Verifying of Participation of Nitric Oxide in Morphine Place Conditioning in the Rat Medial Septum Using Nicotinamide Adenine Dinucleotide Phosphate-Diaphorase (NADPH-d)

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ABSTRACT

**Background:** Role of nitric oxide (NO) in morphine-induced conditioned place preference (CPP) has already been proposed in the rat medial septum (MS), but no molecular evidence has been provided to clear this fact. **Methods:** Effects of intraseptal injections of L-arginine and/or N⁶-nitro-L-arginine methyl ester (L-NAME) on morphine place conditioning in Wistar rats were examined. Morphine (2.5-7.5 mg/kg) was injected s.c. using a three-day schedule of an unbiased place preference. All of the brain samples were examined histochemically by nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d), the main marker for NO activation. **Results:** Morphine induced a significant CPP in the rats. Single injections of L-arginine or L-NAME (0.3, 1.0 and 3.0 µg/rat) did not induce CPP. In addition, co-administration of morphine (5.0 mg/kg) with L-arginine or L-NAME (0.3, 1.0 and 3.0 µg/rat) did not affect morphine response. However, administration of L-arginine (0.3, 1.0 and 3.0 µg/rat) prior to morphine conditioning testing enhanced the expression of morphine response. Moreover, pre-injection of L-NAME (0.3, 1.0 and 3.0 µg/rat) to L-arginine (0.3 µg/rat) did not reverse the response to the agent. The expression of NADPH-d was observed in the rat brain samples treated by L-arginine. A decreased expression of NADPH-d was also observed in rats pre-injected by L-NAME. **Conclusion:** This finding strongly suggests that NO system in the rat MS has an impact on the expression of morphine rewarding, and that the NO participates in place conditioning induced of morphine. *Iran. Biomed. J.* 14 (4): 150-157, 2010

**Keywords:** Morphine, Nitric oxide (NO), Conditioned place preference (CPP), Nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d)

INTRODUCTION

Nitric oxide (NO) is an endogenous highly short-lived molecule [1] which serves as an important regulator of neuronal functions [2, 3]. This molecule is implicated in cellular events which underlie the processes of learning and memory [4]. The molecule NO appears to regulate many processes such as dopamine release from the brain [5, 6] and intercell communication [7-9].

In 1994, Peng et al. [10] reported that a subset of septal neurons in the rat brain may release NO. The expression of NO synthase (NOS) mRNA in the rat brain medial septum (MS) has been observed using *in situ* hybridization [11]. Also, positive nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) [12] as well as NOS immunoreactive neurons [13] have been detected in the MS of the rat brain. Place preference is mainly related to the enhancement of the dopamine release in shell of accumbens by doubling the firing rate of the dopamine neurons of the ventral tegmental area (VTA) [14]. Injection of morphine intra-VTA has been shown to have a reinforcing effect due to the activation of mesolimbic dopamine neurons [15].
Mul-opioid receptors [16] and delta-opioid receptors, located in central glutamate synapses, have been implicated in morphine-induced preference to the place [17]. In addition, the activation of N-methyl-D-aspartate receptors in the nucleus accumbens and VTA [18] has been recognized to have a role in morphine-induced place preference.

It has recently been shown that different neural systems mediate morphine reward [19]. Some researchers proposed that levels of NO play a role in the modulation of dopamine outflow in the mesolimbic and mesocortical reward and motivation circuitries [20]. Manzano et al. [21] more recently demonstrated that the NO pathway is implicated in the development of sensitization to the conditioned rewarding effects of morphine. Also by genetic engineering studies using neuronal NOS (nNOS) knockout mice it has been provided the useful data on the nNOS gene underlying the cue conditioning [22].

Our previous research without molecular evidence supported the fact that L-arginine, a precursor of NO in the rat MS, may modulate the expression of morphine conditioning due to dopamine release [23]. The present experiments were conducted to clearly reveal the involvement of NO of the rat MS in the expression of morphine-induced place conditioning. Therefore, a histochemical study by using the NADPH-d technique was mainly carried out to show the activity of specific NOS at the site. NOS cofactors including nicotinamide adenine dinucleotide phosphate, flavin mononucleotide, and flavin adenine dinucleotide were needed in the processes of generation of NO from L-arginine [24].

NADPH-d and NOS activities are caused by different properties of the same enzyme molecule so that NADPH-d activity can be used as a marker for NOS [25]. A high neuronal activity of NADPH-d has also been marked by NOS isoforms [26, 27]. NADPH-d histochemical reaction is now commonly used to reveal NOS protein [28]. In the present experiments, the involvement of NO in the rat MS in inducing conditioned place preference (CPP) by morphine was examined both behaviorally and by using NADPH-d histo-chemistry, a marker of NOS activation.

**MATERIALS AND METHODS**

**Subjects.** Subjects were male Wistar rats (Pasteur Institute of Iran, Tehran), weighing 220-250 g at the time of the experiment. Animals were housed four per cage in a controlled colony room (temperature 21 ± 2°C) and maintained on a 12 h light/dark cycle with food and water *ad libitum*. The experiments were carried out during the light phase (7.00 am-19.00 pm) of the cycle. Each animal was used once and 8 animals were used in each experiment. The protocol was approved by the Local Ethical Committee of Basic Science Research Center, Shahed University (Tehran, Iran).

**Drugs.** Morphine sulfate (Temad Co., Tehran, Iran), sodium pentobarbital (Sigma, Chemical Co., U.S.A.), L-arginine (Sigma Chemical Co., USA), and N^6^-nitro-L-arginine methyl ester (L-NAME; Research Biochemical Inc, U.S.A.), were prepared freshly in sterile 0.9% NaCl solution. Morphine and pentobarbital were respectively injected s.c. and i.p. in a volume of 1 ml/kg. L-arginine and L-NAME was unilaterally injected into the MS. Vehicle injections were 0.9% physiological saline.

**Surgical procedure.** The animals were anesthetized and placed in a stereotaxic apparatus, while maintaining the incisor bar at approximately 3.3 mm below horizontal zero to achieve a flat skull position. An incision was made to expose the rat skull. A hole was drilled in the skull at stereotaxic coordinates AP + 1.2 mm anterior to bregma, and L + 0.1 mm according to the atlas of Paxinos and Watson [29]. A guide cannula (21 gauges) was inserted into the hole and lowered 6.0 mm below bregma through the hole drilled at the desired coordinates. The guide cannula was anchored by a jeweler’s screw and the incision was closed with dental cement. All animals were allowed to recover for 1 week before behavioral testing began.

**Injection into the MS.** The animals were gently restrained by hand; the dummy cannula was removed out of the guide cannula. For intra-MS injections of drugs, a 5.0-µl Hamilton glass syringe was used. The injection (inner) cannula (27 G) which further (0.5 mm) projected to the tip of the guide was attached to polyethylene tubing (0.6 mm internal diameter) to the Hamilton syringe. The injection volume was 1.0 µl for all groups. Injections were made over a 30 s period, and the injection cannulae were retained in the guide cannulae for an additional 60 s to facilitate the diffusion of the drugs.

**Histological verification.** After behavioral testing,
experimental animals were given over dose of chloroform to collect the brain samples in 10% formaldehyde. The samples were then assessed histologically to show the site of injection and the placements of the cannulae were verified using the atlas of Paxinos and Watson [29].

**Apparatus.** A two compartment CPP apparatus (30 × 60 × 30 cm) was used in these experiments. Place conditioning was conducted using an unbiased procedure, with minor changes to the design previously been described [23]. In this apparatus, rats showed no consistent preference for either compartment.

**Conditioning procedure.** The rats were housed in the colony for at least 1 week prior to commencement of the experiments which consisted of following phases.

**Familiarization.** On day one, the animals were accustomed to the CPP apparatus in 15 min; the removable wall was raised 12 cm, thereby allowing each rat to move freely between the two compartments. Animals were then randomly assigned to groups for place conditioning.

**Conditioning.** This phase consisted of six 45-min sessions: 3 saline and 3 drug pairing. These sessions were conducted twice daily (days 2-4) with 6 h separating each and designed and followed as accurately as been described in detail in a previous report [23].

**Testing or post conditioning.** The test session was carried out on day five, 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 and each of the uninjected animals was allowed free access to both compartments of the apparatus. An observer then assessed the time spent in the morphine- and saline-paired compartments. The scores (in s) represent the time spent in the drug-paired compartment minus that of spent in the side pre-conditioning, and are expressed as mean ± S.E.M.

**Induction and assessment of morphine place conditioning.** Morphine (2.5, 5.0 and 7.5 mg/kg) was administered s.c. according to a 3-day schedule of a place conditioning task to produce a significant CPP; morphine or saline (1 ml/kg, s.c.) was injected once/day during the conditioning. Scores were assessed by measuring the time spent in the morphine-paired compartment of the CPP apparatus in a morphine-free state minus that of spent in the side pre-conditioning. This may eliminate the possibility that morphine-induced motor effects are influencing the response [28, 30]. The data are expressed as mean of scores ± S.E.M.

**Measurement of effects of L-arginine (NO precursor) and L-NAME (NO synthase inhibitor) on the acquisition and expression of morphine CPP.** Single doses (0.3, 1.0 and 3.0 µg/rat) of L-arginine or L-NAME were injected once/day according to a 3-day schedule of conditioning task or once/pre-testing to survey on the drugs’ effects in acquisition or expression of a place conditioning in comparison to the controls. Control groups were simply injected saline (1 µl/rat, intra-nucleus or 1 ml/kg, s.c.). Co-administration of different doses (0.3, 1.0 and 3.0 µg/rat) both of L-arginine and L-NAME with morphine (5.0 mg/kg) during conditioning was used to determine their effects on morphine CPP in rats.

**NADPH histochemistry.** After testing, experimental animals were killed using over dose of chloroform and their brains were collected in 4% buffered formaldehyde for 3-5 days in order to provide an adequate NADPH staining as been described in the literature [31]. The buffer was prepared using an attested protocol [31]: 3.3 g of hydrated monobasic sodium phosphate, 10.8 g of anhydrous dibasic sodium phosphate in 11 ml of water. After being fixed, the unembedded samples in paraffin were sectioned into smaller pieces holding the MS. The pieces were then embedded in paraffin and cut by a microtome (8-40 µ) and collected in the buffer. These slices were later mounted on slides greased by albumin and the slides were then retained at room temperature for about 24 h. Dioxane was used instead of alcohol throughout the processes of dehydration. The prepared slices were floated in a diluent of 0.3% Triton X-100 while being shaken for 5-10 min. The staining was then performed by incubating the slices in a solution containing equal parts of nitro blue tetrazolium (NBT, 0.4 mg/ml in buffer) and NADPH (2 mg/ml in buffer) at 37°C for about 16-18 hours [31]. NBT is a salt that yields an insoluble blue formazan which is visible by light microscopy [24]. For the control groups, the NADPH was excluded and in the control sections no staining was observed.
Dose-response curve for place preference conditioning produced by morphine in rats. Animals (8/group) received s.c. injection of saline or morphine in a 3-day schedule of conditioning as described in Materials and Methods. Figure 1 shows a dose-response curve for place conditioning induced by morphine in rats. Animals, which received saline (1 ml/kg) twice per day, 6 sessions, exhibited no preference for either place cues.

Statistics. One-way analysis of variance (ANOVA) or two-way ANOVA followed by Tukey-Kramer or Newman keul's multiple comparison tests were used to determine the effects of the various treatments upon morphine-induced place conditioning. *P<0.05 was considered as significant and all values are expressed as mean ± S.E.M. The NADPH-d histochemical reaction in at least seven brain sections of each sample was also examined using the light microscope (Olympus Photomicroscopy, USA) at 100 µm² defined as a unit of quantification measurement. Image Tool program (UTHSCSA ImageTool, version 2.03), the free image processing and analysis program for Microsoft Windows, was used for image analysis functions after spatial calibration to provide quantification of the staining results for the area (100 µm²).

RESULTS

Administration of different doses of morphine (2.5, 5.0 and 7.5 mg/kg) during conditioning induced a significant CPP according to the one-way ANOVA [F (3, 28) = 160.14, P<0.0001]. Maximum response was observed at 5.0 mg/kg of the opioid. By considering the result, a dose of morphine (5.0 mg/kg), was employed, during conditioning sessions, for subsequent studies.

Effect of nitric oxide synthesis precursor and inhibitor on the acquisition of morphine-induced CPP. L-arginine, the precursor of NO synthesis and L-NAME, the inhibitor of NOS were used alone or in combination with morphine during conditioning (as described in methods). Figure 2A shows the effect of L-arginine with or without morphine on CPP. Two-way ANOVA indicates no significant difference between the response to L-arginine (0.3, 1.0 and 3.0 µg/rat) with that of L-arginine plus morphine (5.0 mg/kg, P>0.05). Figure 2B shows the effect of L-NAME on CPP. Two-way ANOVA indicates no significant interaction between the responses to L-NAME (0.3, 1.0 and 3.0 µg/rat) in the presence or absence of morphine (5.0 mg/kg, P>0.05).

Effect of nitric oxide synthesis precursor and inhibitor in the expression of morphine CPP. L-arginine and L-NAME were administered on the testing day in a morphine-free state. Figure 3 shows the effect of L-arginine or L-NAME in the expression of morphine CPP. The drugs were administered 1 min before CPP testing on day 5. One-way ANOVA shows that L-arginine (0.3, 1.0 and 3.0 µg/rat) but not L-NAME (0.3, 1.0 and 3.0 µg/rat) increased the expression of morphine CPP [F (3, 28) = 13.824, P<0.001]. Figure 3 also shows the effect of L-NAME on L-arginine-induced increase in the expression of morphine CPP. One-way ANOVA showed that L-NAME (0.3, 1.0 and 3.0 µg/rat) has no significant effect on the response induced by L-arginine (0.3 µg/rat).

Nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d). Light microscopic observations revealed a difference in the expression of NADPH-d in the control samples (Fig.4) to those treated by NO agents (Fig. 5); the samples treated by L-arginine at the test day of conditioning paradigm displayed positive NADPH-d staining. A significant increase in NADPH-d activity was observed (P<0.001) in the L-arginine-treated samples stained by using the NADPH-d histochemistry (Fig. 5).
FIG. 2. Effect of L-arginine (A) or L-NAME (B) with or without morphine on CPP. Animals received saline (1 µl/rat, intra-MS) or NO agent (0.3, 1.0 and 3.0 µg/rat, intra-MS) in the presence or absence of morphine (5.0 mg/kg, s.c.) during conditioning. The data are expressed as mean of scores ± S.E.M. Score is defined as the time spent in the drug-paired place on the testing day minus that of spent in the side pre-conditioning (day 1). ***P<0.001, difference to the saline control group.

DISCUSSION

In the present study, Wistar rats were administered morphine (2.5, 5.0 and 7.5 mg/kg, 3 sessions) s.c. by using an unbiased CPP paradigm. The animals were tested for a CPP in a morphine-free state. Our data indicated that morphine induces a significant CPP. The current study showed that intra-MS administration of single doses of L-arginine (0.3, 1.0 and 3.0 µg/rat), a precursor of NO or L-NAME (0.3, 1.0 and 3.0 µg/rat) an inhibitor of NOS did not elicit any response in the conditioning task. Furthermore, co-administration of L-arginine or L-NAME with morphine (5.0 mg/kg, s.c.) during conditioning had no significant effect on morphine CPP. On the other hand, the administration of L-arginine but not L-NAME pretesting increased the expression of morphine CPP. When L-NAME was pre-administered intra-MS, 1 min before the injection of L-arginine, the response to L-arginine did not reverse. The results are in agreement with those of the previous reports in this respect [23, 32].

FIG. 3. Effects of L-arginine or L-NAME, and those of L-NAME preinjection to L-arginine in the expression of morphine-induced CPP. Animals received morphine (5.0 mg/kg, s.c.) or saline (1 ml/kg, s.c.) in a 3-day schedule of conditioning. On day of testing, saline (1 µl/rat, intra-MS), L-arginine (0.3, 1.0 and 3.0 µg/rat, intra-MS) or L-NAME (0.3, 1.0 and 3.0 µg/rat, intra-MS) was injected 1 min before testing. In pre-injecting of L-NAME to L-arginine, L-NAME (at 0.3, 1.0 and 3.0 µg/rat, intra-MS) prior to L-arginine at 0.3 µg/rat was administered 1 min before injection of L-arginine. Saline control group received saline (1 µl/rat, intra-MS) and the L-arginine control group received L-arginine (0.3 µg/rat, intra-MS) before testing. The data are expressed as mean of scores ± S.E.M. Score is defined as the time spent in the drug-paired place in testing (day 5) minus that of spent in the side pre-conditioning (day 1). **P<0.01 and ##P<0.01, difference to the control group and respective control group, respectively.

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L-NAME has been shown to be an inhibitor of NO synthesis by competing with the precursor L-arginine for NOS [33]. This antagonist has been reported to attenuate morphine dependence in mice [7, 34, 35]. In contrast, there are findings that show the lack of the L-NAME potency in inhibiting NOS activity in some areas of the brain [36].

Other investigators have also postulated that the isof orm-selective NOS inhibitors differ in efficacy [37]. Pre-injection of L-NAME to L-arginine did not show a significant effect on response to L-arginine pre-testing to morphine CPP. This result might be an evidence for a minimal potency of L-NAME in inhibiting the NOS activity. Also, it may indicate the inappropriate dose of antagonist to prevent the stimulated NOS activity [38]. Therefore, the attenuation or blockade of the effect of L-arginine on expression of morphine-induced CPP might require either more potent or higher doses of the inhibitor in the area of interest. In disagreement, L-NAME has recently been proposed as a potent inhibitor of the inducible NOS and a useful new antitumor drug [39]. However, as an alternative, L-arginine, the NO precursor, may cause itself an increase in extracellular dopamine level at the site of interest [5, 6]. But, this study provided an intensive histochemical evidence to involve the septal NO in the process, by using NADPH-d as a histochemical marker for NOS [26]. According to the present work, the positive NADPH-d histochemistry, was observed in the brain samples taken from animals treated by L-arginine (Fig. 5), identifying the activation of NOS at the site of injection. The expression in nitrergic neurons did vary and was decreased in the samples of animals pre-injected with L-NAME in comparison with those of injected L-arginine before testing of

**Fig. 4.** Negative NADPH-d histochemistry in the expression of NOS in brain slices from the rats which received saline (1 µl/rat, intra-MS) on day of the testing. Arrowheads show non-stained septal cells under NADPH-d examination. Line is 20 µ.

**Fig. 5.** Positive NADPH-d histochemistry in the expression of NOS in brain slices from the rats which received L-arginine (0.3 µg/rat, intra-MS) on the day of testing. Animals were administered morphine (5 mg/kg) or saline (1 ml/kg) s.c. in a 3-day schedule of conditioning. L-arginine (0.3 µl/rat, intra-MS) was given on day of the testing in a morphine-free state. Arrows show the septal cell expressing NADPH-d. Line is 20 µ.

**Fig. 6.** NADPH-d histochemistry in the expression of NOS in brain slices from the rats which received L-NAME (0.3, 1.0 and 3.0 µg/rat, intra-MS) prior to L-arginine (0.3 µg/rat, intra-MS) before testing. Animals were administered morphine (5 mg/kg) or saline (1 ml/kg) s.c. in a 3-day schedule of conditioning. L-NAME (0.3, 1.0 and 3.0 µg/rat, intra-MS) was administered 1 min before direct injection of L-arginine into the site prior to testing in a morphine-free state. L-arginine (0.3 µl/rat, intra-MS) was given after the injection of L-NAME. Arrow indicates cells which did not express NADPH-d activity at the site of interest. Line is 20 µ.
morphine response. This finding showed that NO is participated in the expression of the morphine response. This technique also confirmed that the NO system is presented and expressed in the MS of morphine conditioned Wistar rats.

In conclusion, this behavioral measurement only proposed that L-arginine may play an important role in expression of morphine conditioning during testing. The decrease in NOS activity due to prior injection of L-NAME to L-arginine did not attenuate the enhanced expression of morphine response induced by L-arginine. However, the NADPH-d histochemistry demonstrated that the NO as well as the L-arginine is involved in the expression of morphine-induced place preference in adult Wistar rats.

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