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ORIGINAL ARTICLE

Silver nanoparticles (Ag-NPs) in the central amygdala protect the rat conditioned by morphine from withdrawal attack due to naloxone via high-level nitric oxide

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Abstract

Repeated injection of morphine during conditioned place preference (CPP) leads to spatial craving due to high-level nitric oxide (NO) in the central nucleus of amygdala (CeA). Silver nanoparticles (Ag-NPs) can produce oxygen-free radicals that lead to NO formation. We aimed to show the Ag-NPs protective effect on naloxone (NLX)-induced morphine withdrawal in the conditioned rats. Wistar rats (300–350 g) were implanted with cannulae in the CeA. After recovery, they were randomly divided into experimental and saline groups. CPP was conducted by three-phase unbiased program. Morphine (0.5–7.5 mg/kg) was injected subcutaneously (s.c.) once/per day during the conditioning phase. Naloxone (NLX) (0.05–0.4 μg/rat) was given, intra-CeA, 10 min before the CPP test. Ag-NPs (0.0001–0.01 μg/rat) were administered alone or prior to the NLX effective dose (0.4 μg/rat), intra-CeA. Conditioning score and withdrawal signs (wet dog shaking and scratching) were obtained and compared with saline group data. All rats' brains were collected in formalin 10% and after 48–72 h stained with NADPH-diaphorase, the NO marker. All data were analyzed by one-way or two-way ANOVA. Morphine (2.5–7.5 mg/kg, s.c.) induced a significant CPP *vs.* saline (1 mL/kg, s.c.). The single Ag-NPs had no significant effect, whereas the NLX caused meaningful WDS and scratching. However, the NLX pre-treatment in combination with Ag-NPs eliminated these signs. Furthermore, the NO level increased in the CeA. The Ag-NPs may protect the morphine-conditioned rats against the NLX-induced withdrawal symptoms due to high-level NO in the CeA.

Keywords Morphine · Naloxone · Silver nanoparticles · Withdrawal · CeA · NO

Introduction

Opioid drugs have been used as analgesic substances from the past (Emmett-Oglesby et al. 1990). Substances abuse lead to addiction (Niu et al. 2009), and this limits their use in the clinic. Addicts, after years of abstinence, may relapse to seeking the drugs, the behavior which is frequently caused by memory recall of drug rewarding (Taubenfeld et al. 2010). The opioids, such as morphine, are characterized by their ability to bind to opioid receptors (Zhang et al. 2008) throughout the brain (Mansour et al. 1987). They have an adverse effect on the hippocampal formation (Famitafreshi et al. 2015) that is implicated in memory foundation and spatial navigation (Strange et al. 2014). The clinic uses naloxone (NLX) for the treatment of opioid overdosing (Wermeling 2015). The NLX, as a selective fast-acting antagonist of mu-opioid receptors, can also introduce an addicted person with the help of the withdrawal signs (Stinus et al. 2000), which are known as unpleasant symptoms (Koob and Volkow 2010). The antagonist causes dysphoria as well as tension/anxiety in humans (Del Campo et al. 1994). In rodents, it antagonizes the morphine-induced conditioned place preference (CPP) and displays the conditioned place aversion (CPA) (Tzschentke 2007; Morgan and Christie 2011). Evidence has shown that the amygdala plays an essential role in the CPA performance (Lu et al. 2000). The amygdala is known as a major brain structure that functions in emotional learning (Koob 2009; Everitt et al. 1999). Studies by c-Fos mapping have also

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involved that area in the opioid withdrawal (Hamlin et al. 2004). We have demonstrated the role of this region in the NLX-induced avoidance events in the morphine-conditioned rats. We have also shown that by using the nitric oxide (NO) agents within the central nucleus of the amygdala (CeA), the effect would be stopped (Rahimpour et al. 2011). A calcium-/calmodulin-dependent enzyme in the presence of a cofactor, nicotinamide adenine dinucleotide phosphate (NADPH), catalyzes the production of NO during the conversion of two amino acids L-arginine to L-citrulline (Knowles et al. 1989). The CeA and hippocampal CA1 are rich in NADPH that been introduced as a NO marker by Garthwaite and Boulton (1995). We have already shown that NO levels in these areas of the brain are affected by morphine (Karimi et al. 2011). Today, nanoparticles (NPs) are thought to be effective in producing reactive oxygen species (ROS) in some of these brain regions (such as the hippocampus), which contribute to the production of NO (Liu et al. 2012). Today, the nanotechnology industry is also overgrowing and can create novel nanoscale products (1–100 nm) with significant and exciting physical and chemical properties (Nel et al. 2006). Silver has remarkably strong antimicrobial and antifungal properties, and this is the main reason for silver nanoparticles (Ag-NPs) extensive use (Vance et al. 2015; Salata 2004). The Ag-NPs may cause oxidative stress (Johnston et al. 2010) and can spread throughout the brain (van der Zande et al. 2012). While most studies have generally pointed out the toxic effects of high doses of Ag-NPs, but the fact is that these particles are not harmful at low concentrations. It should not be ignored that these particles can affect biological systems due to their presence in health products. Thus, we aimed to assess the effect of short-term administration of nontoxic and low concentrations of these particles on the elimination of NLX-induced morphine withdrawal in the rat. Also, we examined the expression of NO in the CeA as well as hippocampal CA1.

Materials and methods

Animals

Adult male Wistar rats weighing 300–350 g were obtained from Pasteur Institution, Tehran, Iran, and were housed in standard PVC cages with free access to food and water ad libitum. The animal house temperature was maintained at 23 ± 2.0 °C with a 12:12 h light/dark cycle. Each experiment was conducted in 6–8 rats, and each animal was experienced just for once. All rats passed 1 week adaptation before surgery. Behavioral tests were carried out during the light phase of the daily cycle. The guidelines were under the protocol of the Ministry of Health (2019) and approved by the local committee. The rats were randomly categorized into control (saline 0.9%) and dose groups: morphine (0.5, 2.5, 5, 7.5 mg/kg), and NLX (0.05, 0.1, 0.2, 0.4 μg/rat), and Ag-NPs (0.0001, 0.001, 0.01 μg/rat). The dose selection of materials is based on previous studies (Siahposht-Khachaki et al. 2016; Ghazvini et al. 2015). After analyzing the dose-response curves and identifying the most effective doses, the animals received saline (1 mL/kg, s.c.) or morphine (5 mg/kg, s.c.) once daily for three consecutive days (during the conditioning phase). Single NLX was (intra-CeA) injected 10 min before the morphine-induced CPP test. Single Ag-NPs were also administered intra-CeA pretesting. The NPs were furthermore given before the NLX most effective dose (intra-CeA). During these procedures, the control group solely received saline (1 μL/rat, intra-CeA) instead of materials. The CPP apparatus was made and designed based on previously published research (Karami et al. 2002). All behavioral signs were checked by the EthoVision system.

Drugs

Morphine sulfate was purchased from TEMAD Co., and naloxone HCl from Tolid-Daru Co., Tehran, Iran. Ag-NPs were provided by Dr. Hajnorouzi at the department of Physics of Shahed University and presented to us. The particles were produced by the aid of the sonoelectrochemical technique, a method in which both electrolysis and ultrasonic irradiation is used (to see details refer to Tang et al. 2009). They were 60–70 nm in size; 95% of the particles were about 62 nm, and the remaining 5% were slightly larger or smaller. The hydrodynamic diameter and zeta potential of them were characterized by dynamic light scattering (DLS) using a Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd., Worcestershire, UK). Besides describing the stock suspension (10 ppm or 0.01 mg/1 mL), it was also diluted 1:10, 1:100 in distilled water. The suspensions, before use, were ultrasonicated in a water bath for 10 min to prevent adhesion (agglomeration). The placebo was saline (0.9%), which was administered 1 mL/kg, s.c., and1 μL/rat, intra-CeA.

Behavioral signs

After the treatment of rats, they were placed in the CPP apparatus. In the test day, their behaviors (wet dog shaking and scratching) and rate of stopping at each side of the CPP device were recorded by the EthoVision system (equipped with a video camera located 12 cm above the device) for 10 min. All records were then reviewed by a double-blind observer and expressed as mean ± SEM.

Stereotaxic surgery

The animals were anesthetized with ketamine–xylazine and placed in a stereotaxic apparatus with the incisor bar which was set at approximately − 3.3 mm below horizontal zero to achieve the flat skull position. They were then cannulated (23-

Gauge) bilaterally in the central amygdala (AP = −2.12 mm, L = ±4.1 mm; V = 7.8 mm, according to the atlas of Paxinos and Watson (2003). The incision was eventually closed with dental cement. An injection needle (30-Gauge) attached to a 5-μL Hamilton syringe through a polyethylene tubing (internal diameter = 0.3 mm) was inserted into the guide and allowed to inject the materials inside the nucleus. The injection needle was further projected (1 mm) ventral to the tip of the guide one. The duration of intra-CeA infusion was 1 min. It should be noted that the animals needed a week of recovery after surgery, and during this time, they were handled every other day to reduce stress at the time of testing.

CPP apparatus and paradigm

A two-compartment CPP apparatus (30 × 60 × 30 cm) was used in this task. Place conditioning was conducted using previously described design (Karami et al. 2002). The wooden apparatus with two equal-sized compartments was split by a removable wall that was inserted in the middle of the equipment. The chambers, though, were similarly colored white, but they were differently striped black on their walls (vertical vs. horizontal). The parts were also distinguished by texture and olfactory cues. Rats displayed no consistent preference to one side of this device, confirming the unbiased design.

The CPP paradigm

In the first phase (familiarization), the animals were placed in the middle line of the CPP box and they had free access to both compartments for 10 min (to reduce stress and novelty), while the removable wall was raised 12 cm above the floor. During the next conditioning phase, the animals received 1-morphine and 1-saline-paired sessions with a 6-h interval each taken 30 min. During these sessions, the removable wall was closed, and the rats were placed on the side of the saline or drug. In the last (testing) phase, each animal at first received the desired substance (singly or cumulatively), intra-CeA, it was then placed in the CPP box, and surveyed in a morphine-free state (Olmstead and Franklin 1997) for 10 min, while the removable wall was again lifted 12 cm.

Intra-CeA pre-testing injection

In the last phase, the animals were restrained by hand and received the substances alive. Based on previous work (Rahimpour et al. 2011), 1 μL of the volume concentration of the material was injected into the CeA using the injection set up as mentioned above. The duration of the infusion always was 30 s, and the needle was remained in place for an additional 30 s to facilitate the diffusion of the substance (total time = 1 min).

Histology

The rats were sacrificed 2 h after completion of the test, and their brains were fixed in 10% formaldehyde for 48–72 h.

Experimental procedure

Induction of morphine-induced CPP

Different doses of morphine (0.5, 2.5, 5, 7.5 mg/kg) or saline (1 mL/kg) were subcutaneously (s.c.) injected in the rats (n = 6–8/ per dose) by the 3-day schedule of conditioning task.

Effect of pre-testing injection of single naloxone (NLX), intra-CeA, on the morphine CPP and induction of behavioral withdrawal signs

On day 5, the NLX (0.05–0.4 μg/rat) was administered, intra-CeA, 10 min before the test. The animals were then tested in the CPP box timing 10 min. Control group received saline (1 μL/rat, intra-CeA) instead of NLX.

Effect of pre-testing intra-CeA injection of Ag-NPs; either alone or before the administration of NLX (cumulative NLX/ag-NPs)

To assess the effects of single Ag-NPs, intra-CeA, on the CPP induction by morphine, the Ag-NPs (0.0001, 0.001, 0.01 μg/rat), was pretesting injected (control group received saline, 1 μL/rat, intra-CeA). The rats then were tested in the box timing 10 min. For cumulatively injection of NLX/ Ag-NPs, first, the particles were given to the rats, intra-CeA, and 10 min later, they were administered the NLX, intra-CeA. Eventually, they were tested for 10 min. The control group received saline (1 μL/rat, intra-CeA) instead of the materials,

Histological verification

The animals were killed with the overdose of ketamine-xylazine 2 h past of the behavioral testing. To verify, an ink (0.5 μL of 1% aquatic methylene blue solution) was injected into the per side guide cannula, using a 30-gauge injection cannula that projected 1 mm ventral to the tip of the guide. The animals then were sacrificed, and their brains were removed and fixed in 10% formalin solution for 48–72 h. Brain slices were taken through the brain areas of cannula placements. Data from brains with injection sites located outside the CeA were excluded from the statistical analyses. The coronal sections (4–5 μm) were finally cut and after 24 h (keep in room temperature) stained either with Cresyl violet or silver nitrate. We also examined the NOS positive activation reaction using the NADPH-diaphorase (NADPH-d) histochemistry. The sections were finally observed under the

optical photomicroscope (Olympus) equipped with the Image tool program (UTHSCSA Image Tool, version 2.03, USA) to assess the NO expression in the target areas.

Cresyl violet staining

Deparaffinized, rehydrated sections were placed in jars full of Cresyl violet (0.1%) during 15–20 min. The sections following ordinary washing and dehydrating protocols were mounted by Entellan (Merck, Germany), and lastly coverslipped.

Silver nitrate staining

Autometallography (AMG) For localization of exogenously administered Ag-NPs, according to previously published work (Miller et al. 2016), the deparaffinized, rehydrated sections were placed in the clean Coplin jars, and the AMG developers were added. The jars containing the slides and developers were wrapped with foil and put into a gently shaking 26 °C water bath. The entire water bath was covered with foil to decrease light penetration. After that (45 to 90 min), the developing solution was replaced with a 5% sodium thiosulfate solution for 10 min, and washed. Test slides were sequentially removed from the solution to determine the optimal incubation time for a particular treatment regimen and tissue. The slides were counterstained by hematoxylin and after dehydration, coverslipped. For dry silver staining (Klein 1958), the deparaffinized, rehydrated sections were instead placed into 37 °C water bath, and silver nitrate 0.1% was added. The entire water bath was similarly covered with foil to decrease light penetration. After the incubation (60 to 90 min), the slides were washed. They were then exposed to heavy sunlight for 15–20 min, and washed by tap water, and coverslipped.

NADPH histochemistry

All animals' brains, in the end, were examined for NOS positive activation reaction. They were fixed in 4% buffered formaldehyde, and after 24 h, they were cut into small pieces, which included the target areas. The brain samples were then paraffinated, and they were cut serially by the rotary microtome; the slices were collected in the buffer. The slices were mounted on albumin greased slides and retained at room temperature for about 24 h. The prepared thin parts were studied at first by a light microscope, and the routine histology protocol was then performed (dehydration). The sections after rinsing in buffer were colored by the NADPH-d technique to demonstrate the neuronal NOS possessing NADPH-d activity. These slices were placed for a while (1–2 min) in diluents of 0.3% Triton-X 100 in phosphate buffer and shacked. The slices were then incubated in a solution containing equal proportions of nitro-blue tetrazolium (NBT) (0.4 mg/mL in buffer) and NADPH (1 mg/mL in buffer) for 16–20 h at 37 °C. NBT is a salt that yields an insoluble blue formazan that is visible by a light microscope. These slides without agitation were kept in a wet chamber. This protocol as used as for the control and the experimental sections except that the NADPH was excluded in the control (Bredt et al. 1990). Nothing was observed in the control sections.

Statistical analysis

Change in place preference (sec) and behavior between the first and the last phases was comparatively calculated for all animals by one-way or two-way ANOVA followed by Tukey-Kramer *post hoc* test. *P*-values less than 0.05 ($p < 0.05$) were considered to be statistically significant. The data were expressed as mean ± SEM. To quantify, the histological features of the brains' slices at the areas of 100 μm^2 were analyzed by the Image Tool program.

Results

Verification of microinjection site in the central amygdala (CeA)

Data reveal the injection site (the CeA) using intra-CeA injection of 1 μL (0.5 μl/per side) of the methylene blue solution provided by infusion set up that was as similar as the set used for the material injection, intra-CeA (Fig. 1).

Induction of morphine place conditioning

Figure 2 shows the morphine dose response in the rat. Administration of morphine (0.5–7.5 mg/kg, s.c.) resulted a significant response [$F(4, 25) = 3.302, p < 0.05$]. The Tukey's *post hoc* analysis showed the between-group differences. Based on the results, morphine's most effective dose (5 mg/kg, s.c.) was used for subsequent studies.

Effect of morphine on behavioral signs

Figure 3 shows the impact of different doses of morphine (0.5–7.5 mg/kg, s.c.) on behavioral indications in Wistar rats (wet dog shaking or scratching). Administration of morphine had no significant effect ($p > 0.05$) on the signs in comparison with the control.

Effect of naloxone (NLX) on the expression of morphine response in the rat place conditioning procedure

Figure 4 shows the effect of single NLX injection before morphine response testing. Pre-testing administration of NLX

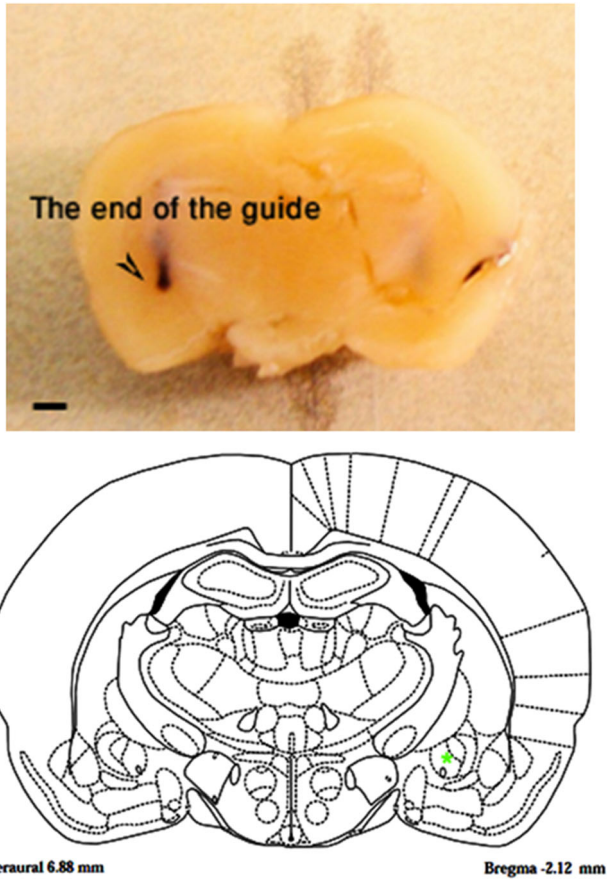

Fig. 1 (Above) Cannula placement in CeA; evidence by the ink injection (intra-CeA, 1 μL/rat or 0.5 μL/per side) with the use of the injection set up as same as that used for the infusion of the substance, intra-CeA (AP: − 2.12). (Down) Verification of injection site at the stereotaxic coordinates under the rat's brain atlas by Paxinos and Watson (2003). Line is 100 μ

(0.05–0.4 μg/rat, intra-CeA) in morphine (5 mg/kg, s.c.) conditioned rats resulted a significant effect compared to the

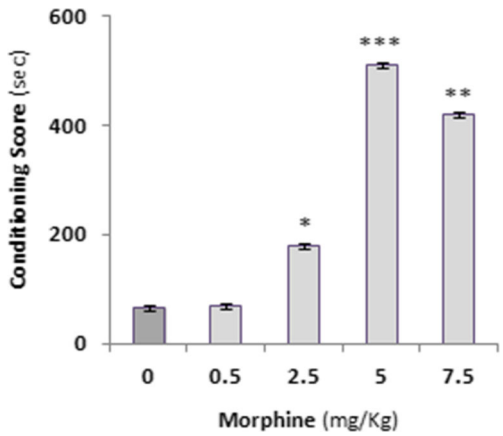

Fig. 2 Morphine dose-response: the rats were administered morphine (0.5–7.5 mg/kg, s.c.) by a 3-day conditioning schedule. The saline group (legend 0) and morphine doses groups' data are expressed as Mean ± SEM. Based on the Tukey's *post hoc* results, the most effective dose (5 mg/kg, s.c.) of morphine is very significantly different from the 0 (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$)

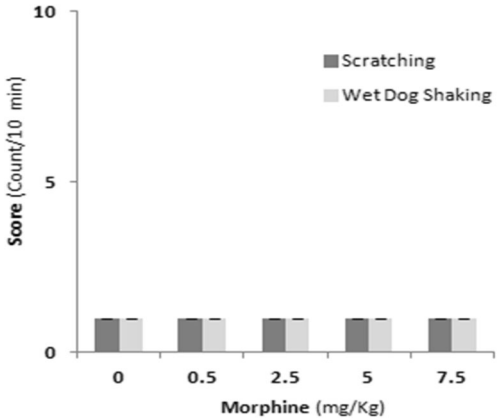

Fig. 3 This Figure shows morphine-induced behavioral signs in opioid-naive male Wistar rats. Morphine (0.5–7.5 mg/kg, s.c.) or saline (1 mL/kg, s.c.) was given during the 3-day schedule of the unbiased conditioning paradigm. Data are expressed as a count of behavioral signs/10 min ± SEM. Tukey's *post hoc* analysis showed no difference between morphine doses and saline groups

control group receiving saline 1 μL/rat instead of the NLX [F (4, 25) = 3.107, $p < 0.05$]. In view of the Tukey's *post hoc* results, the NLX's most effective dose (0.4 μg/rat, intra-CeA) was used for subsequent studies.

Effect of naloxone (NLX) on the expression of behavioral signs in conditioned model

Figure 5 shows the effect of injection of NLX (0.05–0.4 μg/rat, intra-CeA), once, before morphine response testing, on scratching and wet dog shaking (WDS). Pre-testing administration of the NLX in morphine (5 mg/kg, s.c.) conditioned animals resulted in a significant effect. As analysis shows, the antagonist NLX (0.05–0.4 μg/rat, intra-CeA) significantly induced withdrawal scratching [F (4, 25) = 3.321, $p < 0.05$] as well as WDS [F (4, 25) = 6.320, $p < 0.0001$]. By the Tukey's

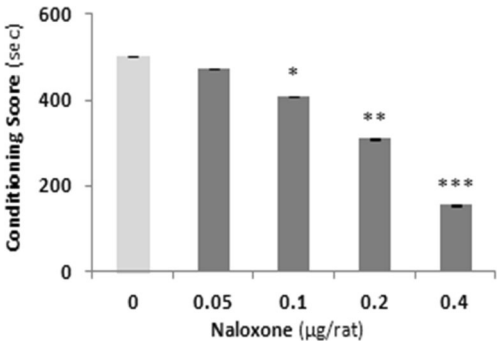

Fig. 4 This Figure shows the NLX response in morphine-conditioned male Wistar rats. Morphine (5 mg/kg, s.c.) was given in a 3-day schedule of the unbiased conditioning paradigm. The NLX prior to the testing (10 min) was injected, intra-CeA, for once. Data are expressed as conditioning score/10 min ± SEM. Tukey's *post hoc* analysis showed a difference between the NLX and saline data group receiving 1 μL/rat, intra-CeA, instead of NLX (legend 0). The *post hoc* analysis indicated the between-groups' differences from the control (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$)

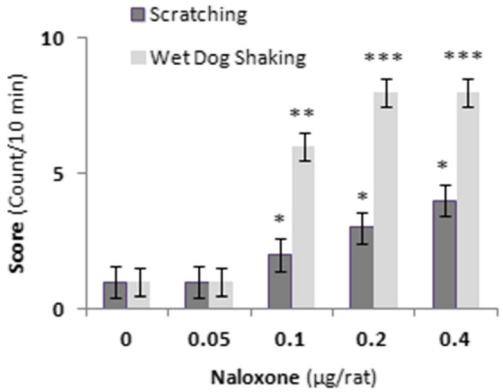

Fig. 5. NLX (0.05-0.4 μg/rat, intra-CeA) was given 10 min before the morphine 5 mg/kg response testing. Data are expressed as the number of behavioral signs/10 min ± SEM. Tukey's *post hoc* analysis showed the differences: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ *vs.* control that passed the morphine conditioning, but, pre-testing, it received saline (1 μL/rat), instead of the antagonist

post hoc test, the differences between the NLX doses and the control group (1 μL/rat, intra-CeA, instead of NLX) were achieved.

Effects of intra-CeA injection of single Ag-NPs or cumulative Ag-NPs/ naloxone (NLX) on the response of the morphine administered animals

Pre-testing injection of single doses of Ag-NPs (0.0001–0.01 μg/rat, intra-CeA) resulted in an insignificant effect in the morphine (5 mg/kg, s.c.)-treated animals. However, the bilateral injection of Ag-NPs (0.0001–0.01 μg/rat, intra-CeA) before (10 min) injection of the effective NLX (0.4 μg/rat, intra-CeA) prior to the morphine 5 mg/kg (s.c.) response testing resulted in a significant effect based on the ANOVA [$F(3, 15) = 3.825$, $p < 0.01$] (Fig. 6). The two-way ANOVA revealed the interaction between the Ag-NPs and the NLX. Further analysis indicated that the Ag-NPs might reverse the response to the NLX dose. Based on the Tukey's *post hoc* test, a dose of Ag-NPs (0.01 μg/rat) was surprisingly high effective when paired with the NLX effective dose (0.4 μg/rat).

Effect of single Ag-NPs or cumulative Ag-NPs/ NLX injection in the CeA on the expression of behavioral signs in conditioned model

Only the injection of Ag-NPs (0.0001–0.01 μg/rat, intra-CeA) prior to the NLX (0.4 μg/rat, intra-CeA) before testing of morphine 5 mg/kg (s.c.) response leads to significant behavioral effect [$F(3, 15) = 2.685$, $p < 0.05$]. So, the injection of single Ag-NPs did not affect the behavioral signs (Fig. 7).

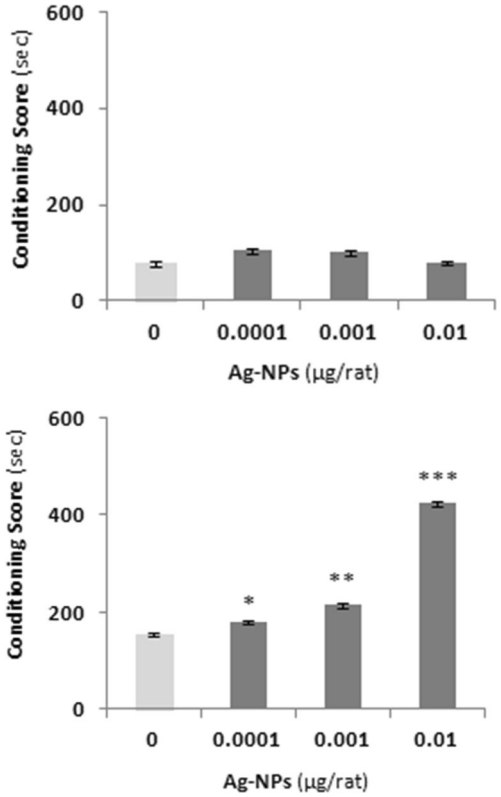

Fig. 6 Responses induced by single Ag-NPs (0.0001–0.01 μg/rat, intra-CeA) or cumulative Ag-NPs/ NLX, pre-testing of morphine response. Ten minutes later, the rats were tested in a morphine-free state. Data are expressed as mean ± SEM. Although, the Tukey's *post hoc* analysis revealed no difference between the single Ag-NPs treatments and saline legend 0; however, it illustrated a significant difference between the cumulative group *vs.* control (receiving saline 1 μL/rat, intra-CeA, instead of the Ag-NPs)

Effects of intra-CeA injection of Ag-NPs on NOS activation in naloxone (NLX) induced withdrawal symptoms

Complementary data taken by the light microscope revealed the NADPH-d difference ($t = 4.529$, sig (2-tailed) = 0.001) between the CeA (but not the CA1) of the Ag-NPs administered samples and saline (Fig. 8).

Discussion

The purpose of this research was to study the effect of silver nanoparticles (Ag-NPs) on the naloxone (NLX) induced withdrawal signs in the morphine-conditioned rats. Based on the results, the Ag-NPs, when injected in the rat CeA, before the NLX, pretesting of morphine-induced CPP, had a protective effect against the withdrawal signs induced by naloxone. They furthermore caused the positive NADPH-d NOS activation reaction in the target area.

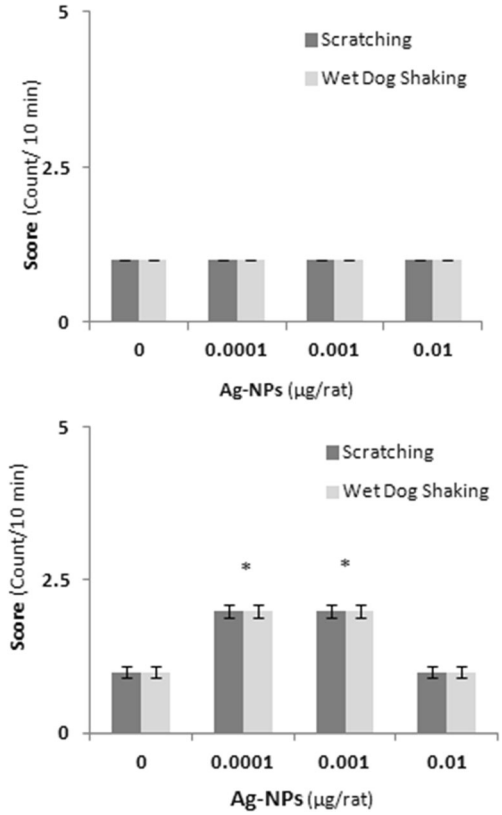

Fig. 7 Response induced by pretesting administration of single Ag-NPs (0.0001–0.01 µg/rat, intra-CeA) or cumulative Ag-NPs (0.0001–0.01 µg/rat, intra-CeA)/ NLX (0.4 µg/rat, intra-CeA) in the morphine conditioned rats. Ten minutes later, the responses were measured. The control group (legend 0) either in the single or cumulative group passed the procedure but, it solely received saline (1 µL/rat, intra-CeA). Data are expressed as the count of behavioral signs/10 min ± SEM. $*p < 0.05$ shows a difference from the control

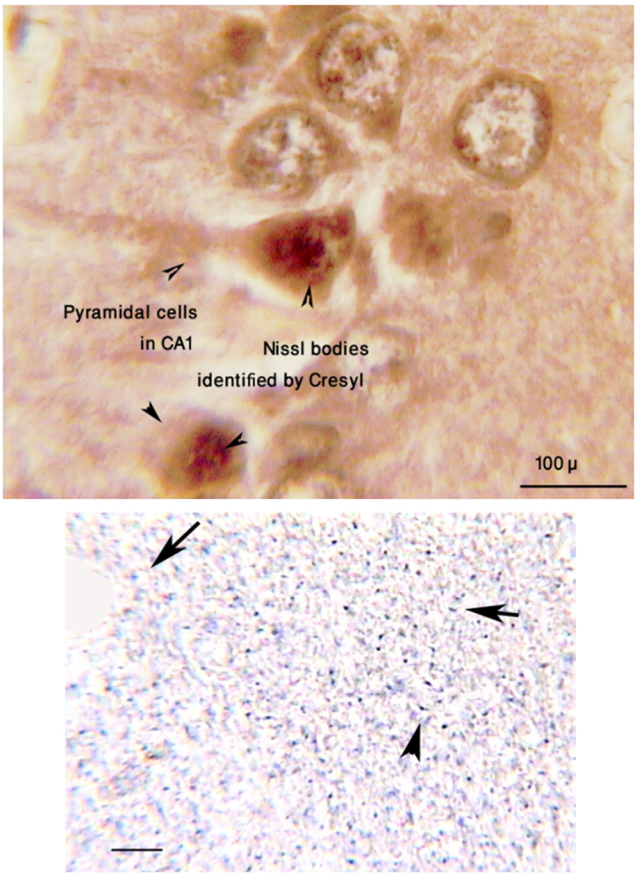

Fig. 8 The place neurons of the CA1 identified by the NADPH-diaphorase did not show the NOS activation response (Above). The CeA of the Ag-NPs group, however, illustrated the positive NADPH-d reaction (Down). The line is 100 µ

We know that the development of morphine-induced conditioned place preference (CPP) requires dopaminergic transmission from the ventral tegmental area (VTA) to the hippocampus (Esmaeili et al. 2012). It has already been indicated that the amygdala is implicated in morphine induced-dependence (Koob et al. 1992) because of projections toward the nucleus accumbens (NAc). Furthermore, the glutamatergic projections of VTA into the amygdala are graded as the main component in the expression of morphine dependence (Tzschentke and Schmidt 2003). According to this study, injections of morphine (0.5 2.5, 5, 7.5 mg/kg, s.c.) dose-dependently induced a significant CPP in male Wistar rats. This effect, in one hand, may display the role of the mu- and delta-opioid receptors, which mediate morphine-induced reinforcing in the rat (Zhu and Pan 2005). In the other hand, it may be related to several neuronal systems, including tegmental pedunculopontine glutamate and GABA-B synapses, which mediate the systemic morphine rewarding (Heinmiller et al. 2009). It has also already been indicated that the inhibition of GABAergic neurons via activation of the mu-opioid receptor allows dopaminergic neurons to release more dopamine into the reward pathway (Johnson and North 1992). The neuromodulator dopamine (DA) has been involved in the mechanism of dependence on the drug (Kalivas and Volkow 2005). We injected the mu-opioid competitive antagonist, the NLX pretesting of morphine conditioning to induce the withdrawal behavioral signs in the morphine-conditioned rats. According to our data, the NLX (0.1–0.4 µg/rat, intra-CeA), when administered before testing of morphine 5 mg/kg (s.c.) response, induced a significant place aversion, reflecting the opioid system involvement, and accords with a previous finding (Skoubis et al. 2001). The previous studies have also shown an aversive response to the morphine due to the blockade of mu-receptors by the NLX (Bie et al. 2009). Furthermore, it has been evidenced that the CeA is involved in place aversion induced by NLX in the single-dose morphine-treated rats (Ishida et al. 2008). Other researchers by the model of NLX-induced withdrawal have similarly indicated an increase of GABA release in the CeA in the morphine-dependent rats (Bajo et al. 2014). It has already been shown that the administration of competitive morphine antagonist, the NLX, reverses the action of morphine (Piepponen et al. 1997). It has also been indicated that the antagonist acute

application can prevent the effects of acute morphine agonists on GABAergic transmission (Bajo et al. 2014). Thus, our findings may further suggest inhibition of the CeA opioid system by the NLX since its pretesting injection (0.1–0.4 μg/rat, intra-CeA) completely increased the wet dog shaking (WDS) and scratching in the morphine-conditioned model. This study aimed to involve the NLX in the rat CeA in the expression of behavioral withdrawal signs of morphine conditioning. To discuss mechanism, based on our achievements, morphine may cause the mu- or delta- or kappa-opioid receptor excitation coupled with the G protein complex as been already proposed (Snyder and Pasternak 2003). It has previously been demonstrated that the mu-opioid receptor activation in the VTA causes the release of DA in the NAc via excited dopaminergic neurons (Di Chiara and Imperato 1988). However, the activation of kappa-receptors has been suggested to suppress the unpleasant mu/ delta-mediated side effects (Narita et al. 2001). The delta- and kappa-opioid receptors have slightly been implicated in the expression of convulsions and WDS (Lee et al. 1989). Furthermore, the interconnections between the CeA and the NAc have formerly been indicated as the important substrates of compulsive behaviors (Marroni et al. 2007). So, the morphine response (2.5–7.5 mg/kg, s.c.) may reveal, in one hand, an interaction between the mu-opioid receptors and morphine in the target areas. On the other hand, by the other findings, the excitatory effects occurring during opioid conditioning may be mediated by a Gi/o complex (Wang and Burns 2006) that remains elusive. To detail the mechanisms, we tried to identify the effects of nontoxic Ag-NPs on the NLX function in the rats treated with morphine during the conditioning. The single injection of the particles, based on our findings, neither induced tissue damage nor caused significant change in the morphine-induced CPP. A previous study has discussed that the cytotoxicity due to nanoparticles is strongly influenced by their size, and the smaller Ag-NPs (10 nm size) are more poisonous than the larger ones (50 and 100 nm) (Feng et al. 2015). By the researcher data, the accumulation of the particles in the tissues is dose-dependent (Sardari et al. 2012). To date, a large number of in vitro studies have reported that the Ag-NPs at relatively high doses produce toxicity in the liver and other organs (Ahamed et al. 2010), while the report on the nontoxic dose of these particles is negligible. We hence aimed to evaluate the potential effects of non-cytotoxic Ag-NPs on the NLX function in a conditioned model. Though the effects of the Ag-NPs have not been extensively studied to date, however, others have given different concentrations of Ag-NPs inhalation to Sprague-Dawley rats repeatedly for 6 h/ day, for 5 days/ week, and for 4 weeks, and they did not find a significant toxicity (Ji et al. 2007). Beside, other investigators have demonstrated the positive effects of the particles on neuronal growth (Alon et al. 2013). Our experiments, in contrast, were carried out with low doses of the Ag-NPs and intra-CeA, and again no dangerous effect in the morphology of the cells of that nucleus was shown. However, as we expected, the cumulative but not the single injection of the Ag-NPs, pretesting of morphine CPP, had a dose-dependent effect both on the drug response and the behavioral withdrawal scratching and WDS signals. These data may indicate that the Ag-NPs (0.0001–0.01 μg/rat, intra-CeA) are noneffective on the morphine reinforcing in the rat. Still they protect the animal against the adverse effects of the NLX, the finding which is important in eliminating undesirable morphine withdrawal symptoms. This finding may correlate the presence of the particles in the CeA with the increase in the level of DA, likely by inhibition of GABA neurons (Skoubis et al. 2001), or with the NO production via the oxygen-free radical components. A previous work has shown that the intermediate concentrations of the Ag-NPs can enhance learning and memory formation in the aquatic organisms (Young et al. 2017). In contrast, the others have reported an obstruction effect on the rats' memory (Bagheri-abassi et al. 2015), thus, the other main point is the concentration of the particles. And about the mechanism of action, we say that the Ag-NPs as biomolecules may interact with the G-protein-coupled receptors in the cell membrane (Hild et al. 2010), which are the main binding sites for the morphine as well as the NLX. About the behavioral signs (WDS and scratching), we suggest that the kappa- and delta- than mu-opioid receptors are likely activated in the cumulative Ag-NPs/ NLX route that is unclear. For further discussion on the role of the NO system, let us say that some studies have proposed the role of several neurotransmitter systems on the expression of morphine place conditioning (Zarrindast and Rezayof 2010). We have previously shown that the NO participates in the morphine-induced CPP (Karami et al. 2014). The role of NO in the CeA on the induction of CPA by the NLX has already been proposed by this laboratory (Rahimpour et al. 2011). In the present study, we furthermore evaluated the NO involvement in the CeA in the absence or presence of the Ag-NPs. Considering the present NADPH-d data, the NO expression by the CeA neurons had a significant increase in group that received the Ag-NPs prior to the NLX, pretesting of morphine CPP. It may again show that the NO in the CeA plays a role in expression of withdrawal aspects of morphine conditioning and accords with a previous result (Werkheiser et al. 2009). We may also propose a decreased affinity of mu-receptors following exposure to the Ag-NPs. The calcium-dependent glutamate pathway is likely the other route. As we review the previous studies, we find that the calcium is involved in morphine dependence and withdrawal (Seth et al. 2011), and glutamate plays the role through the activation of the NMDA receptor subtypes (Ohno et al. 1995). These effects are also due to the NO synthase activity (Ohno et al. 1995). Since the NO is a powerful mediator of dopaminergic synapses (Wiesinger 2001), so, as the suggestions for the subsequent studies, we may look at such mechanisms that

likely interfere with Ag-NPs to protect the morphine-conditioned animal against the unpleasant withdrawal features.

Conclusion

As we know in the clinic for morphine or narcotics quit while taking the less toxic derivatives of morphine a low dose of NLX is given to prevent tolerance to the derivatives, but, this antagonist (the NLX) itself causes the symptoms which are often undesirable (see Wermeling 2015). We have found that due the use of NLX in combination with Ag-NPs, the NLX-induced adverse effects reduced, which can be advantageous in the future since the NLX-NPs compound can be introduced for the treatment.

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Authors' contribution M Karami designed research. M Rahimpour conducted experiments. A Hajnorouzi generously provided Sliver nanoparticles. M Karami and M Rhimpour analyzed data. M Rahimpour wrote the initial manuscript. M Karami carefully studied the MS and corrected the syntax errors. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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