Iranian Biomedical Journal 15 (3): 92-99 (July 2011)

Reversal Effect of Intra-Central Amygdala Microinjection of L-Arginine on Place Aversion Induced by Naloxone in Morphine Conditioned Rats

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Received 15 February 2011; revised 5 July 2011; accepted 10 July 2011

[DOR: 20.1001.1.1028852.2011.15.3.6.4]

[DOI: -]

ABSTRACT

Background: Role of nitric oxide (NO) on expression of morphine conditioning using a solely classic task has been proposed previously. In this work, the involvement of NO on the expression of opioid-induced conditioning in the task paired with an injection of naloxone was investigated. **Methods**: Conditioning was established in adult male Wistar rats (weighing 200-250 g) using an unbiased procedure. Naloxone (0.05-0.4 mg/kg, i.p.), a selective antagonist of mu-opioid receptor, was administered once prior to morphine response testing. NO agents were administered directly into the central amygdala (CeA) prior to naloxone injection pre-testing. **Results**: Morphine (2.5-10 mg/kg, s.c.) produced a significant dose-dependent place preference in experimental animals. When naloxone (0.05-0.4 mg/kg, i.p.) was injected before testing of morphine (5 mg/kg, s.c.) response, the antagonist induced a significant aversion. This response was reversed due to injection of L-arginine (0.3-3 μg/rat), intra-CeA prior to naloxone administration. However, pre-injection of L-NAME (intra-CeA), an inhibitor of NO production, blocked this effect. **Conclusion**: The finding may reflect that NO in the nucleus participates in morphine plus naloxone interaction. *Iran. Biomed. J. 15 (3): 92-99, 2011*

Keywords: Morphine, Naloxone, L-arginine, L-NAME, Place aversion

INTRODUCTION

Morphine is a drug of wide therapeutic usage that has primarily pain relieving effect [1]. However, chronic use of the opioid leads to physical and psychological dependences, which are characterized by the expression of withdrawal symptoms on cessation of the drug administration [2]. In addition, evidence shows that morphine has a major site of action at midbrain [3], which is implicated in reward circuitry [4].

Place conditioning, which is related to enhance in dopamine (DA) release in shell of accumbens by doubling the firing rate of ventral tegmental area DA neurons [5], is a simple and an effective method to assess the rewarding properties of drugs [6, 7]. The majority of morphine-central actions such as place preference are mediated by mu-opioid receptors [6]. Binding of morphine with mu-opioid receptors at midbrain may lead to dopamine release at the end of the neuronal projections in amygdala, which induce a rewarding effect of opioid [3, 8]. According to a previous study, Swiss-Webster mice which rendered dependent on morphine after receiving naloxone, showed a significant increase in mu-opioid receptor expression during opioid withdrawal in some brain areas including amygdala [9]. Opiate withdrawal symptoms can be precipitated by an opiate antagonist either after a short-term infusion or even after a single dose of an opiate both in humans and in animals [10]. Naloxone, a pure narcotic antagonist, is shown to antagonize the opioid effect by competing for the same receptor sites [11]. It has been notified that infusion of naloxone into ventral tegmental area or periaqueductal gray blocked the acquisition of morphine place preference [12]. After systemic injection of naloxone, on the contrary, the expression of morphine conditioned place preference both in mice and rats increased [13]. Since morphine may induce place preference through interactions by different subtypes of

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mu-opioid receptor [11], we studied a place conditioning produced of morphine in the task paired with an injection of naloxone. This procedure provided evidence to more address the dependenc on the drug.

Nitric oxide (NO) is an endogenous and enzymatically generated molecule of great pharmacological interest and physiological importance [14]. The retrograde neurotransmitter, NO, is shown to produce postsynaptically in response to activation of central excitatory amino acids [15], and plays a role in the regulation of behavior [16]. NO is implicated in the actions of opioids [17]. Morphine is able to stimulate the release of NO from hippocampus and amygdala tissues in a naloxone and L-NAME sensitive manner, the finding which provides evidence that morphine biosynthesis occurs in neural tissues, and that the release of NO in limbic system may involve in the modulation of psychological dependence induced by morphine [18]. In contrast to studies on chronic as well as acute dependence on opioids, no knowledge is known about the neural mechanisms mediating psychological dependence due to morphine therapy, which is used to establish place preference. In light of these data, we propose that morphine withdrawal may, in part, involve activation of NO synthesis in central amygdala (CeA). This site was selected because of receiving massive reward pathways [3] amongst the subdivisions of amygdala complex. Furthermore, this site is implicated in place aversion induced by naloxone [10].

MATERIALS AND METHODS

Subjects. Adult male Wistar rats (weighing 200-250 g, the Pasteur Institute of Iran, Tehran) were housed in standard plastic cages in groups of 2 in a controlled colony room (temperature 21 ± 3°C). The animals 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. Six to eight animals were used per group and each animal was tested once. At the end of each experiment, the rats were decapitated with chloroform overdose. All experiments were conducted in accordance with standard ethical guidelines and approved by the Local Ethical Committee of Shahed University (Tehran).

Drugs. Morphine sulphate (TEMAD Co., Tehran, Iran) was prepared freshly in sterile 0.9% NaCl solution, and injected s.c.. The injection volume of naloxone hydrochloride (Tolid-Daru Co., Tehran, Iran) was 1 ml/kg for all groups. Naloxone was injected i.p.. Vehicle injections were 0.9% physiological saline. L-arginine (Sigma Chemical Co., USA) and N^G-Nitro-L-arginine Methyl Ester (L-NAME; Research Biochemical Inc., USA) were also prepared freshly in sterile 0.9% NaCl solution. NO agents, L-arginine and L-NAME, were bilaterally injected into the area of interest (central-amygdala) at the volume of 1.0 µl/rat. Vehicle injections were at the appropriate volume of 0.9% physiological saline. Ketamine (Veterinary organization of Iran) at 100 mg/kg i.p. was used to anesthetize the experimental animals.

Place conditioning apparatus. A two-compartment conditioned place preference apparatus (30 × 60 × 30 cm) was used in these experiments. Place conditioning was conducted using an unbiased program with a little change in design as described previously [6]. The apparatus was divided into two equal-sized compartments. In the middle of the apparatus, a removable door was designed. Both compartments were completely white colored but differently striped black (vertical vs. horizontal). The compartments were also distinguishable by texture and olfactory cue. To provide the tactile difference between the compartments, one of the compartments had a smooth floor, while the other one had a grinded floor. A drop of menthol was placed at the corner of the compartment with a textured floor to provide the olfactory difference between the compartments. In this apparatus, rats showed no consistent preference for either compartment. All experiments were recorded using a video camera (model LVC-DV323ec of an Auto Iris Lg) located 120 cm above the apparatus. The video tapes were then observed by an observer, which was unfamiliar to the experiments.

Surgery. The animals were anesthetized and placed in a stereotaxic apparatus with the incisor bar set at approximately 3.0 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 of Paxinos and Watson [19]. Two guide cannulae (21 gauge) were inserted into the holes. For animals receiving bilateral injections into the central amygdala, the guide cannulae were lowered 6 mm below bregma through the holes drilled at the desired coordinates. The guide cannulae were anchored with a jeweler's screw, and the incision was closed with dental cement. After surgery, dummy inner cannulae, that extended 0.5 mm beyond the guide cannulae, were inserted into the guide cannulae and left in the place until injections were made. All animals were allowed to recover for 1 week before behavioral testing began.

Intra-central amygdala injection. The animals were gently restrained by hand; the dummy cannulae were

[DOR: 20.1001.1.1028852.2011.15.3.6.4]
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removed from the guide cannulae. Drugs were injected directly into the nucleus through guide cannulae by using injection needles (27 gauge) and connected by polyethylene tubing (0.3 mm internal diameter) to a 5.0-µl glass Hamilton syringe. The injection needles projected a further 1.8 mm ventral to the tip of the guides. The injection volume was 1.0 µl for all groups. Injections were made over a 30-s period, and the injection cannulae were left in the guide cannulae for an additional 60 s to facilitate the diffusion of the drugs.

Histological verification. After completion of behavioral testing, animals were killed with an overdose of chloroform. Ink (0.5 µl of 1% aquatic methylene blue solution) was injected into the guide cannulae using 27-gauge injection cannulae that projected a further 1.8 mm ventral to the tip of the guides, to aid in histological verification. The brains were removed and fixed in 10% formalin solution for 48 h before sectioning. Sections were taken through the brain areas of cannulae placements, and the cannulae placements were verified (Fig. 1) using the atlas of Paxinos and Watson [19]. Data from rats with injection sites located outside the nucleus were excluded of the analyses.

Statistical analysis. The one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison tests was used to determine the effects of the various treatments on morphine-induced place conditioning. *P* values less than 0.05 were considered as significant.

Experimental procedure. The conditioning paradigm consisted of three phases:

Pre-conditioning (familiarization): On day 1 (before conditioning phase), animals received one habituation session. They were placed in the middle line of the apparatus and allowed free access to the entire apparatus for 10 min, while the removable door was raised 12 cm above the floor. The time spent by rats in each compartment was recorded by an observer who was blind to the treatments after reviewing the camera tapes. None of the groups displayed a significant preference for one of the compartments, confirming that this procedure is unbiased.

Conditioning. This phase, consisted of 3-saline and 3-drug pairings, was started one day after familiarization and lasted 3 days. The animals were daily injected with drug or saline and the drug was administrated once with a 6-h interval to saline injection. Control groups received saline (1 ml/kg) twice a day with a 6-h interval. The duration of all conditioning sessions was 30 min. During these sessions, the removable wall was inserted along the seam separating the two compartments. For each drug dose, animals were randomly assigned into groups of 6-8 rats. The groups were then injected with drug and confined in a compartment for drug pairing (drug-paired side). The drug treatment for half of the group was paired with one compartment, while for the other half it was paired with the other compartment [6]. The presentation order of morphine and saline was counterbalanced for each drug dose.

Testing (post-conditioning). Test sessions were carried out on day 5, one day after the last conditioning session, in a morphine-free state. Each animal was tested only once. For testing, the removable wall was raised 12 cm above the floor and each of the uninjected animals was allowed free access to both compartments of the apparatus for 10 min. The time spent in both compartments was then assessed.

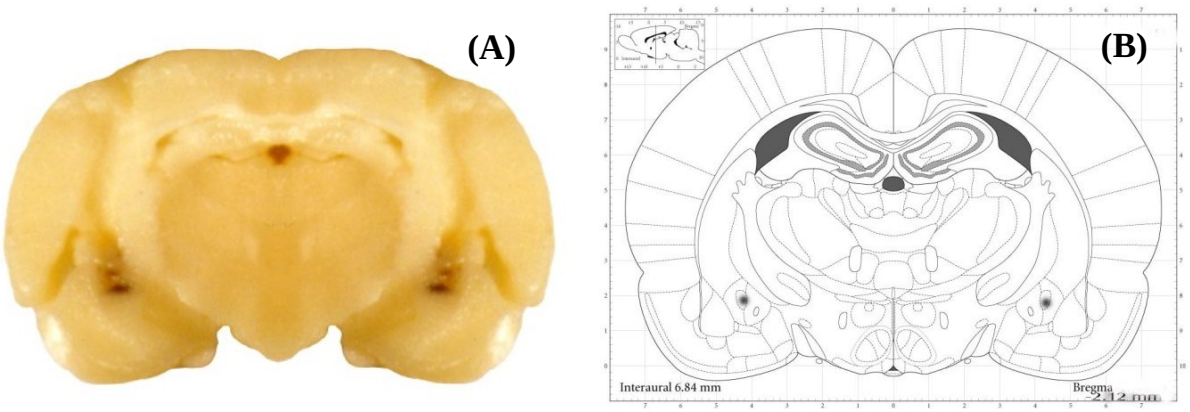

Fig. 1. Histological verification of data. **(A)** Cannulae placements in central amygdala, evidenced by ink injection (1 µl/rat) using the same set up as used for intra-central amygdala injection of drugs (AP: _2.12); **(B)** verification of injection site at the stereotaxic coordinates in accordance with rat's brain atlas by Paxinos and Watson [19].

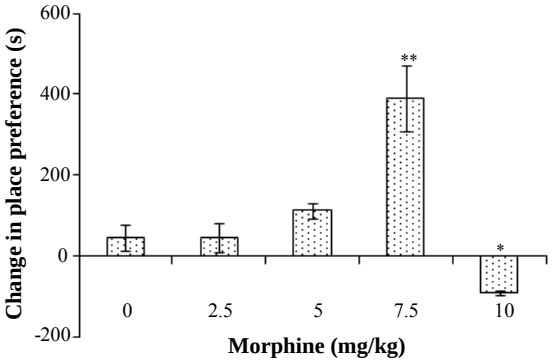

Fig. 2. Dose response to morphine-induced conditioned place preference in opioid-naive male rats. Morphine (2.5-10 mg/kg) or saline (1 ml/kg) was given s.c. in a 3-day schedule of an unbiased conditioning paradigm. The control group (legend 0) received saline (1 ml/kg, s.c.) twice daily for 3 days. The data are expressed as mean of change in place preference ± S.E.M. Change in place preference is defined as the time spent in the drug-paired place on day of testing minus the time spent in the same place pre-conditioning. Tukey-Kramer post hoc analysis showed the differences to control legend 0 (**P*<0.05 and ***P*<0.01).

The time spent in the drug-paired compartment on testing day minus that spent in the same compartment on day of familiarization, representing the score of change in place preference (in second), was expressed as mean ± SEM.

Induction and assessment of morphine place conditioning. The effects of s.c. administration of morphine (2.5, 5 , 7.5 and 10 mg/kg) on induction of place conditioning in animals were determined. Each morphine dose or saline was injected in a 3-day schedule of conditioning task as described in detail in the materials and methods. The time spent in the drug-paired compartment on day of testing minus the time spent in the same compartment on day of familiarization was calculated to assess the conditioned place preference induction. Each dose of drug was tested in 6-8 animals which were examined only once. Animals were tested in a morphine-free state. This may eliminate the influence of morphine-induced motor effects on the response [12, 20].

Measurement of effects of Naloxone (an antagonist of mu-opioid receptor) in a morphine conditioning procedure. Naloxone (0.05-0.4 mg/kg, i.p.) was injected once 10 min prior to testing on day of morphine place conditioning testing. The time spent in the drug-paired compartment on day of testing minus the time spent in the same compartment on day of familiarization was calculated to assess the response induction by the drugs. Animals were tested in a morphine-free state.

Measurement of effects of NO agents either alone or in combination with naloxone in the morphine conditioning task. L-arginine or L-NAME (0.3-3 µg/rat, intra-central amygdala) was injected alone pre-testing of morphine response. The agents (0.3-3 µg/rat, intra-central amygdala) were also pre-administered (1-2 min) to naloxone before morphine response testing to determine the interaction of naloxone and these agents. Naloxone (0.1 mg/kg, i.p.) was injected 10 min prior to the testing of morphine (5 mg/kg, s.c.) response. Control groups were simply received saline (1 µl/rat intra-CeA or 1 ml/kg, i.p) in respect to the drug injection order on day of testing.

RESULTS

Dose-response induced by morphine in Wistar rats. Figure 2 shows the effect of morphine in rats. Administration of morphine (2.5-10 mg/kg, s.c.) resulted a significant response compared to the saline group ($F_{3,20} = 4.461$; $P<0.05$). In view of the results, morphine (5 mg/kg, i.p.) was used for subsequent studies.

Effect of naloxone on expression of morphine response in the place conditioning procedure in rat. Figure 3 shows the effect of naloxone single injection prior to morphine response. Pre-testing administration of naloxone (0.05-0.4 mg/kg, i.p., in experimental

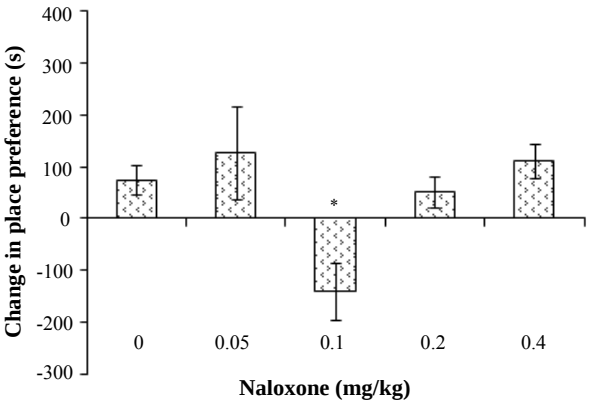

Fig. 3. Response to naloxone in morphine-treated male rats. Naloxone (0.05-0.4 mg/kg) was given i.p. prior to testing of morphine (5 mg/kg, s.c.) place conditioning in an unbiased conditioning paradigm. After 10 min, the rats were tested in a morphine-free state. Control group solely received saline (1 ml/kg, i.p.) in testing day. The data are expressed as mean of change in conditioning ± S.E.M. Change in place preference is defined as the time spent in the drug-paired place on day of testing minus that time spent in the same place pre-conditioning. Tukey-Kramer post hoc analysis showed a difference to control legend 0 (**P*<0.05).

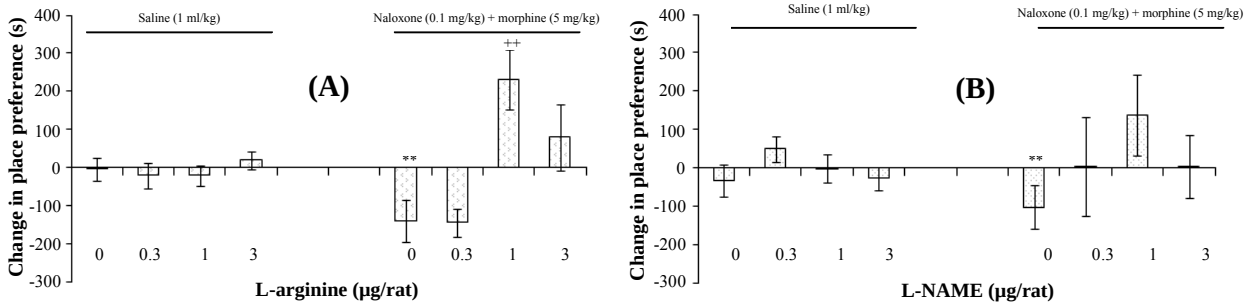

Fig. 4. Responses induced by an injection of **(A)** L-arginine or **(B)** L-NAME (0.3-3 µg/rat, intra-central amygdala) both in saline group and prior to naloxone-treated group before morphine response testing. Ten minute later the rats were tested in a morphine-free state. Data are expressed as mean of change in conditioning ± S.E.M. Change in place preference is defined as the time spent in the drug-paired place on day of testing minus the time spent in the same place pre-conditioning. Tukey-Kramer post hoc analysis showed differences to control legend 0 in saline (**P<0.01) or naloxone (++P<0.01) groups.

animals which were morphine (2.5-10 mg/kg, s.c.) injected, during the conditioning task, resulted a significant effect compared to the control group ($F_{4,25}$ = 3.904; P<0.05). In view of the results, naloxone (0.1 mg/kg, i.p.) was used for subsequent studies.

Effect of NO producer on naloxone-modulated morphine response in the place conditioning in rat. Pre-testing injection of single doses of L-arginine or L-NAME (0.3, 1.0 and 3.0 µg/rat, intra-central amygdala) resulted no significant effect in the animals which were simply saline (1 ml/gk, s.c.) injected during the conditioning procedure (Fig. 4). On the other hand, bilateral injection of L- arginine but not L-NAME (0.3, 1.0 and 3.0 µg/rat, intra-central amygdala) before injection (1-2 min) of naloxone prior to morphine testing response resulted (Fig. 5) a significant effect compared to the control group ($F_{3,20}$ = 7.196, P<0.01). Analysis by two-way ANOVA revealed the interaction between L-arginine and naloxone ($F_{drug(1,40)}$ = 0.115, P>0.05; $F_{dose(3,40)}$ = 7.797, P<0.0001: $F_{drug*dose(3,40)}$ = 4.813, P<0.001). Further analysis indicated that L-arginine reversed the response to naloxone. This effect was blocked by L-NAME (1 µg/rat, intra-central amygdale) prior to the injection of effective dose of L-arginine (1 µg/rat, intra-central amygdale) before administration of naloxone pre-testing ($F_{2,15}$ = 15.968, P<0.0001) (Fig. 6).

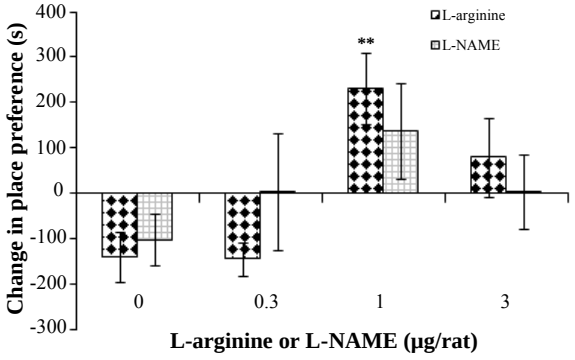

Fig. 5. Response induced by L-arginine or L-NAME (0.3-3 µg/rat, intra-central amygdala) prior to naloxone (0.1 mg/kg, i.p.) injection before morphine response testing. The rats were tested 10 min later in a morphine-free state according to the schedule of conditioning paradigm. The control group legend 0 was injected saline (1 µl/rat, intra-nucleus or 1 ml/kg, i.p.) instead of L-arginine or L-NAME. Data are expressed as mean of change in conditioning ± S.E.M. Change in place preference is defined as the time spent in the drug-paired place on day of testing minus the time spent in the same place pre-conditioning. Tukey-Kramer post hoc analysis showed a significant difference to control legend 0 (***P<0.01)*.

DISCUSSION

Injections of morphine (2.5, 5 , 7.5 and 10 mg/kg, s.c.) in the present study induced a significant dose-dependent conditioned place preference in male Wistar rats. This effect may bear in mind the role of the mu- and delta-opioid receptors, which mediate morphine rewarding in rat [21, 22]. It is also related to several neuronal systems, including tegmental pedunculo-pontine glutamate and GABA-B synapses which mediate the systemic morphine rewarding [23].

Naloxone (0.05-0.4 mg/kg, i.p.) injection prior to morphine (5 mg/kg) response testing induced a significant place aversion according to our data reflecting an opioid system involvement [24]. Previous studies proposed that an aversive effect of naloxone can be seen by using either a biased paradigm [25] or a balanced biased procedure with no strong preconditioning preferences [26]. On the other hand, these experiments may indicate an independence on the type of paradigm (biased or unbiased) designed to study the interaction between morphine and

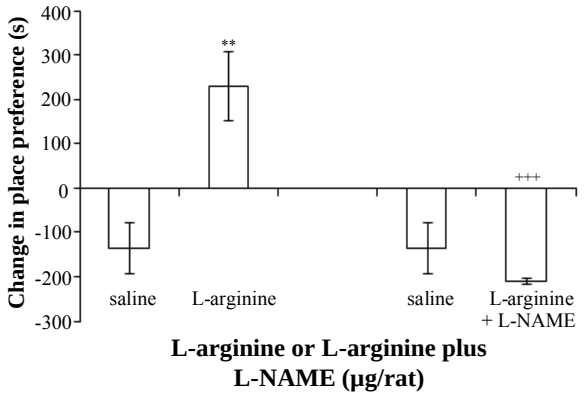

Fig. 6. Response induced by L-NAME (1 µg/rat, intra-central amygdala) known as an inhibitor of NO synthase. The inhibitor was preinjected to L-arginine (1 µg/rat, intra-central amygdala) prior to naloxone injection. The rats pre-administered by NO-agents, then were injected naloxone (0.1 mg/kg, i.p.) prior to morphine response testing. Ten minutes later, the animals were tested in a morphine-free state according to the schedule of conditioning paradigm. Controls legend saline were injected saline (1 µl/rat, intra-nucleus or 1 ml/kg, i.p.) instead of NO agents prior to testing. The data are expressed as mean of change in conditioning ± S.E.M. Change in place preference is defined as the time spent in the drug-paired place on day of testing minus the time spent in the same place pre-conditioning. Tukey-Kramer post hoc analysis showed differences to respective controls (**P<0.01 and +++P<0.001).

naloxone in the conditioning as an aversive effect of naloxone pre-testing to morphine response was seen after using an unbiased conditioning procedure in this research. In addition, it may indicate an involvement of the same receptor subtypes. In contrast, reinforcing property of naloxone using i.p. administration of naloxone has been reported previously [27]. At present, no molecular evidence is provided to elucidate the effect exactly. It can be only noted that this effect may be mediated through activation of the same subtype opiate receptors [7] as that type takes part in avoiding property of drug abused. This dose of the opioid was ineffective in producing of place preference as our data proposed. To involve the same type receptor signalling in producing of the associated learned behavior, the ineffective dose effect of the opioid was challenged by pairing the drug response testing with a single injection of a low dose of the antagonist, naloxone. Our results may indicate the same type opioid receptor involvement because of morphine dose effect potentiation due to naloxone pairing. Other studies indicated that high doses of naloxone inhibit the expression of morphine place conditioning in mice [28, 29]. In a survey, the magnitude of naloxone potency to precipitate morphine withdrawal been reported as depended to acute morphine dose [30]. These studies do not clearly match ours as we performed a 3-day schedule of place conditioning. This procedure was included 3-session morphine therapy. As another explanation, we may express that different subtypes of opioid receptors are involved in the effect. Because a role of kappa-opioid receptor has previously been demonstrated in morphine dependence [31] and naloxone withdrawal [32].

Present observations showed that injection of L-arginine (0.3-3 µg/rat, intra-central amygdala) before pre-testing injection of naloxone produced a significant effect on the response to the antagonist. However, single injection of the drug showed no significant effect on the control task. Previous studies proposed the role of several neurotransmitter systems in the expression of morphine place conditioning [6, 33]. It is well known that NO participates in morphine-induced conditioned place preference [33]. It is recently noted that both mu- and GABA (A)-receptors in the CeA inhibit the behavior induced by withdrawal from acute morphine treatment [34]. There is also evidence expressing that CeA is involved in place aversion induced by naloxone in single-dose morphine-treated rats [10]. In contrast, Ishida *et al.* [10] used a single-session morphine treatment to survey on the acute dependence, but in this study repeated morphine injection was performed to associate between primary unconditioned properties of the environment to the conditioned stimulus [35].

Present finding may indicate that both opioid and non-opioid mechanisms mediate the effect. These data may participate the NO of CeA in the interaction between naloxone and morphine to reveal the psychological dependence. These data showed no significant effect of L-NAME, a highly selective neuronal NOS inhibitor, injected both in the control and prior to naloxone paired task. This part of the finding may indicate that the experience is not solely related to the antagonist effcet. However, the inhibitor when was injected before to L-arginine effective dose (1 µg/rat, intra-central amygdala) prior to naloxone reversed the effect induced of the NO precursor, L-arginine. This fact may suggest the NO formation due which the naloxone paired conditioning was potentiated. This finding may support the recent data identifying that different neural systems [36] both opioid and non-opioid mechanisms mediate morphine reward and its spontaneous withdrawal signs. However, to date, the role of opioid system is not obviously defined. The present study may clearly indicate an interaction between naloxone and morphine, and that the NO as a highly active neurotransmitter may modulate dynamically the opioid and antagonist effects in the CeA which remains to be studied at the molecular level.

[DOR: 20.1001.1.1028852.2011.15.3.6.4]
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ACKNOWLEDGMENTS

This study was supported in part by Deputy of Research at Shahed University, Tehran, Iran (document no. 7941 for the research plans).

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