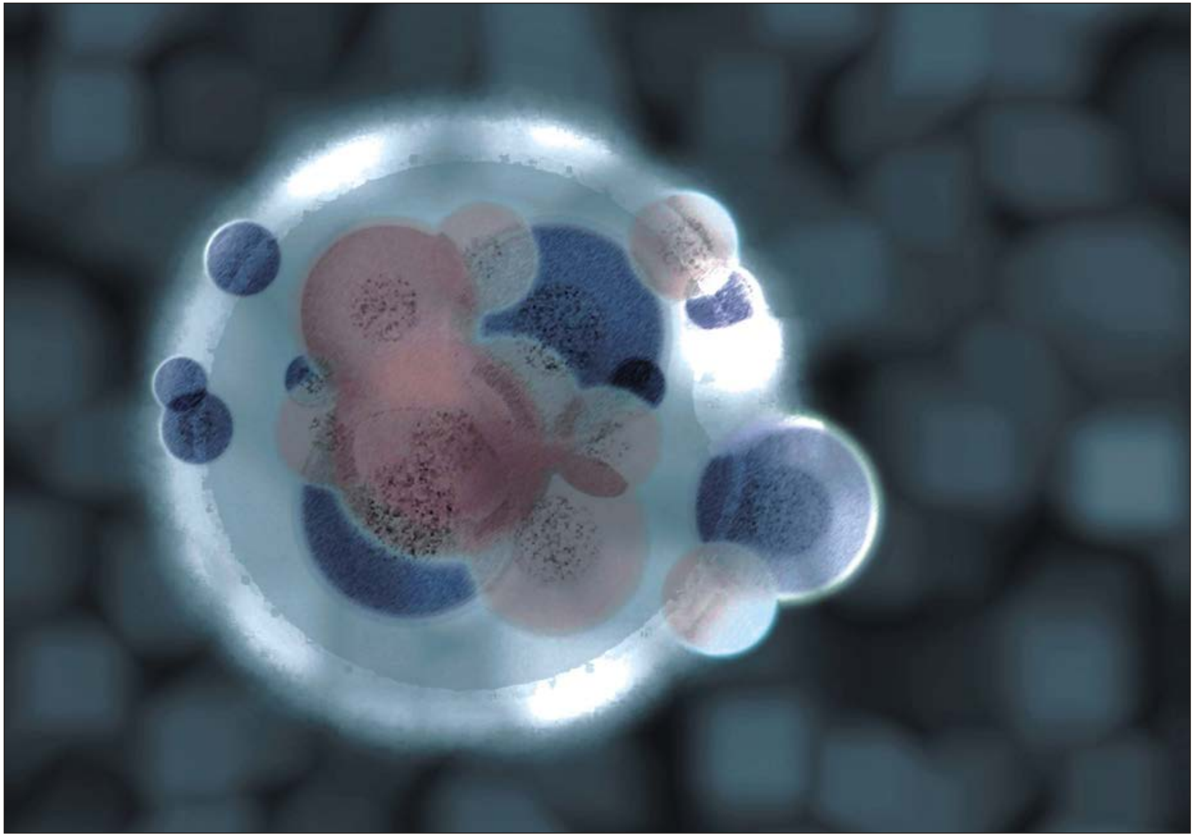

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Nitric Oxide in the Hippocampal Cortical Area 1 (CA1) Augments the Naloxone-induced Place Aversion

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Abstract: It has been shown that naloxone induces place aversion in the conditioning task. We examined the involvement of Nitric Oxide (NO) in naloxone- induced place aversion. The experiment was conducted in male Wistar rats (weighing 200-250 g) using an unbiased conditioning program. Control group solely received saline (1 mL kg^{-1}, i.p.) throughout the procedure. The experimental animals were injected naloxone (0.1-0.4 mg kg^{-1}, i.p.) once per day during the phase of conditioning. L-Arginine (0.003-3 μg rat^{-1}), the precursor of NO, was microinjected intra-CA1 prior to testing of naloxone response. A selective inhibitor of neuronal NO synthase (nNOS), L-NAME (0.03-3 μg rat^{-1}), was pre-microinjected in testing day. Naloxone induced a significant place aversion in the experimental animals. The main narcotic drug also caused an increase in Wet Dog Shaking (WDS). The drug use, however, attenuated the compartment entering. The place aversion induced of naloxone was highly potentiated due to microinjection of L-arginine intra-CA1. This injection also increased the compartment entering and the rearing but not the WDS. The potentiated aversion response induced of L-arginine pre-testing was blocked by pre-microinjection of L-NAME. The NOS inhibitor L-NAME moreover restored the WDS induced by naloxone. The molecule NO in the CA1 likely interacts with naloxone in induction of place aversion.

Key words: Naloxone, nitric oxide, CA1, place aversion, behavior

INTRODUCTION

Drug-induced behaviors reveal the neural pathways in processing of sensory information (Hills *et al*., 2004). The opioid drugs activate the reward circuits which positively grade the sensory information in brain (Cowen and Lawrence, 1999; Olson *et al*., 1993; Vaccarino and Kastin, 2001; Vanderschuren *et al*., 1997). The opioids act through specific interactions with mu (μ)-, delta (δ)- and, kappa (κ)-opioid receptors (Mattes *et al*., 1996; Kieffer, 1999; Le Merrer *et al*., 2009) which are mainly distributed in brain's motivational areas (Harrison *et al*., 1998).

The reward and place conditioning are mediated by mu (μ)- and delta (δ)- opioid receptors (Wang *et al*., 2005). The nucleus accumbens in the reward system (Spyraki and Fibiger, 1988) and hippocampus are interconnected (Everitt and Robbins, 2005).

The Nitric Oxide (NO) in the hippocampal CA1 area influences the place preference induced of morphine (Zarrindast *et al*., 2002). This neural messenger (Manzanedo *et al*., 2004) is primarily produced by converting of L-arginine to L-citrulline via nNOS in response to the stimulation of N-methyl-D-aspartate (NMDA) glutamate receptors (Garthwaite and Boulton, 1995).

The naloxone, a selective antagonist of morphine neutralizes the opioid effect through competing for the same receptor sites (Wang *et al*., 2005). The injection of naloxone in morphine-dependent mice causes an increase of mu-opioid receptors in the limbic area during the opioid withdrawal (Diaz *et al*., 2006). We examined the NO interaction in the CA1 with the narcotic drug naloxone in induction of the place aversion. We additionally measured the behavioral signs induced of drug.

MATERIALS AND METHODS

Animals: Adult male Wistar rats weighing 200-250 g (Bought of Pasteur Institute, Tehran, Iran) were housed in the colony room under the standard temperature 21±3°C and fed by food and water *ad libitum*. The experiments were carried out during the light phase of the 12 h light/dark cycle. The animals were tested once and after completion of the experiments they were exposed to the overdose of chloroform. All experiments were done in

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accordance with the guidelines of laboratory animals' care and use approved by the local committee of ethics.

Drugs: Naloxone hydrochloride (purchased from Tolid Daru Co., Tehran, Iran) was intraperitoneally (i.p.) injected. Vehicle was 0.9% physiological saline (1 mL kg^{-1}, i.p.). L-Arginine (Sigma Chemical Co., USA) and N^G-Nitro-L-arginine Methyl Ester (L-NAME; Research Biochemical Inc., USA) were bilaterally injected (μg rat^{-1}) into the CA1. Vehicle for intra-CA1 was 0.9% physiological saline (1 μL rat^{-1}). Ketamine (Bought of Veterinary organization of Iran) (100 mg kg^{-1} i.p.) and xylazine (20 mg kg^{-1}) were used for anesthetizing the experimental animals.

Stereotaxic surgery: After the collective injection of ketamine and xylazine the animals were placed in a stereotaxic apparatus which was set at approximately 3.3 mm below horizontal zero to achieve a flat skull position. An incision was made to expose the rat's skull. Two holes were drilled in the skull at stereotaxic coordinates for the CA1 (AP = -3.8 mm posterior to bregma and L = ±2.2 mm) in accord with the atlas of Paxinos and Watson (2005). Two guide cannulae (21-Gauge) were inserted into the holes; the cannulae were lowered 2 mm below the bregma. They were then anchored with a jeweler's screw. Finally, the incision was closed with the dental cement. After completion of the surgery, a dummy inner cannula was inserted into each guide cannula. One week after the animals' recovery the behavioral testing began.

Intra-CA1 injection: The dummy cannulae were gently removed from the guide cannulae. Drugs were directly injected into the CA1 using the injection set up: the injection needles (27-Gauge) which passed over 1.0 mm ventral to the tip of the guides were connected to a 5.0 μL glass Hamilton syringe by polyethylene tubing (0.3 mm internal diameter). All injections were done slowly throughout a 30 sec period.

Conditioning place preference apparatus and paradigm

Place conditioning apparatus: A two compartment Conditioned Place Preference (CPP) apparatus (30×60×30 cm) was used in these experiments. Place conditioning was conducted using an unbiased program with a little change to the design previously been described (Zarrindast *et al*., 2002; Karami *et al*., 2002). The apparatus was divided into two equal-sized compartments in which a guillotine door was inserted. Both compartments were completely colored white but differently striped black (vertical vs. horizontal). The compartments were also distinguishable by texture and olfactory cues. To provide the tactile difference between the compartments, one of the compartments floored smooth vs. the other compartment which grinded. A drop of natural aqueous rose extract was placed at the corner of the compartment equipped with a textured floor, to provide the olfactory difference between the compartments. In this apparatus, rats presented no consistent preference for one of the compartments confirming the unbiased procedure. 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 blind to the experiments.

Conditioning paradigm: The conditioning paradigm consisted of 3 phases.

Pre-conditioning (Familiarization) phase. On day 1, animals were placed (10 min) in the apparatus while the guillotine door was raised 12 cm above the floor to move freely in the apparatus. The time spent by rats into each compartment was recorded using the Ethovision system.

Conditioning phase: This phase was started a day after habituation. The conditioning phase daily consisted of 3-saline and 3-drug pairings with a 6 h interval between the sessions. Drug administration in conditioning phase was carried out during the light phase of a 12 h light/dark cycle (e.g., at 09:00 am and 03:00 pm). Control groups received saline (1 mL kg^{-1}, i.p.) twice a day. All conditioning sessions lasted 40 min. For each drug dose, each group of animals (6 rats) was randomly assigned into two subgroups. After the drug injection each subgroup of animals was confined in one compartment (drug-paired side). The presentation order of drug or saline was counterbalanced.

Post-conditioning (testing) phase: Test sessions were carried out in last day, a day after the last conditioning session in a drug-free state. Each animal was tested only once. During the testing phase the guillotine door was raised 12 cm above the floor and the animals freely accessed in the apparatus for 10 min. The time spent in both compartments was recorded by Ethovision system. The time (sec) spent in the drug-paired compartment in testing day was then calculated. The score was expressed as Mean±SEM.

Experimental design

Induction of place aversion by naloxone: The effects of administration of naloxone (0.1-0.4 mg kg^{-1}, i.p.) in

induction of place aversion in animals were determined. The naloxone was injected using a 3-day schedule of conditioning task as detailed in above section. The score was presented as Mean±SEM.

Effect of injection of nitric oxide agent intra-CA1 on expression of naloxone response: Different doses of L-arginine (0.003-3 μg rat^{-1}) and cumulatively L-arginine/ L-NAME (0.03-3 μg rat^{-1}) were bilaterally administered prior to naloxone response testing (the last day).

Histological verification: After completion of behavioral testing, the animals were exposed to overdose of chloroform. The animals' brains after injection of ink (0.5 μL of 1% aqueous solution of methylene blue/per side of the CA1) were removed. The specimens were fixed in a 10% formalin solution for 48 h. The brain slices were taken through the cannulae placements and the placements were verified by Paxinos and Watson (2005). Data from rats with injection sites located outside the appropriate area were excluded from the statistical analyses (8 rats).

Statistical analysis: All results are expressed as Mean±SEM (standard error of mean). One- and/or two-way analysis of variance (ANOVA) followed by appropriate *Post-hoc* analysis (Tukey's test) for multiple comparisons were used. p-values less than 0.05 ($p<0.05$) were considered as significant.

RESULTS

Histological verification of microinjection sites in the CA1: Figure 1 reveals the injection site intra-CA1 after administration of 1 μL of methylene blue solution.

Dose-response of naloxone in conditioning place preference paradigm: Figure 2 shows the effect of different doses of naloxone (0.1-0.4 mg kg^{-1}, i.p.) in Wistar rats in the conditioning task. Administration of naloxone resulted in a significant response in comparison with the saline group ($F_{4,25}$ = 4.363, $p<0.01$). The opioid induced a meaningful aversion response in rats. In view of the results after the Tukey analysis, a naloxone dose (0.1 mg kg^{-1}) was used for the subsequent studies.

Effect of NO agents on the expression of naloxone response in the place conditioning procedure: Figure 3 shows the effect of injection of L-arginine prior to testing of naloxone response. Pre-testing administration of the NO producer resulted in a significant effect as compared to the control group. The ANOVA indicated a significant difference ($F_{4,25}$ = 12.724, $p<0.01$). Further analysis demonstrated a dose effect for the NO precursor (0.003 μg rat^{-1}, intra-CA1).

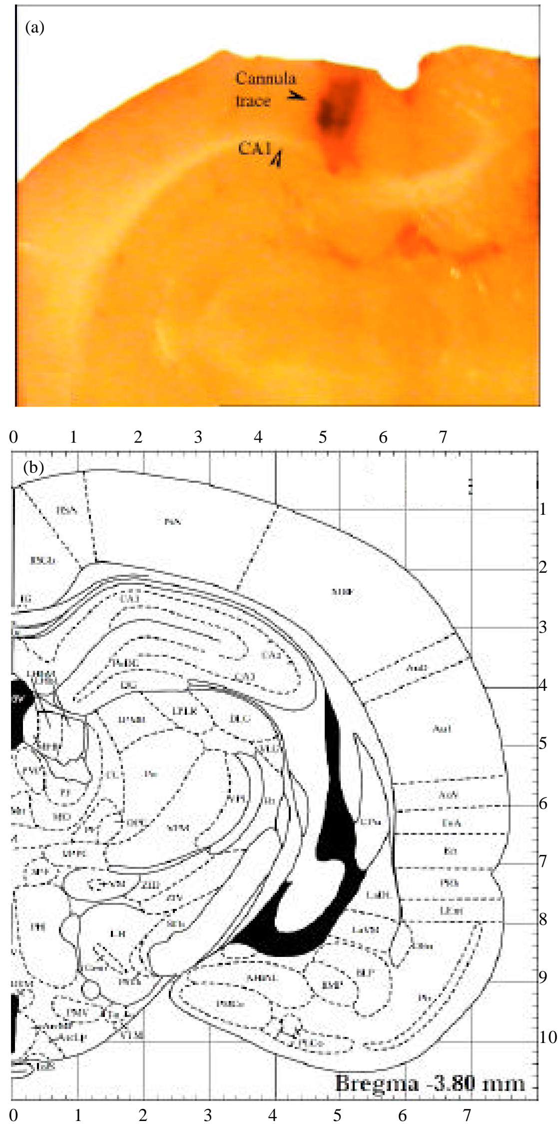

Fig. 1(a-b): (a) Evidence of cannulae placements in CA1. After testing, the animals were exposed to overdose of chloroform. The animals' brains after injection of ink (0.5 μL of 1% aqueous solution of methylene blue/per side of the CA1) were removed. The specimens were fixed in a 10% formalin solution for 48 h. The brain slices were taken through the cannulae placements and (b) Verification at AP: -3.8 by Paxinos and Watson (2005)

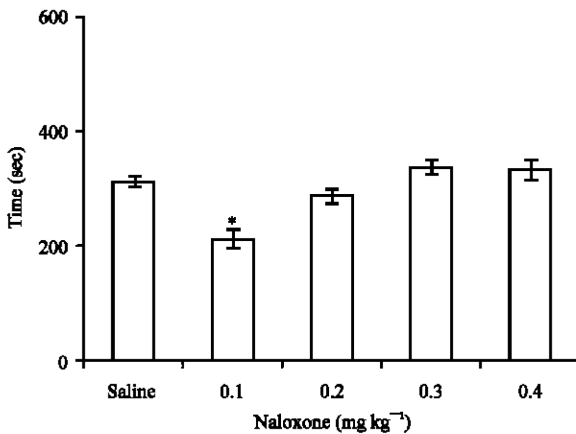

Fig. 2: Dose response to naloxone in male Wistar rats. Naloxone (0.1-0.4 mg kg^{-1}) or saline (1 mL kg^{-1}) was given intraperitoneally (i.p.) using a 3-day schedule of an unbiased conditioning paradigm. The control group received saline (1 mL kg^{-1}, i.p.), twice daily throughout the conditioning phase. Data are expressed as mean of time spent in drug-paired side±SEM, Tukey-Kramer *post hoc* analysis showed the differences: *p<0.05 difference to control (saline treated group)

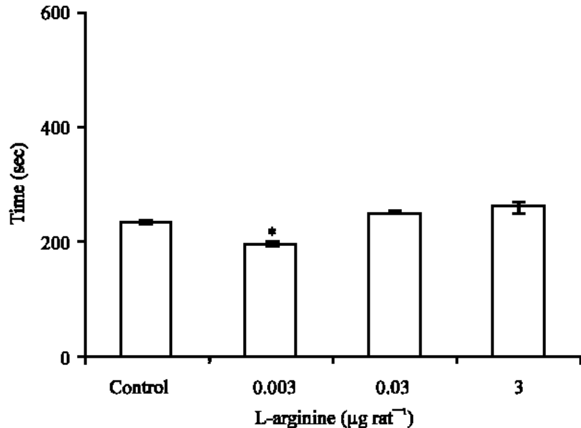

Fig. 3: Response curve to L-arginine (0.003-3 µg rat^{-1}, intra-CA1) in naloxone treated male rats. Naloxone (0.1 mg kg^{-1}) was given intraperitoneally (i.p.) throughout the conditioning phase once per/day. The naloxone given rats after 10 min were placed in the box for 40 min. On day 5, the animals were microinjected L-arginine promptly after which they were tested. Control group though was given naloxone (0.1 mg kg^{-1}, i.p.), during the conditioning phase but received saline (1 µL, intra-CA1) pre-testing. Data are expressed as mean of time spent in drug-paired side±SEM. *p<0.05 difference to control based on *post hoc* Tukey

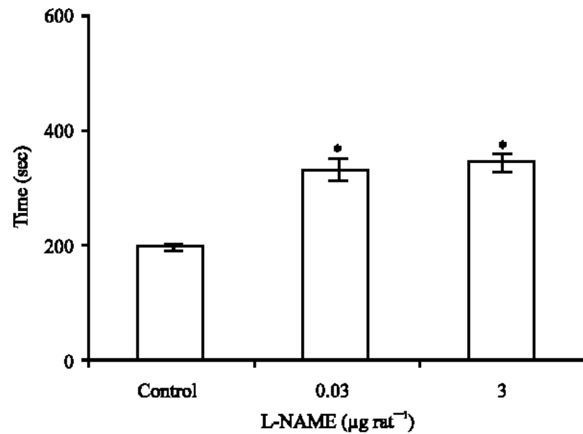

Fig. 4: Response curve to L-NAME (0.03-3 µg rat^{-1}, intra-CA1). The animals were injected naloxone (0.1 mg kg^{-1}, i.p., once per/day) in the conditioning phase. Before of testing they were given L-NAME (0.03-3 µg rat^{-1}); they were then injected L-arginine (0.003 µg rat^{-1}). Control group though received naloxone (0.1 mg kg^{-1}, i.p.) during the conditioning phase but simply received saline (1 µL, intra-CA1) pre-testing. Data are expressed as mean of time spent in drug-paired side±SEM *p<0.05 difference to control according to the *post hoc* Tukey

Effect of injection of L-NAME in the CA1 on the response to L-arginine pre-testing of naloxone-induced response: Pre-testing injection of L-NAME (0.03-3 µg rat^{-1}, intra-CA1) prior to L-arginine in testing day resulted the significant effect ($F_{2,15}$ = 5.005, p<0.05) in the animals (Fig. 4).

Effect of naloxone on behavioral signs in male Wistar rats: Repeatedly injections of naloxone (0.1-0.4 mg kg^{-1}, i.p.) through the conditioning phase in rats caused a decrease in compartment entering ($F_{4,25}$ = 3.757, p<0.05) and significantly increased the wet dog shaking ($F_{4,25}$ = 4.020, p<0.01). This treatment, however, did not induce meaningful change in rearing or grooming. Further analysis indicated the respective dose effects (0.1 and 0.2 mg kg^{-1}) (Fig. 5).

Effect of intra-CA1 injection of L-arginine on behavioral signs in naloxone treated rats: The naloxone (0.1 mg kg^{-1}, i.p.) received animals which were microinjected L-arginine showed the increased compartment entering ($F_{4,25}$ = 7.361, p<0.01) and rearing ($F_{4,25}$ = 3.890, p<0.05). This treatment, however, did not induce significant change in other signs. *Post hoc* analysis indicated the dose effect (0.003 µg rat^{-1}) (Fig. 6).

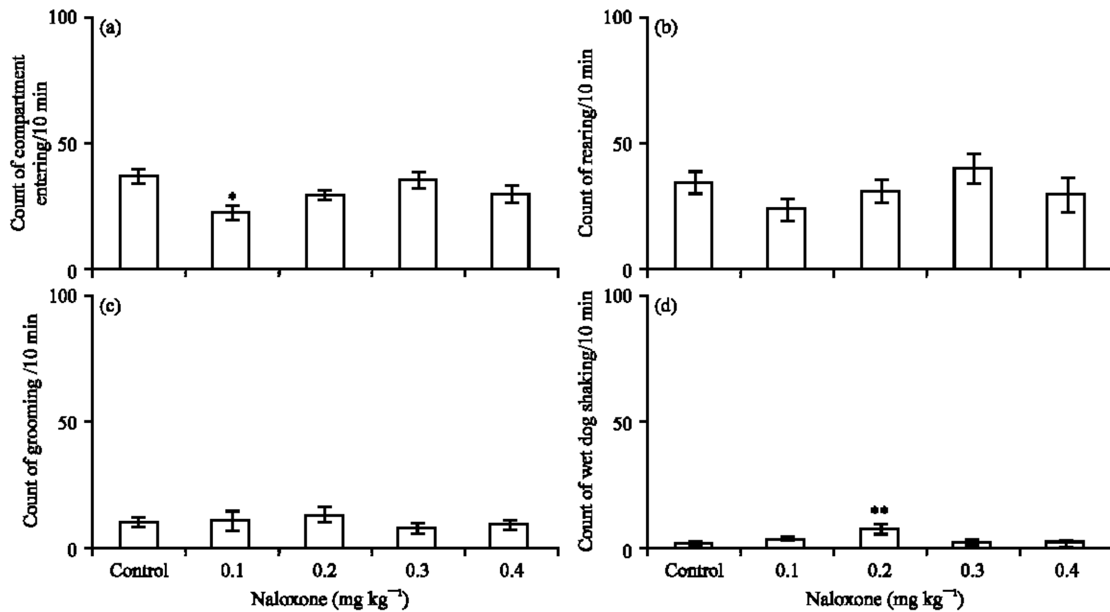

Fig. 5(a-d): Naloxone-induced behavioral signs in male Wistar rats. The drug (0.1-0.4 mg kg^{-1}, i.p.) or saline (1 mL kg^{-1}, i.p.) was given in a 3-day schedule of an unbiased conditioning paradigm. Control group only received saline (1 mL kg^{-1}, i.p.), twice daily for 3 days. Data are expressed as mean of count of behavioral signs per 10 min±SEM. Tukey *post hoc* analysis showed the differences to control (*$p < 0.05$, **$p < 0.01$)

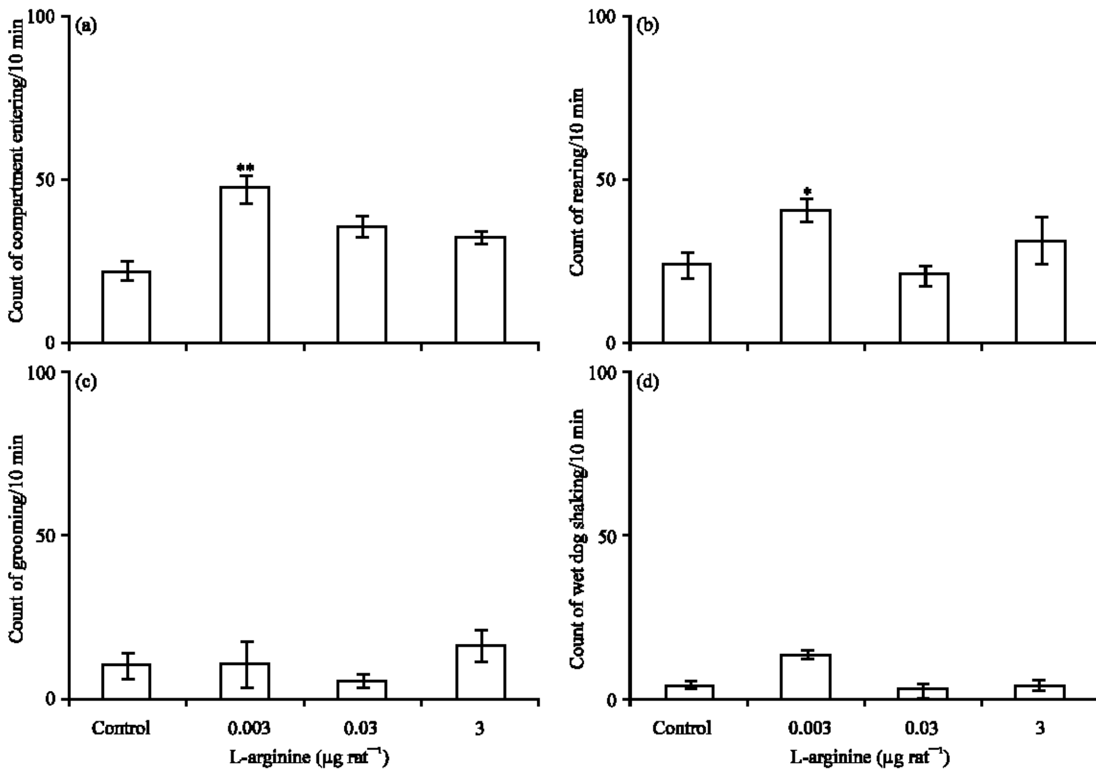

Fig. 6(a-d): The behavioral signs in naloxone (0.1 mg kg^{-1}) received rats which received L-arginine (0.003-3 μg rat^{-1}, intra-CA1) pre-testing. Control group simply received saline (1 μL rat^{-1}, intra-CA1) instead of L-arginine. Data are expressed as mean of number of behavioral signs per 10 min±SEM. Tukey *post hoc* analysis showed the differences to the control: *$p < 0.05$, **$p < 0.01$

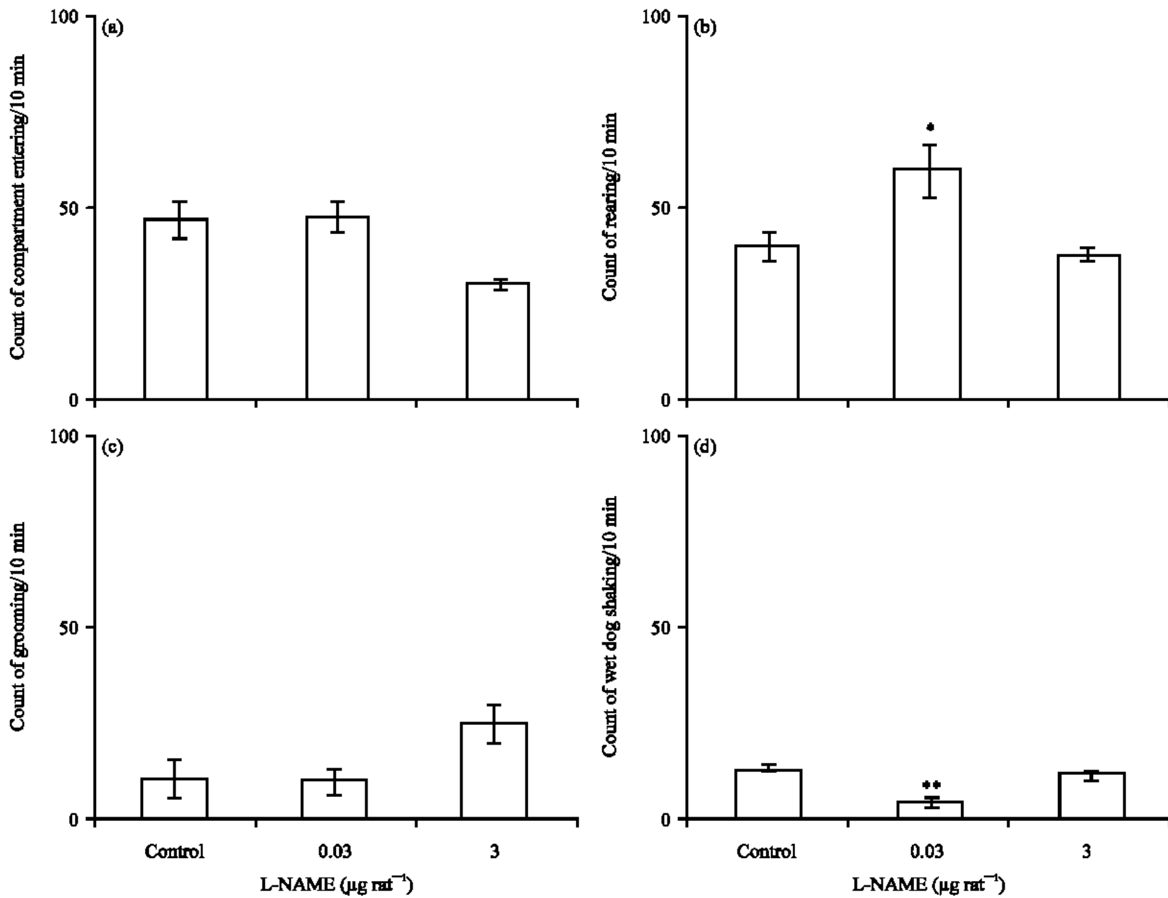

Fig. 7(a-d): The signs induced by microinjection of L-NAME (0.03-3 μg rat^{-1}, intra-CA1) prior to L-arginine (0.003 μg rat^{-1}, intra-CA1) pretesting of response to naloxone (0.1 mg kg^{-1}, i.p.). The control group though received naloxone (0.1 mg kg^{-1}, i.p.) throughout the conditioning phase but received saline (1 μL rat^{-1}, intra-CA1) instead of the NO agents. Data are expressed as mean of count of behavioral signs per 10 min±SEM. *p<0.05, **p<0.01 differences to control based on Tukey *post hoc*

Effect of pre-microinjection (intra-CA1) of L-NAME prior to L-arginine on behavioral signs in naloxone treated rats: The naloxone (0.1 mg kg^{-1}, i.p.) received animals were pre-microinjected L-NAME (0.03-3 μg rat^{-1}) to L-arginine. The inhibitor injection though augmented the cumulative effect of L-arginine and naloxone on rearing ($F_{4,25}$ = 4.433, p<0.05), but, decreased the wet dog shaking ($F_{4,25}$ = 18.432, p<0.01) with no significant effect on other signs (Fig. 7).

DISCUSSION

According to present data naloxone (0.1-0.4 mg kg^{-1}, i.p.) induced a significant place aversion in male Wistar rats. Microinjection of L-arginine (intra-CA1) prior to testing of response to naloxone potentiated the aversive response induced of naloxone. The response to the NO agent L-arginine was blocked by pre-injection of L-NAME (intra-CA1). The narcotic drug naloxone also caused an increase in Wet Dog Shaking (WDS), the response which was blocked by L-arginine microinjection. Pre-microinjection of L-NAME, however, reversed the L-arginine effect on WDS. The NO agent, L-arginine, increased both the compartment entering and rearing.

Naloxone is known as a competitive antagonist of opioid receptors which produces place aversion and increases the climbing and rearing behavior in morphine treated rats (Quock *et al*., 1987). The rewarding pathway projecting from Ventral Tegmental Area (VTA) to the hippocampus along with the Nucleus Accumbens (NAc), amygdala and prefrontal cortex (Ikemoto and Panksepp, 1999) certainly mediates the reinforcing properties of abuse drugs (Wise and Bozarth, 1987). This property is antagonized by naloxone as been previously proposed

(Quock *et al*., 1987). The activation of VTA dopaminergic neurons into the hippocampus facilitates the induction of Long-term Potentiation (LTP) and enhances learning (Lisman and Grace, 2005). Thus, the avoiding response to the naloxone as a simple learning process is depended on activation of this functional loop (Rossato *et al*., 2009). Also, the NMDA receptors of VTA dopaminegic neurons (Engblom *et al*., 2008; Argilli *et al*., 2008) and D1/D5 dopamine receptors are involved in the process (Lemon and Manahan-Vaughan, 2006; Navakkode *et al*., 2007). Considering that the microinjection of L-arginine, intra-CA1, prior to testing of naloxone-induced response potentiated the response we may discuss that the Nitric Oxide (NO) interacts with the naloxone in the CA1. It should be notified that the immunoreactivity of Nitric Oxide Synthase (NOS) in the area of interest has been previously detected (Rodrigo *et al*., 1994). The NO is formed by its producing enzyme after activation of NMDA receptors (Moncada *et al*., 1991). According to our results the compartment entering of naloxone given rats showed a significant decrease in a comparison to the control. Previous studies in agreement have demonstrated a depressive effect of morphine on locomotor activities of rodents (Szekely *et al*., 1980; Browne and Segal, 1980; Manzanedo *et al*., 1999; Belknap *et al*., 1998; Rodriguez-Arias *et al*., 2000). The rearing behavior has previously been used as a measure of locomotor effects following opioid administration (Milman *et al*., 2006; Patti *et al*., 2005). Other data have provided a dose dependent decrease in rearing behavior in morphine received animals (Milman *et al*., 2006; Patti *et al*., 2005; Kuzmin *et al*., 2000). In the present results the rats' locomotor activities obtained by count of the compartment entering and rearing increased due to the microinjection of L-arginine pre-testing, though, the rearing in the naloxone treated rats showed no change significantly. Thus, we may propose that the NO signals the motor responses to the naloxone. Reports have indicated the grooming and Wet Dog Shaking (WDS) as the associated signs with the opiate withdrawal syndrome (Koob *et al*., 1992). As another data the grooming behavior of naloxone received rats showed no change, but, the WDS was increased. We may argue that the motivational and motor responses to naloxone are mediated by the NO. This ending is proposed by considering that the response to L-arginine was reversed in presence of L-NAME. Other signal molecules may involve in motor effects of m- and k-opioid agonists (Matsumoto *et al*., 1988).

CONCLUSION

The molecule NO in the CA1 likely participates in signaling of naloxone-induced behavioral action.

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