Crombag HS, Bossert JM, Koya E, Shaham Y. Review. Context-induced relapse to drug seeking: a review. *Philosophical transactions of the Royal Society of London Series B, Biological sciences*. 2008;363(1507):3233-43. <https://doi.org/10.1098/rstb.2008.0090>
54. Jacobs S, Cui Z, Feng R, Wang H, Wang D, Tsien JZ. Molecular and genetic determinants of the NMDA receptor for superior learning and memory functions. *PloS one*. 2014;9(10):e111865. <https://doi.org/10.1371/journal.pone.0111865>
55. Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B, Seeburg P.H. Heteromeric NMDA Receptors: Molecular and Functional Distinction of Subtypes. *Science*. 1992;256(5060):1217-1221. DOI: 10.1126/science.256.5060.1217
56. Cui Z, Wang H, Tan Y, Zaia KA, Zhang S, Tsien JZ. Inducible and reversible NR1 knockout reveals crucial role of the NMDA receptor in preserving remote memories in the brain. *Neuron*. 2004;41(5):781-93. [https://doi.org/10.1016/S0896-6273\(04\)00072-8](https://doi.org/10.1016/S0896-6273(04)00072-8)
57. Kalev-Zylinska M.L, Symes W, Young D, During M.J. Knockdown and overexpression of NR1 modulate NMDA receptor function. *Molecular and Cellular Neuroscience*. 2009;41(4):383–396. <https://doi.org/10.1016/j.mcn.2009.04.003>
58. Xin Jin & Rui M. Costa. Start/stop signals emerge in nigrostriatal circuits during sequence learning. *Nature*. 2010;466(7305):457–462. <https://doi.org/10.1038/nature09263>
59. Müßig L, Richlitzki A, Rößler R, Eisenhardt D, Menzel R, Lebouille G. Acute Disruption of the NMDA Receptor Subunit NR1 in the Honeybee Brain Selectively Impairs Memory Formation. *Journal of Neuroscience*. 2010;30(23):7817-7825. <https://doi.org/10.1523/JNEUROSCI.5543-09.2010>
60. Caron MG and Wightman RM. To learn, you must pay attention: Molecular insights into teachers' wisdom. *PNAS*. 2009;106 (18):7267-7268. <https://doi.org/10.1073/pnas.0903306106>

61. Li P, Wang PJ, Zhang W. Prenatal Exposure to Ultrasound Affects Learning and Memory in Young Rats. *Ultrasound in Medicine & Biology*. 2015;41(3):644-653. <https://doi.org/10.1016/j.ultrasmedbio.2014.09.015>
62. Fleischmann R, Prüss H, Rosche B, Bahnemann M, Gelderblom G, Deuschle K, Harms L, Kopp U, Ruprecht K. Severe cognitive impairment associated with intrathecal antibodies to the NR1 subunit of the N-Methyl-D-Aspartate Receptor in a patient with Multiple Sclerosis. *JAMA Neurology*. 2015;72(1):96-99. doi:10.1001/jamaneurol.2014.1817
63. Weiss F, Ciccocioppo R, Parsons LH, Katner S, Liu X, Zorrilla EP, et al. Compulsive drug-seeking behavior and relapse. Neuroadaptation, stress, and conditioning factors. *Annals of the New York Academy of Sciences*. 2001;937:1-26. <https://doi.org/10.1111/j.1749-6632.2001.tb03556.x>
64. Kalivas PW, Peters J, Knackstedt L. Animal models and brain circuits in drug addiction. *Molecular interventions*. 2006;6(6):339-44. <http://dx.doi.org/10.1124/mi.6.6.7>
65. Job MO. Injection of Cocaine-Amphetamine Regulated Transcript (CART) peptide into the nucleus accumbens does not inhibit caffeine-induced locomotor activity: Implications for CART peptide mechanism. *Pharmacology Biochemistry and Behavior*. 2016; 148:8-14. <https://doi.org/10.1016/j.pbb.2016.05.001>
66. Alasmari F, Goodwani S, McCullumsmith RE, Sari Y. Role of glutamatergic system and mesocorticolimbic circuits in alcohol dependence. *Progress in Neurobiology*. 2018;171:32-49. <https://doi.org/10.1016/j.pneurobio.2018.10.001>

Figures

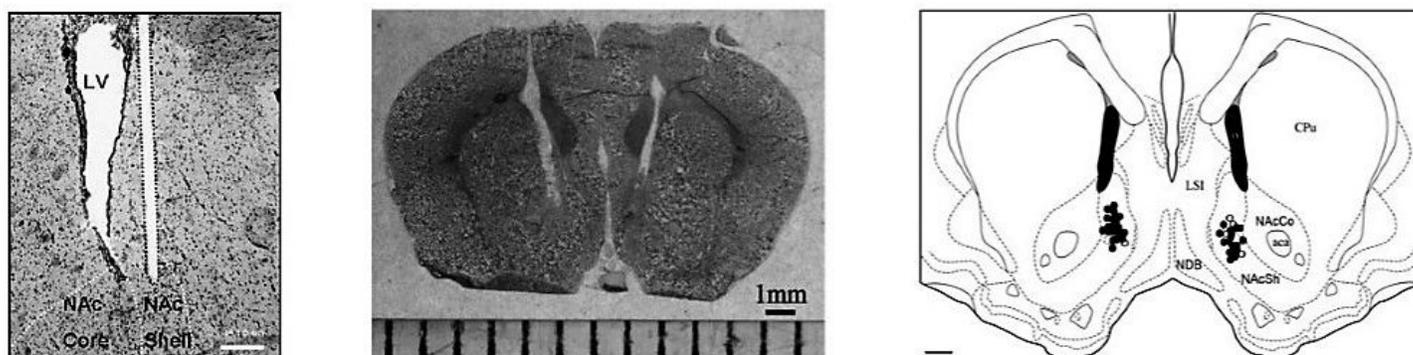


Figure 1

The site of drug injection in the NAc shell.

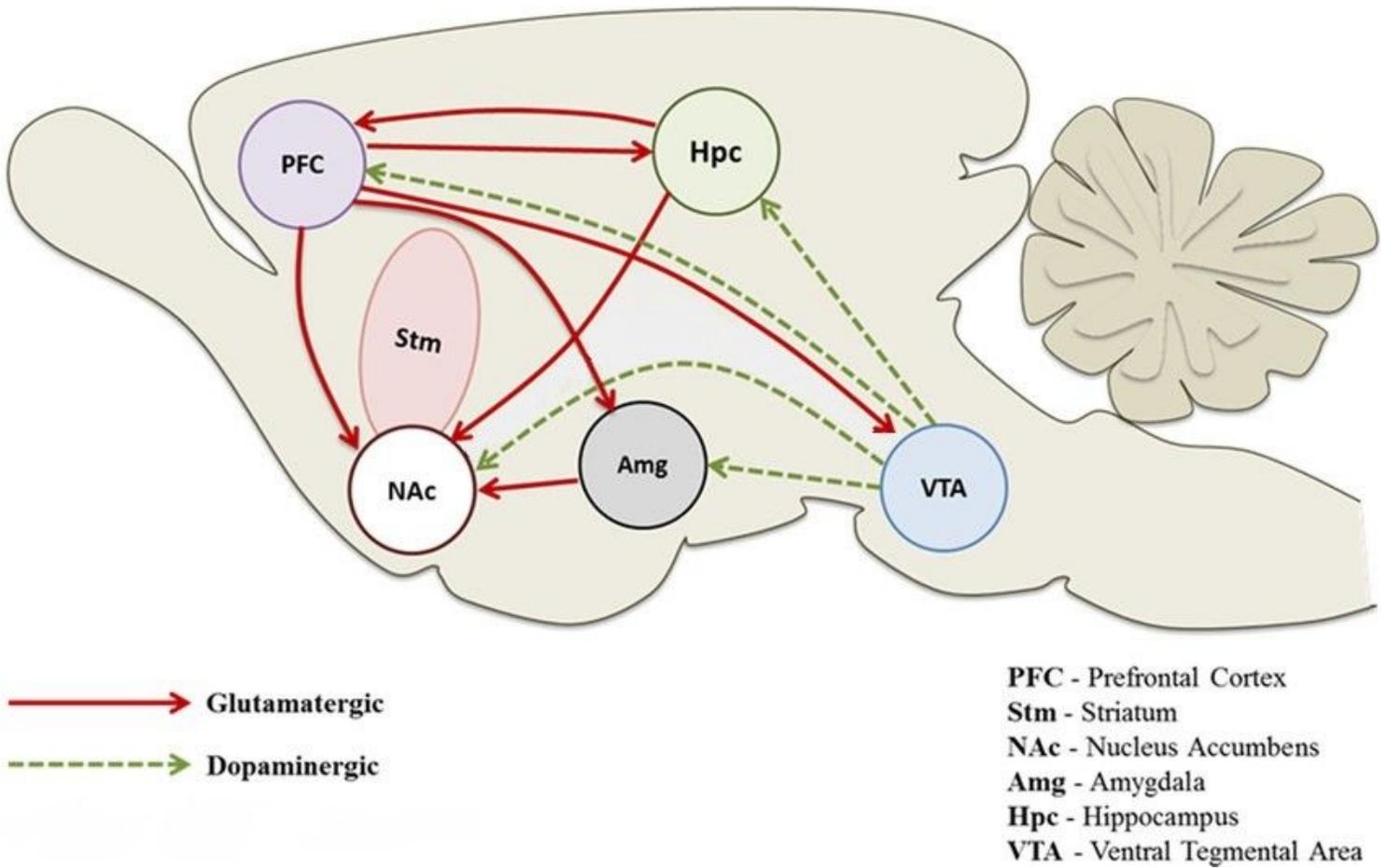


Figure 2

Schematic diagram of the main brain sites of the reward system and their dopaminergic and glutamatergic connections [66] (Modified).

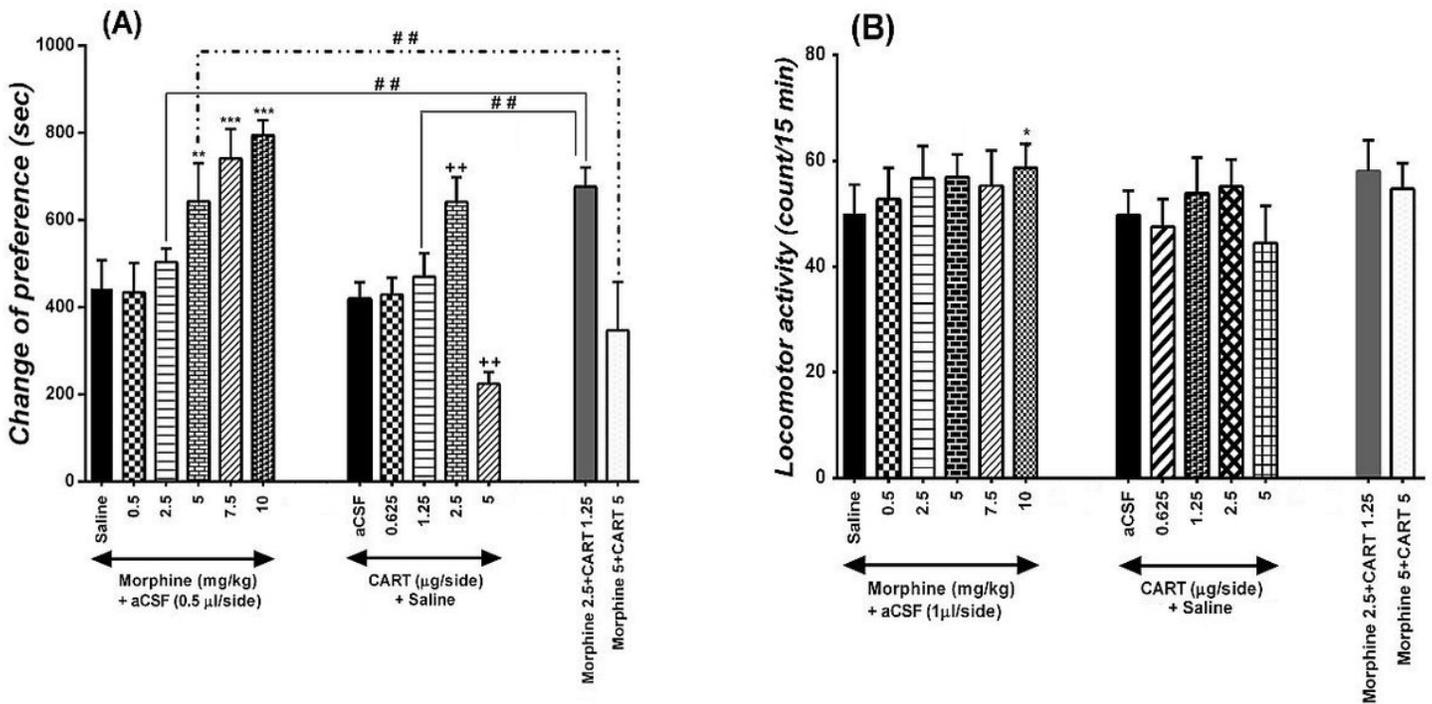


Figure 3

Effect of morphine, CART, and morphine + CART on the acquisition of place preference (a) and exploratory behaviors (b). Panel a represents the effects of drugs in a three-day schedule of conditioning on place preference. The locomotor activity was evaluated during the testing day (Panel b). Data are expressed as mean \pm S.E.M. ($n = 8$ in each group). ** $P < 0.01$ and *** $P < 0.001$ different from the saline group, ++ $P < 0.01$ different from the aCSF group, ## $P < 0.01$ different from the indicated group.

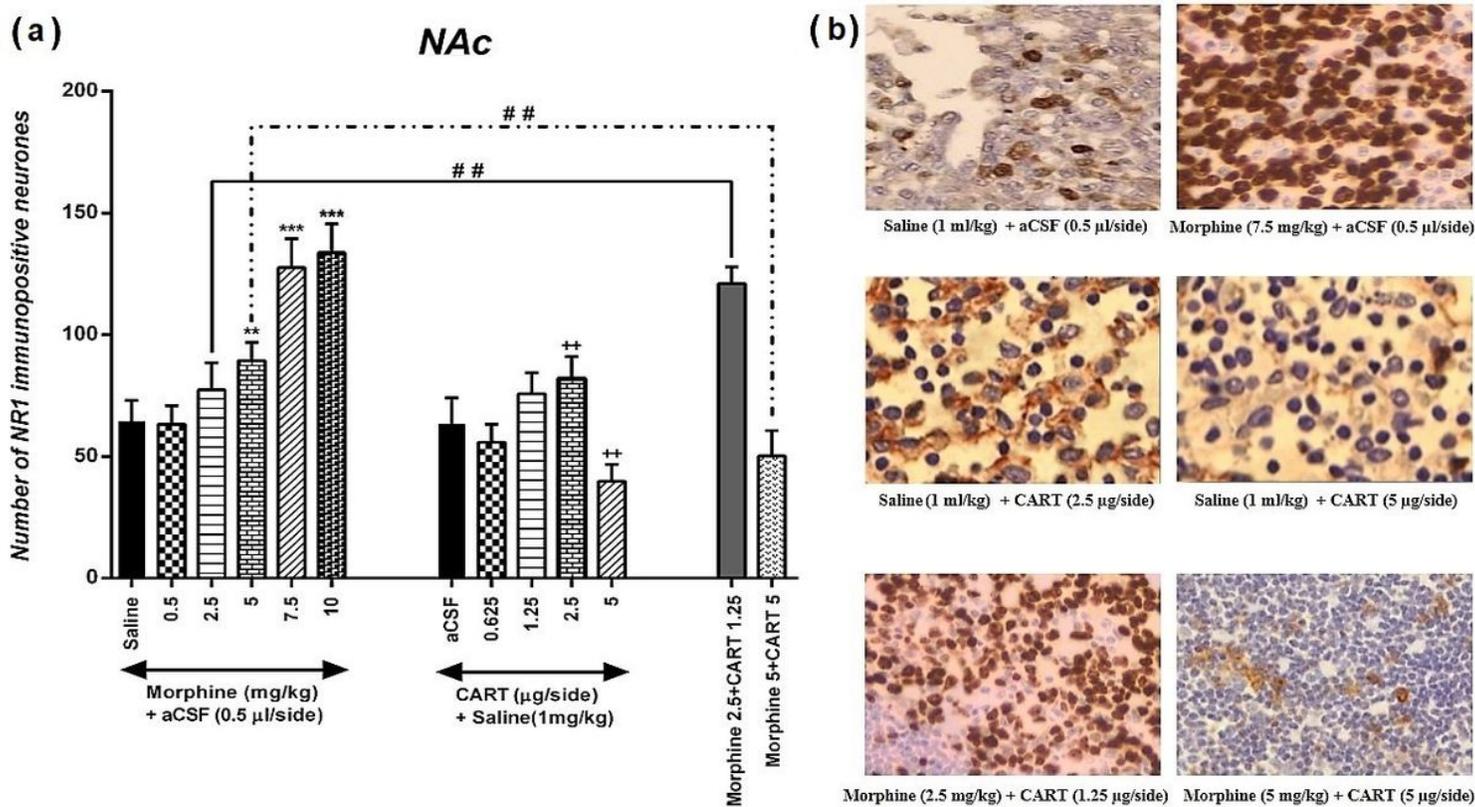


Figure 4

Number of NR1 immunopositive neurons in the NAc after place-conditioning with morphine, CART, and morphine + CART (panel a). Data are expressed as mean \pm S.E.M. ($n = 5$ in each group). ** $P < 0.01$ and *** $P < 0.001$ different from the saline group, ++ $P < 0.01$ different from the aCSF group, ## $P < 0.01$ different from the indicated group. Some IHC images of the groups are presented in panel b.

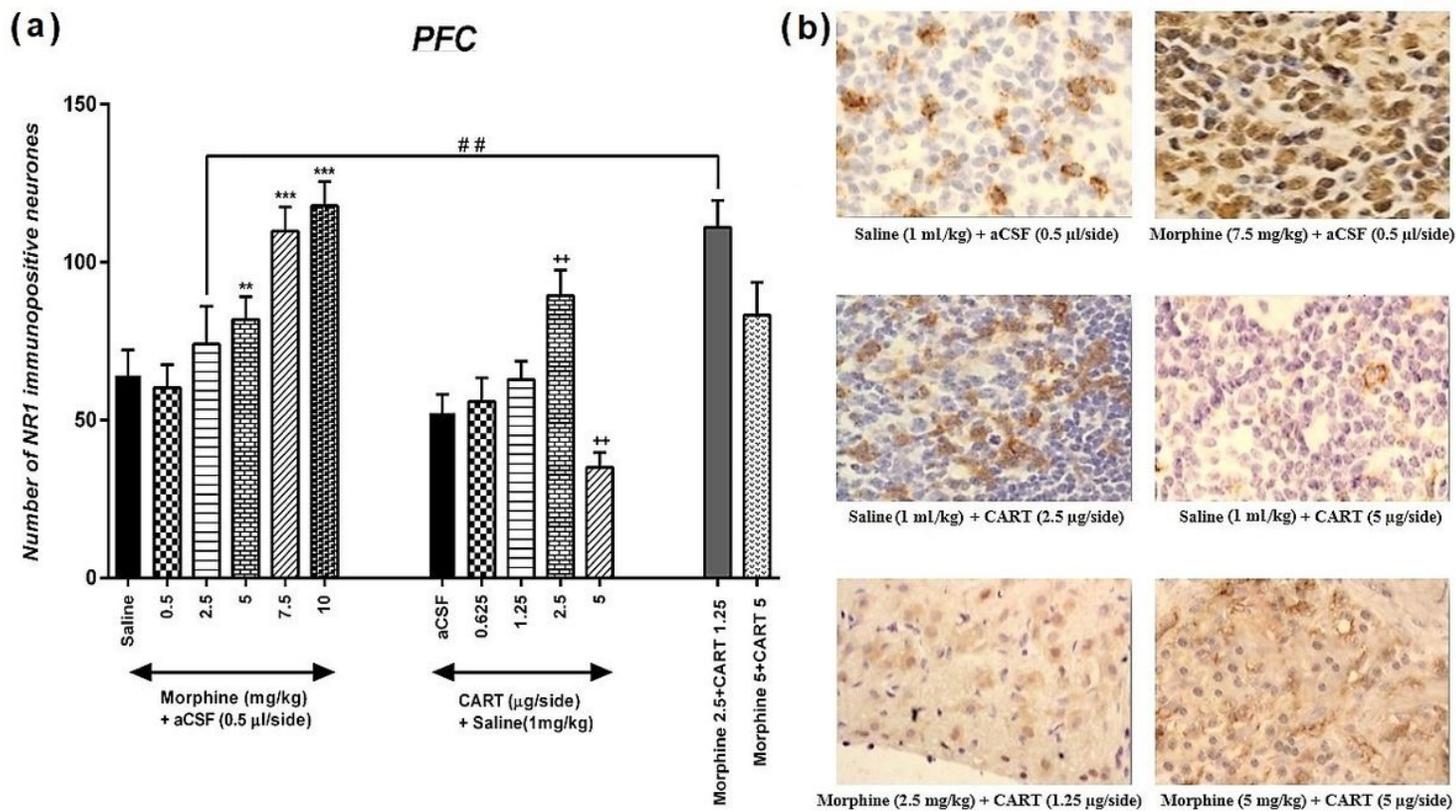


Figure 5

Number of NR1 immunopositive neurons in the PFC after place-conditioning with morphine, CART, and morphine + CART (panel a). Data are expressed as mean \pm S.E.M. ($n = 5$ in each group). ** $P < 0.01$ and *** $P < 0.001$ different from the saline group, ++ $P < 0.01$ different from the aCSF group, ### $P < 0.01$ different from the indicated group. IHC images of some groups are shown in panel b.

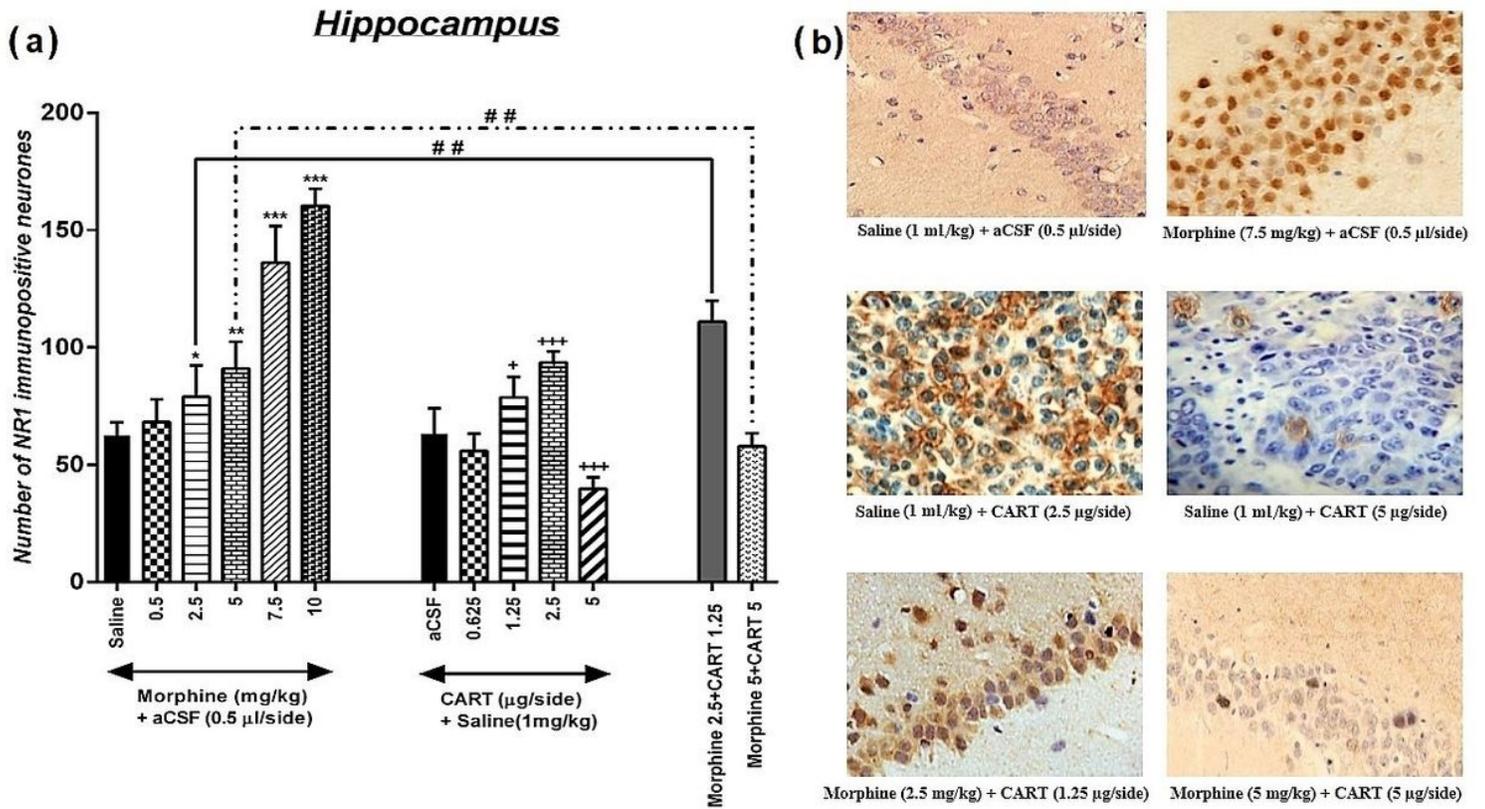


Figure 6

Number of NR1 immunopositive neurons in the hippocampus after place-conditioning with morphine, CART, and morphine + CART (panel a). Data are expressed as mean \pm S.E.M. ($n = 5$ in each group). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ different from the saline group, + $P < 0.05$ and +++ $P < 0.001$ different from the aCSF group, ## $P < 0.01$ different from the indicated group. IHC images of some groups are presented in panel b.

Supplementary Files

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