Nitric oxide in central amygdala potentiates expression of conditioned withdrawal induced by morphine

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ABSTRACT

Objective: The aim of this study was to evaluate if nitric oxide (NO) in the central amygdala (CeA) is involved in the expression of withdrawal aspects induced by morphine.

Materials and Methods: Male Wistar rats (weighing 200-250 g) were bilaterally cannulated in the CeA and conditioned to morphine using an unbiased paradigm. Morphine (2.5-10 mg/kg) was subcutaneously injected once a day throughout the conditioning phase of the procedure. This phase also included 3-saline paired sessions. Naloxone (0.1-0.4 mg/kg, intraperitoneally [i.p.]), an antagonist of opioid receptors, was administered i.p. 10 min prior to testing of morphine-induced withdrawal features. The NO precursor, L-arginine (0.3-3 µg/rat) was intra-CeA injected prior to testing of naloxone response. To evaluate the involvement of NO system an inhibitor of NO synthase (NOS), N⁶-nitro-L-arginine methyl ester (L-NAME) (0.3-3 µg/rat), was injected ahead of L-arginine. Control group received saline solely instead of drug. As a complementary study, the activation of NOS was studied by nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d).

Results: Morphine induced a significant increase in wet dog shaking and grooming behaviors compared with controls. Injection of naloxone pre-testing of morphine response significantly reversed the response to morphine. However, pre-microinjection of L-arginine intra-CeA recovered the response to morphine. Injection of L-NAME intra-CeA ahead of L-arginine though had no effect behaviorally, but inhibited the NOS which has been evidenced by NADPH-d.

Conclusion: The present study shows that NO in the CeA potentiates the expression of conditioned withdrawal induced by morphine paired with naloxone.

KEYWORDS: Central amygdala, morphine, naloxone, nitric oxide, withdrawal behavior

Introduction

Conditioning task is known as a simple learning process through which a conditioned response to a neutral stimulus after multiple pairing with an unconditioned stimulus is shown. This program causes adaptation to the drug presence, consequently, it changes the brain functioning. Thus, stopping the drug use causes some signs of psychological withdrawal.

Grooming is a major activity during the non-sleeping phase in rats. This feature often displays as a reaction to the unexpected stimuli or stress. The other aspect of withdrawal, the wet dog shaking (WDS) is characteristically observed in morphine dependent rats. This signal is differently listed as a sign of seeking behavior.

A single injection of naloxone, which is commonly used to counter the effects of opiate overdose augments the mu opioid receptor expression during the opioid withdrawal in some brain areas including amygdala.

Nitric oxide (NO), a main retrograde neurotransmitter, as has been shown by conditioned place preference (CPP) is implicated in the rewarding actions of opiates. Morphine stimulates release of NO in the hippocampus and amygdala in a naloxone-and N⁶-nitro-L-arginine methyl ester-(L-NAME) sensitive route however, the molecule NO in the central amygdala (CeA) plays no role in rearing and sniffing in morphine.
conditioned rats. This molecule acts as a modulator of morphine-induced psychological dependence in the limbic system.

This laboratory has recently indicated that morphine usage as much as is needed to establish a place preference may lead to psychological dependence on morphine. Study aimed to examine the withdrawal aspects of morphine conditioning. The task was paired with a single injection of naloxone pre-testing. This procedure moreover sought to involve NO in the rat CeA in the conditioned leaving aspects. Complementary evidence was provided using nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d), the main marker of activation of NO synthase (NOS).

Materials and Methods

Subjects

Adult intact male Wistar rats weighing 200-250 g (Pasteur Institute, Tehran, Iran) were housed in standard plastic cages in a controlled colony room (temperature 21 ± 3°C). They were maintained on a 12-h light/dark cycle (lights on at 07.00 a.m.) with food and water ad libitum. The experiments were carried out during the light phase of the cycle. Each group of animals was tested only once. They were then decapitated at the end of each experiment to obtain the rats’ brains. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996). The trials were also approved by local ethical committee (Document No: 7941).

Drugs

Morphine sulfate (TEMAD, Co., Tehran, Iran) prepared in sterile 0.9% NaCl solution was injected subcutaneously (s.c.). Naloxone hydrochloride (Tolid-Daru Co., Tehran, Iran) was administered intraperitoneally (i.p.). L-arginine (Sigma Chemical Co., USA) and (L-NAME; Research Biochemical Inc., USA) were bilaterally injected in the area of interest (CeA). Vehicle was 0.9% physiological saline. A combination of ketamine (100 mg/kg) and xylazine (20 mg/kg) purchased from Veterinary Organization of Iran was used i.p. to anesthetize the experimental animals.

Stereotaxic Surgery

The anesthetized animal was placed in a stereotaxic apparatus, with the incisor bar set at approximately 3.3 mm below horizontal zero to achieve a flat skull position. An incision was made to expose the rat skull. Two holes were drilled in the skull at stereotaxic coordinates: AP = −2.12 mm posterior to bregma and L = ±4.1 mm according to the atlas. Two guide cannulae (21-Gauge) were inserted into the holes. The guide cannulae were lowered 6 mm below bregma through the holes drilled at the desired coordinates. They were anchored with a jeweler’s screw. The incision was then closed with dental cement. Finally dummy inner cannulae were inserted into the guide cannulae. All animals were allowed to recover for 1 week before behavioral testing began.

Intra-CeA Injection

The animals were gently restrained by hand in order to remove dummy cannulae. NO agents were injected into the nucleus by injection needles (27-Gauge) through guide cannulae. The needles with polyethylene tubing (0.3 mm internal diameter) were connected to 5.0-μL glass Hamilton syringe. They were more projected (1.8 mm) ventral to the guides. A volume, 1.0 μL (0.5 μL/side), was microinjected in the CeA through the injection cannulae over a 30-s period.

CPP Apparatus and Paradigm

Place conditioning apparatus

A two-compartment CPP apparatus (30 cm × 60 cm × 30 cm) was used in these experiments. Place conditioning was conducted using an unbiased program, with a design which has previously been described. The apparatus was divided into two equal-sized compartments with a guillotine door in the middle of apparatus. Both compartments were completely colored white but they were differently striped black (vertical vs. horizontal). They were also cleared by texture (floored smooth with a drop of natural aqueous rose extract placed in vs. grinded) and olfactory cues. In this apparatus, rats displayed no consistent preference to one side of the apparatus, confirming that the procedure was unbiased. All experiments were recorded using an EthoVision system equipped with a video camera located 120 cm above the apparatus. The files were then reviewed by an observer who was unfamiliar with the experiments.

Conditioning paradigm

The three-phase paradigm was as follows:

Pre-conditioning (familiarization) phase

On day 1, animals received one habituation session after placement in the middle line of the apparatus. The animals were allowed free access to the entire apparatus for 15 min, whereas the guillotine door was raised 12 cm above the floor. The number of grooming or WDS in both compartments throughout the phase was recorded with an EthoVision system. The video files were then reviewed by an observer who was unfamiliar with the treatments.

Conditioning phase

This phase was conducted in-saline and 3-drug pairings after familiarization. Conditioning to morphine (2.5-10 mg/kg) was induced by s.c. injection of the opioid drug once daily during the 3-day conditioning phase; saline (1 mL/kg, s.c.) was also injected once/day throughout the phase with 6 h interval to drug-pairing. Control groups solely received saline (1 mL/kg, s.c.) twice/day. All conditioning sessions were 45 min long. During these sessions the guillotine door was closed. For each drug dose, animals were randomly assigned into groups of eight rats. Each group was then subdivided into two sub-groups of four rats; each subgroup after injection of the drug was confined in one compartment (drug-paired side). The sub-group after saline giving was placed in the opposite part (saline-paired side). The drug treatment for each of sub-group regarding the presentation order of morphine and saline was counterbalance paired with one compartment.

Post-conditioning (testing) phase

Test sessions were carried out on day 5, 1 day after the last conditioning session, in a morphine-free state. Each animal was tested only once. For testing, the guillotine door was raised 12 cm above the floor and intact animals were allowed to move freely in the box for 15 min. The number of grooming or WDS was then counted and what that was obtained in the drug-paired compartment on testing day was subtracted from that of familiarization shown as the number of behavior count/15 min.
Experimental Procedure

Induction and assessment of morphine behavioral signs

Effect of morphine (2.5, 5, 7.5 and 10 mg/kg; s.c.) on induction of withdrawal aspects in animals were conditioned with morphine was determined. Morphine or saline were repeatedly injected in accordance to the conditioning protocol detailed above. Behavioral signs were calculated as described elsewhere [Figure 1]. Each drug dose was tested in eight animals only once. Animals were tested in a morphine-free state in order to eliminate the influence of morphine-induced behavioral effects on the response.[18]

Effects of naloxone, an antagonist of mu opioid receptor on behavioral signs in the rats conditioned to morphine

An antagonist of opioid receptors, naloxone (0.1-0.4 mg/kg, i.p.) was administered i.p. 10 min prior to testing of morphine response to clarify the withdrawal aspects of morphine conditioning. The behaviors were determined as detailed in above sections. The control group received saline instead of the antagonist (1 mL/kg, i.p.) in the testing day.

Effects of intra-CeA no agents in combination with single injection (i.p.) of naloxone on the behavioral signs in the morphine conditioned rats

To determine the effects of intra-CeA L-arginine (0.3-3 μg/rat) on behaviors in the naloxone paired morphine conditioning task, the agent was bilaterally administered 1-2 min prior to naloxone injection (0.4 mg/kg, i.p.) on the day of testing. Control groups simply received saline (1 μL/rat, intra-CeA) instead of the NO agent. To involve the NO system in the observed behaviors, L-NAME (0.3-3 μg/rat) before L-arginine was microinjected prior to naloxone injection pre-testing i.p. The control rats instead received saline (1 μL/rat, intra-CeA).

NADPH histochemistry

As the complementary study, all the experimental animals after the experiments were decapitated and their brains were stored in 4% buffered formaldehyde to provide an adequate NADPH staining. After 3-5 days, the samples were cut into small pieces that had the injection site. These slices were then mounted on albumin greased slides and retained at room temperature for about 24 h. The prepared sections were first studied by light microscopy then the routine histology protocol was performed (clearation, dehydration). After rinsing sections in buffer they were colored by NADPH-d technique to demonstrate the neuronal NOS possessing NADPH-d activity. The prepared slices were placed (1-2 min) in diluent of 0.3% Triton-X 100 in phosphate buffer under shaking. Then staining was performed by incubating the slices in a solution containing equal proportions of nitro-blue tetrazolium (NBT) (0.4 mg/mL in buffer) and NADPH (1 mg/mL in buffer) for 16-20 h at 37°C. NBT is a salt that yields an insoluble blue formazan that is visible by light microscopy. A similar procedure was used for the sections on the slides. These slides without agitation were kept in wet chamber. This protocol was used for the control sections except that the NADPH was removed from it. In the control sections, no staining was observed.

Statistical Analysis

All results were expressed as the average number of behavior counted in 15 min ± standard error of mean (SEM). In order to compare the behaviors displayed in all groups (vehicle and experimental groups), one-way analysis of variance followed by appropriate post-hoc Tukey-Kramer test was used. The P < 0.05 was considered as significant. To quantify the histological data in the site of injection the stained brains’ slices were examined micrographically by video-light microscope (Olympus); areas of 100 μm² were considered as quantitative measurement units and they were analyzed by Image Tool program (UTHSCSA ImageTool, version 2.03, USA), the free image processing and analysis program for Microsoft Windows. This program after spatial or density comparison between test and control samples provided a significant data ± SEM.

Results

Histological Verification of Microinjection Sites in the Central Amygdale

Data reveal the injection site using intra-CeA injection of 1 μL (0.5 μL/side) of a methylene blue solution by same injection
Effect of Morphine on Behavioral Signs

Figure 1 shows the effect of different doses of morphine (2.5-10 mg/kg, s.c.) on behavioral signs in Wistar rats. Administration of morphine had significant effect on the WDS behavior ($F_{4,35} = 3.476; \ P < 0.05$) in comparison to the control group. The morphine effect on the grooming behavior was also significant ($F_{4,35} = 3.110; \ P < 0.05$). In view of the post-hoc analysis the opioid at 7.5 mg/kg (s.c.) induced a meaningful increase in number of drug-induced behavior. Hence, the dose effect was chosen in the subsequent studies.

Effect of Naloxone on The Expression of Behavioral Signs in Conditioned Model

Figure 2 shows the effect of injection of naloxone (0.1-0.4 mg/kg, i.p.) once prior to morphine response testing. Pre-testing administration of naloxone in morphine (7.5 mg/kg, s.c.) conditioned animals resulted in a significant effect when compared to the control group. As analysis shows the antagonist, naloxone (0.1-0.4 mg/kg, i.p) significantly caused a change in morphine dose (7.5 mg/kg)-induced WDS ($F_{4,30} = 4.020; \ P < 0.05$) and grooming ($F_{4,30} = 8.265; \ P < 0.001$).

Supplementary Figure: (a) Cannula placement in central amygdala (CeA) evidenced by ink injection in a volume of 1 μL/rat with the same set up as used for CeA injection of drugs (AP: −2.12). (b) Arrowhead shows a positive nicotinamide adenine dinucleotide phosphate-diaphorase in the CeA of rats that received the NO precursor, L-arginine, before testing of naloxone paired morphine response. Line is about 50 μm.

Based on Tukey’s post-hoc analysis, a dose of naloxone (0.4 mg/kg) was paired with morphine effective dose (7.5 mg/kg) for subsequent studies.

Effect of NO Production in the CeA on Naloxone Paired Morphine Response

Bilateral injection of L-arginine (0.3, 1.0 and 3.0 μg/rat, intra-CeA) prior to naloxone (0.4 mg/kg, i.p.) before testing of morphine response (7.5 mg/kg, s.c.), lead to significant effect ($F_{3,21} = 5.708; \ P < 0.05$) [Figure 3] on WDS and grooming ($F_{3,21} = 6.901; \ P < 0.01$). On the other hand, pre-injection of L-NAME (0.3, 1.0 and 3.0 μg/rat, intra-CeA) prior to L-arginine before testing of behavioral signs due to morphine-naloxone combination showed no significant response to L-arginine dose effect (1 μg/rat, intra-CeA) [Figure 4].

Effects of Intra-CeA Injection of NO Agents on NOS Activation in Drug Administered Animals

Complementary data provided by light microscopy revealed a NADPH-d difference ($F_{6,28} = 9.636; \ P < 0.001$) of in L-arginine administered samples [Supplementary Figure b] with respect to control. Furthermore, a significant difference in NADPH-d activation between L-NAME samples than single L-arginine was observed ($F_{6,28} = 13.632; \ P < 0.01$).

Discussion

This study was aimed to involve the NO in the rat CeA in expression of WDS and grooming as withdrawal aspect induced by morphine conditioning. As a main finding, morphine (2.5, 5, 7.5 and 10 mg/kg, s.c.) induced a significant increase in WDS and grooming in male Wistar rats at higher doses. Fog in accordance has indicated a similar dose effect of morphine in induction of grooming. Others accordingly have shown that much higher doses of morphine (10-20 mg/kg) suppress the grooming. To discuss the mechanism it has been shown that morphine causes an excitation of G-protein coupled by mu, delta and kappa opioid receptor. The mu opioid receptor activation in the ventral tegmentum area (VTA) causes the release of dopamine in nucleus accumbens via excited dopaminergic neurons. The activation of kappa-receptors, on the other

Figure 2: Naloxone (0.1-0.4 mg/kg) was given intraperitoneally 10 min pre-testing of morphine response (7.5 mg/kg, subcutaneously). Data are expressed as number of behavioral signs/15 min ± standard error of mean. Tukey post hoc analysis showed the differences: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ difference to control that passed the morphine conditioning but only received saline (1 mL/kg) instead of antagonist before testing.
hand, suppresses the unpleasant mu/delta-mediated side effects. This morphine dose effect, so, may disclose an interaction between the mu opioid receptors and morphine in brain areas. The delta and kappa opioid receptors accordingly have slightly been implicated in the expression of these types of behaviors. Furthermore, the connections of CeA are known as the important substrates of compulsive behaviors.

Naloxone as a competitive opioid antagonist was chosen to induce the withdrawal behavioral symptoms. Although, naloxone pretesting injection (0.1-0.4 mg/kg) completely suppressed the grooming behavior in morphine-conditioned model, but it inhibited the WDS only at higher doses (0.4 mg/kg).

The previous studies in accordance have shown an aversive response to morphine due to blockade of mu receptors by naloxone.

In this study, we further assessed the effects of NO agent intra-CeA on expression of withdrawal signs in a naloxone-paired morphine conditioning testing. Injection of L-arginine (0.3-3 μg/rat, intra-CeA) pre-testing, prior to naloxone effective dose (0.4 mg/kg), showed significant effect on grooming behavior. In addition, the agent significantly enhanced the WDS behavior. We have already evidenced a role for NO in the CeA in morphine-induced place preference. The amygdala projections toward the nucleus accumbens are also implicated in morphine induced-dependence. Furthermore, the glutamatergic projections of VTA into amygdala are graded as main component in the expression of morphine dependence. Our result, thus, in accordance to a previous investigation may provide a finding that the NO in the CeA plays an important role in expression of withdrawal aspects of morphine conditioning. However, in other set of our experiments, the injection of L-NAME (0.3-3 μg/rat, intra-CeA) prior to L-arginine microinjection (1 μg/rat, intra-CeA) did not block the L-arginine dose effect in comparison to control. To involve accurately the NO in the phenomenon we complementary evidenced the NOS activation in the nucleus by using a main
marker, NADPH-d. Positive reaction to the NADPH-d in brain slices of rats receiving NO. Hence, the intra-CeA injection of L-NAME prior to L-arginine though had no effect behaviorally, but the NADPH-d revealed a NOS inhibition in the L-arginine received specimen in contrast to single L-arginine recipient. The NO expressing neurons in the nucleus CeA, may regulate the production of NO in the nucleus due to mu receptor activation by morphine. This finding may support a recent data after Vargas-Perez et al. (2009).\[25]\n
**Conclusion**

This work suggests that the NO in the CeA potentiates the expression of conditioned withdrawal aspects in naloxone-paired morphine conditioning tasks.

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**References**