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RESEARCH ARTICLE

The silver nanoparticles induce c-Fos expression in the central nucleus of amygdala that relief the aversive effect of naloxone in the morphine relied animal

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ABSTRACT

Objective(s): Silver nanoparticles (Ag-NPs) that are used daily in care service can enter the body and create free radicals. Despite the toxicity at high concentrations, these particles are non-toxic and useful at low concentrations. Thus, we investigated the effectiveness of nontoxic Ag-NPs to inhibit the avoiding effect of naloxone (NLX) and low level of c-Fos in the testing of morphine-induced conditioned place preference (CPP) in rat.

Methods: The animals (weighing 300-350 g) were surgically equipped by cannulae at the CeA (AP=-2.12 mm; L= ±4.1 mm; V= 7.8 mm). The paradigm was done by using a three-stage technique. Morphine (0.5-7.5 mg/kg) was injected subcutaneously (s.c.) in the second stage. NLX (0.4 µg/rat) was microinjected, intra-CeA, 10 min before the test. Ag-NPs (0.01 µg/rat) were injected before the NLX. The saline (1 µL/rat) was injected, intra-CeA, in the control group. c-Fos level was quantified immunohistochemically in rats at the end of the experiments.

Results: The CeA and hippocampal cornu ammonis 1 (CA1) of rats that treated by NLX showed low c-Fos protein levels during testing, whereas levels of protein were high in the brains of morphine conditioned rats. Interestingly, both areas (CeA and CA1) showed similar increases in protein levels when the injection of NLX was combined with the Ag-NPs. However, these regions were not significantly different in the single Ag-NPs receiving and control groups.

Conclusions: This indicates that the two regions interact with each other when NLX is injected and that in presence of Ag-NPs the protein levels are elevated in the regions.

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INTRODUCTION

So far, we have heard about the undesirable effects of morphine withdrawal and this is a tool to show the extent of dependence. Researchers have demonstrated that the reward of drugs is related to the dopamine pathway[1]. The mu-type of opioid receptors are abundant in those areas of the brain that are associated with reward, motivation, and learning; they are molecular integrating switches for the convergence of planned or unplanned stimulation in reward[2]. Among the areas, the central and baso-lateral amygdala (CeA and BLA) are involved in stimulus-reward associations[3]. The conditioned place preference (CPP) is a dominant trend in the study of drug reward in rodents since the animal during conditioning learns to associate a context with the rewarding effects of drug[4]. Morphine withdrawal has somatic and motivational symptoms[5]. In rodents, the opiate dependence aspects can be shown with the aid of naloxone (NLX), which display several motivational features such as conditioned place

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aversion (CPA)[6].

Silver is a metal with antimicrobial properties that has led to widespread use of silver nanoparticles (Ag-NPs) in the healthcare industry[7]. The Ag-NPs can penetrate the blood-brain barrier (BBB) [8] and they likely accumulate in various brain regions. Despite the toxicity of these particles in large quantities[9], their effect under very small amounts has been less studied. The nuclear protein c-Fos together with other nuclear proteins such as Jun regulate the early genes transcription[10]. It has recently been demonstrated that hippocampal immediate early gene (IEG)-positive cells as well as IEG-positive neurons in other brain areas, including the amygdala and neocortex, cooperate in the memory formation and recall[10]. Also, following the activation of neurons, the c-Fos mRNA (protein level) is increased rapidly in the amygdala, which is involved in opiate withdrawal[11]. Considering the importance of the communication between the hippocampus and the amygdala, this study aimed to measure c-Fos expression both inside the CeA and CA1 of conditioned rats to morphine upon administration of NLX, intra-CeA, during the morphine-induced CPP testing. We also assessed the morphine withdrawal sign due to the antagonist usage. We eventually compared the protein expression as well as the sign result in the two regions following pre-testing injection of NLX with the combination of Ag-NPs.

MATERIALS AND METHODS

Animal subject and groups

In this study adult male animals (Wistar rats with 300-350 g body weights) were purchased from Pasteur Institution of Iran, Tehran, Iran. They were placed in standard PVC cages under freely access to water and food *ad libitum*. They were housed at 24±2 °C temperature with 12:12 h light/dark cycle, and 60% humidity, and appropriate air exchange rate. All animals, before surgery, were managed to cope with the experimens. In this research work each animal was used just for once. Conditioning stage was done in the light phase. The current experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals. The present protocol was also confirmed by the local animal ethics committee. The animals were randomly categorized into groups of six rats per dose effect; morphine (0.5, 2.5, 5, 7.5 mg/kg) was administered s.c. daily once for three days in order to establish CPP. NLX (0.4 µg/rat, intra-CeA) was injected pre-testing of morphine-induced CPP in order to display the morphine withdrawal (the conditioned place aversion: CPA). Ag-NPs (0.01 µg/rat, intra-CeA) were pre-administered to the NLX (0.4 µg/rat, intra-CeA) in testing. Control group solely received sterile saline (0.9%, 1 µL/rat). The animals were placed in a CPP apparatus and their stay time and signs were recorded during 10 min testing. The recorded data were compared with the control.

Surgery

Anesthetized animal with ketamine (100 mg)-xylazine (20 mg) were placed in a stereotaxic apparatus and cannulated (21-Gauge) bilaterally in the CeA and in view of the atlas of Paxinos and Watson coordinated (Anterior posterior= -2.12 mm posterior to bregma and lateral= ±4.1 mm and dorsoventral= 7.8 mm)[12]. This device is set to -3.3 mm to prepare a flat skull position. The guide cannulae were put on skull and the opening was cemented with cold- poly methyl methacrylate. The injection dental needle (27-Gauge) projected 1 mm from the guide cannula was equipped with a 5- µL Hamilton syringe and allowed to inject intracranial (intra-CeA) within 60 sec. All animals passed surgery then after a week (recovery) they were examined with the CPP protocol just for once.

Place conditioning

CPP apparatus

It was a two-part wooden box (30× 60× 30 cm), which is compatible with the previously defined design[*c.f.* 13]: This device has two equal parts with a movable wall, which is inserted in the mid-point of the box. The box was colored white, but, the parts were differently striped black (vertical *vs.* horizontal). They were also differently textured (smooth *vs.* gridded). The rats showed no prominent preference to one side of the apparatus, thus, the paradigm was unbiased. An EthoVision system equipped with a video camera located 120 cm above the apparatus was designed to record animal movements in the box. All records were finally reviewed by a double -blind observer to the experiments.

Conditioning to morphine

One week after the purchase of the rats from the Pasteur Institute of Iran (they were adapted to the local animal care center), the animals were

involved in the process. They were first familiarized with the CPP apparatus on day one taken 10 min. Then, from day two to four, they were daily injected morphine and placed in one part of the box. They were finally tested for 10 min in the last test day, the day 5. To detail the process, in the pre-conditioning (familiarization) phase, animals were familiarized with box for 10 min. In this stage each animal had access to move freely into the whole device and the mobile wall was raised up (12 cm above the floor). In the 2nd stage (days 2 to 4), the animals were injected morphine (0.5-7.5 mg/kg, s.c.)/saline once per day with a 6 h interval. After treatment, each rat was placed only in one part of the CPP apparatus (drug-paired or saline-paired) for 30 min during which the gate was closed. In the last phase, which during the opioid morphine should not be prescribed, each animal received saline or desired substance, intra-CeA, and it was tested for once. In this phase (testing), each animal again could freely move to both parts of the box (10 min). The stop time in drug-paired part of the CPP apparatus was calculated between the two phases of familiarity and the test (in sec) and shown as the difference in the place preference.

Materials

Morphine sulfate was taken from Temad Co., Tehran, Iran and NLX HCl from Tolid-Daru Co., Tehran, Iran. Ag-NPs were donated by Dr Abazar Hajnorouzi at Shahed University (Department of Physics). The Ag-NPs at 60-70 nm, were prepared by the electrosonochemical method and demonstrated using TEM being homogenous in size and spherical in shape. About 95% of the particles were approximately 62 nm in diameter, with the remaining 5% being larger or smaller, were examined using a transmission electronic microscope (Zeiss-EM10C-80KV). The hydrodynamic diameter and zeta potential of Ag-NPs were characterized by dynamic light scattering (DLS) using a Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd., Worcestershire, UK). In addition to examining the stock suspension (0.01 mg/1 mL), it was sonicated in a water bath for 10 min before use.

Statistical Analysis

In the CPP paradigm, the preferred place was our measurement criterion. So changes in place preference between the familiarization and testing was measured in sec and compared by ANOVA. Between groups' variances was compared by Tukey's *post-hoc.* The results were expressed as mean ± SEM. P-values less than 0.05 (p <0.05) were significant. Tissue images of brain sections were also statistically analyzed using the software (UTHSCSA Image Tool, version 2.03, USA).

Immunohistochemistry of c-Fos protein

At the end, the experimental animals were euthanized and their brains were collected in 10% formaldehyde. After 48-72 h the brain sections (3-4 μm) were taken and processed by c-Fos Immunohistochemistry. In view of the instructions of this method the sections were treated with the appropriate dilution of the primary antibody (1:100 dilution in PBS containing 0.3% Triton X-100, 0.05% sodium azide and 2% normal goat serum) for overnight at room temperature and washed three times with PBS and incubated with secondary antibody (biotinylated goat anti-rabbit IgG, 1:100 dilution in PBS with 0.3% Triton X-100) for 40 min at room temperature. The sections then were collected in PBS containing 0.4% avidin-biotinylated horseradish peroxidase complex for another 30 min. Immunoreactivity was shown using a glucose oxidase diaminobenzidine-nickel method. The sections were placed in xylene and after gluing with the help of entellan (Merck) cover slipped. The result of this method was numerically obtained by counting the positive reacting cells from the central areas of 6-8 sections/per rat under magnifications (from 100X to 400X) and statistically shown as mean±S.E.M. .

RESULTS AND DISCUSSION

Histological evidence of Microinjection Sites in the CeA

To verify the area of interest, we used a methylene blue solution which was injected (1 μL/rat or 0.5 μL/side, intra-CeA) with the same set-up that was used for the injection of drugs into the brain. These results showed that the injections were performed at the designated site.

c-Fos expression due to morphine response

Use of morphine (0.5-7.5 mg/kg, s.c.) resulted a meaningful response [$F(4, 25) = 3.002$, $p < 0.05$] compared to the saline group (1 mL/kg, s.c.). Based on the response, a dose of the drug (5 mg/kg, s.c.) was chosen for later studies (Fig. 1). Also, the c-Fos test led to interesting results; the expression of this protein (c-Fos) increased in animals due

to morphine- induced spatial preference (Fig. 2A). It has been previously mentioned that the amygdala may correlates the aspects of NLX-caused morphine withdrawal (*i.e.* CPA) in animals receiving the drug repeatedly[5,6]. Furthermore, it has been evidenced that the morphine-induced CPP develops by dopaminergic transmission from ventral tegmentum to the limbic areas including hippocampus and amygdale[1,2]. In the present study, usage of morphine (0.5-7.5 mg/kg, s.c.) in the CPP, induced a significant and dose dependent place preference in male Wistar rats. This effect may show the role of mu-type of opioid receptors, which mediates the reward induced by morphine in the rat[14]. We can also attribute the reinforcement to the increase in level of dopamine in reward pathway. Looking at the previous works, due to the activation of mu-type opioid receptors, the GABAergic neurons inhibit and dopamine level goes up[15].

c-Fos expression in conditioned rat to morphine receiving intra-CeA injection of single NLX

Fig. 2 shows the effect of injection of NLX alone on c-Fos expression in the conditioned rat to morphine (5 mg/kg, s.c.). Administration of NLX (0.4 µg/rat, intra-CeA), showed a significant reduction effect (Fig. 2B) in a comparison to morphine 5 mg/kg, s.c. (Fig. 2A). In view of the data, the NLX (0.4 µg/rat, intra-CeA) was used for later injection of the NLX in combination with Ag-NPs. The fact that prior administration of NLX (0.4 µg/rat, intra-CeA), as a competitive mu-opioid antagonist, caused a significant CPA is primarily reflecting an opioid system involvement. The studies accordingly have shown an opposed response to morphine due to the blockade of mu-receptors by the NLX[5,6]. There is recent evidence that involves the CeA in the CPA-induced of NLX in morphine-received rats[16]. The CeA is the main efferent pathway of the amygdala complex and here we experienced that morphine-induced CPP can cause high appearance of c-Fos in the nucleus (75%) as well as CA1 (65%). Our results

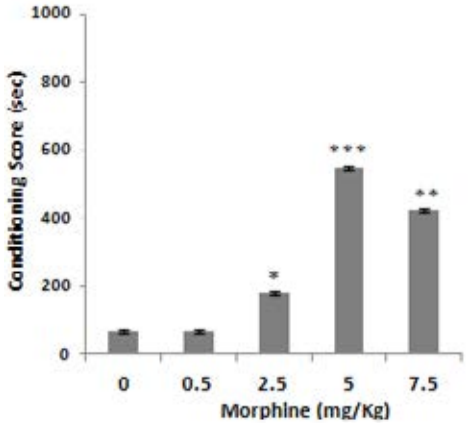

Fig. 1. Dose response of morphine in induction of CPP in opioid-naive male rats. Morphine (0.5-7.5 mg/kg) or saline (1 mL/kg) was given s.c. in a 3-day schedule of an unbiased conditioning paradigm. The control group (legend 0) received saline (1 mL/kg, s.c.) twice daily for 3 days. The data are expressed as mean of change in place preference ± SEM. Change in place preference is defined as the time spent in the drug-paired (defined) place on day of testing minus that spent in the same place pre-conditioning. Tukey-Kramer *post hoc* analysis showed the differences to the control legend 0 (*p <0.05, **p <0.01 and ***p <0.001).

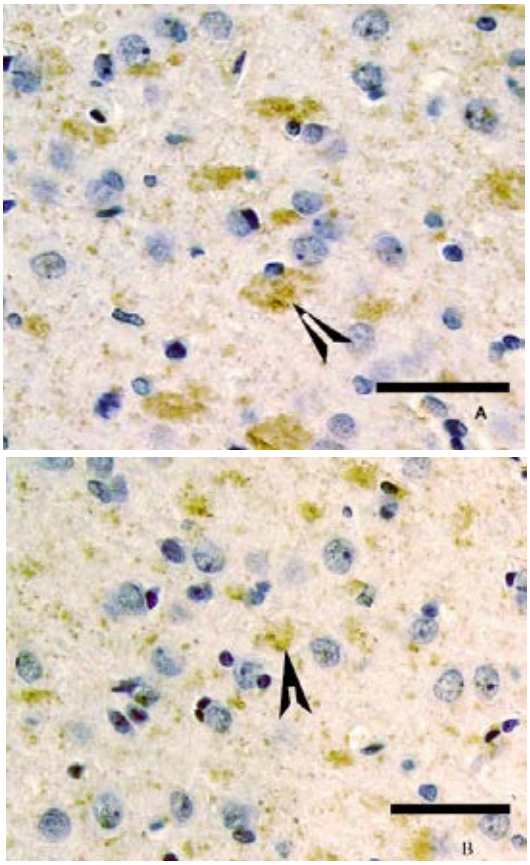

Fig. 2. Naloxone (NLX) effect on c-Fos expression in the rat conditioned to morphine. NLX (0.4 µg/rat) was given intra- CeA prior to testing of morphine (5 mg/kg, s.c.) place conditioning. 10 min later, the rats were tested in a morphine-free state. Control group solely received saline (1 µL/rat, intra-CeA) instead of NLX in the day of test. Image analysis showed a difference to control legend "A". (A) Morphine conditioned group, (B) NLX treated group. Line is 100 µ.

may indicate that the level of c-Fos protein in the conditioned group is the result of the morphine treatment. By referring to the other finding, many abused drugs can activate receptors in specific brain regions that contribute to drug abuse[2]. It also happens similarly in the expression of intermediate early genes like c-Fos[17]. Changes in early gene expression like c-Fos may involve in central nervous system (CNS) alterations which may lead to addiction to drug[17]. In this research, the CeA, following morphine withdrawal induced by acute administration of NLX, showed less activation for c-Fos in the CeA (53%) and the CA1 (43%) as compared with the morphine-conditioned rats, and it accords with the previously described work[18]. As an outcome, part of the result is due to activation of opioid system in the CeA. On the other hand, we should consider that the opiate withdrawal is problematic; even daily stress reduces c-Fos in the CeA. These effects may be related to the adaptive changes within the opioid system[19]. It is also possible that changes to the opioid system will have no effect on this factor[20]. So a decrease in c-Fos in the CeA in the NLX treated rats may not be due to the effects of NLX on opioid receptors. We must also consider the number of these receptors in the CeA which is not notable compared with many other brain areas[2]. It is better to say that the amygdala is more involved in the emotional aspects of opioid withdrawal especially in associative-learning processes[21]. Our findings may further show that repeated injection of morphine likely modulates the neurotransmission in the CeA in the/or despite the presence of NLX. The present data for the first time demonstrate that the NLX-induced morphine withdrawal in morphine conditioned rat is able to modify the c-Fos expression within the CeA as well as CA1.

c-*Fos expression in conditioned rat to morphine receiving intra-CeA injection of Ag-NPs prior to NLX*

Fig. 3 shows the effect of pre-injection of Ag-NPs (0.01 µg/rat, intra-CeA) before injection of NLX (0.4 µg/rat, intra-CeA) in morphine conditioned rats (Fig. 3B). Although, injection of Ag-NPs (0.01 µg/rat, intra-CeA) pre-testing alone (Fig. 3A) showed no significant effect, but when it was injected earlier (10 min) than NLX (0.4 µg/rat), pre-testing, resulted in a significant increase in c-Fos expression. Indeed, the Ag-NPs (0.01 µg/rat) reversed the response to NLX. In addition, the percentages of protein expression between all groups were statistically significant compared with the control ($p < 0.05$) (Fig. 4). As an interpretation, perhaps this result is due to the distribution of opioid receptors in the CeA and CA1 of rats and that in the use of NLX, the connection between them and morphine stopped. Undoubtedly, this has undesirable consequences. In order to prevent this effect, we used low non-toxic doses of Ag-NPs, intra-CeA, before injection of NLX, pre-testing. The Ag-NPs alone did not induce tissue damage because toxic effect of the nanoparticles is mainly influenced by the size of the particles[22]. Massive evidence have shown that high doses of Ag-NPs are harmful for liver and other organs especially with reactive oxygen

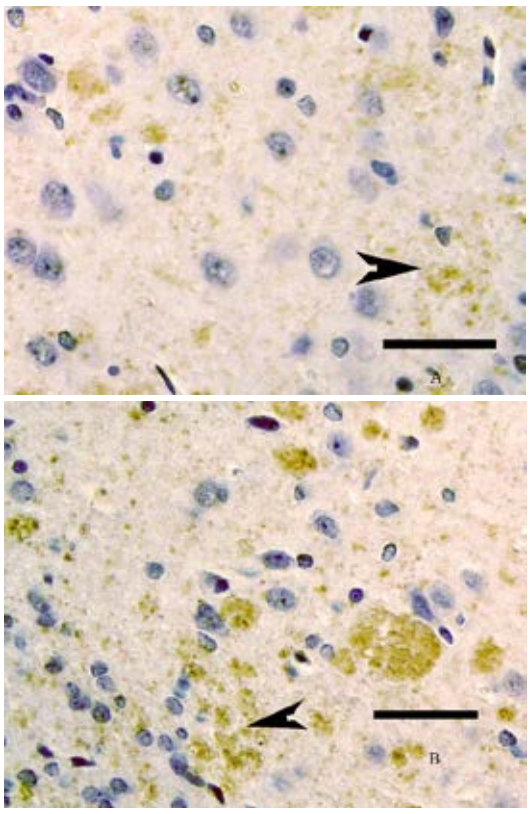

Fig. 3. Effect of ccumulative NLX/Ag-NPs on c-Fos expression in rat conditioned to morphine. The Ag-NPs (0.01 µg/rat) were given prior to the NLX (0.4 µg/rat, intra-CeA) prior to test of morphine (5 mg/kg, s.c.) place conditioning. 10 min after the injection of Ag-NPs, the rats were given the NLX and 10 min later, they were tested in a morphine-free state. Control group solely received saline (1 µL/rat, intra-CeA) instead of Ag-NPs. The image analysis showed a difference between combined injections NLX/Ag-NPs (B) to the single particles "A". Line is 100 µ.

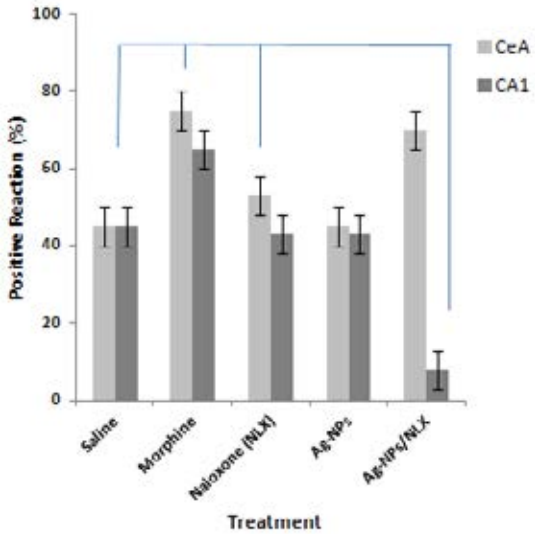

Fig. 4. The percent of c-Fos Protein expression between the control rats to other treatment groups is shown as mean ± SEM. The *post hoc* analysis showed the differences to the control (*p <0.05, **p <0.01 and ***p <0.001).

substrates (ROS) and apoptosis[23]. But the effect of their low concentration on living organisms is really unknown. Therefore, in this project we studied effects of low doses Ag-NPs at the cellular levels on NLX-induced morphine withdrawal. No change in the amygdala was observed after the use of these 62 nm particles. Behavioral analysis of the present study demonstrates that the injection of Ag-NPs (0.01 µg/rat, intra-CeA) with morphine (5 mg/kg, s.c.) had no significant effect on spatial preference in rats, but, it induced c-Fos expression in the CeA (45%) and CA1 (43%) compared to the saline control. There is interaction between Ag-NPs and biomolecules[24], so these particles may cause changes in dopamine levels due to the elimination of GABAergic transport in the CeA. As our findngs, administration of Ag-NPs (0.01 µg/rat, intra-CeA) prior to the NLX (0.4 µg/rat, intra-CeA) in morphine conditioned rats significantly decreased NLX-induced place aversion and facilitated place preference in rats along with the high c-Fos expression within the CeA (70%) not in CA1 (8%). This finding may indicate that the presence of Ag-NPs in CeA may inhibit binding of NLX with the opioid receptors, which belong to G-protein coupled family. Previous studies have indicated that the Ag-NPs interact with G protein receptors in cell membrane and this feature makes these particles unique[25]. This event may change morphine dependence symptoms in the NLX-induced morphine withdrawal. Here (in accumulative injection of NLX/Ag-NPs), we paid attention to c-Fos, and it is probably quite effective in this response. A previous study similarly revealed an increase in c-Fos level on exposure to non-cytotoxic Ag-NPs, which strongly suggests that c-Fos is involved in the motivation signs of morphine reward. The change of mu-receptor activation in the presence of Ag-NPs yet may cause change in c-Fos expression level.

CONCLUSION

Our data may demonstrate that the Ag-NPs express c-Fos mRNA within the CeA as well as CA1 that can relief the aversive effect of NLX in the morphine conditioned animal.

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CONFLICT OF INTEREST

The authors report no conflicts of interest in this work.

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