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Morphine-induced place preference: Involvement of cholinergic receptors of the ventral tegmental area

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Abstract

In the present study, the effects of intra-ventral tegmental area injections of cholinergic agents on morphine-induced conditioned place preference were investigated by using an unbiased 3-day schedule of place conditioning design in rats. The conditioning treatments with subcutaneous injections of morphine (0.5–7.5 mg/kg) induced a significant dose-dependent conditioned place preference for the drug-associated place. Intra-ventral tegmental area injection of an anticholinesterase, physostigmine (2.5 and 5 μg/rat) or nicotinic acetylcholine receptor agonist, nicotine (0.5 and 1 μg/rat) with an ineffective dose of morphine (0.5 mg/kg) elicited a significant conditioned place preference. Furthermore, intra-ventral tegmental area administration of muscarinic acetylcholine receptor antagonist, atropine (1–4 μg/rat) or nicotinic acetylcholine receptor antagonist, mecamylamine (5 and 7.5 μg/rat) dose-dependently inhibited the morphine (5 mg/kg)-induced place preference. Atropine or mecamylamine reversed the effect of physostigmine or nicotine on morphine response respectively. The injection of physostigmine, but not atropine, nicotine or mecamylamine, into the ventral tegmental area alone produced a significant place aversion. Moreover, intra-ventral tegmental area administration of the higher doses of physostigmine or atropine, but not nicotine or mecamylamine decreased the locomotor activity. We conclude that muscarinic and nicotinic acetylcholine receptors in the ventral tegmental area may critically mediate the rewarding effects of morphine.

Keywords: Morphine; Physostigmine; Atropine; Nicotine; Mecamylamine; Conditioned place preference; Ventral tegmental area; (Rat)

1. Introduction

Considerable evidence indicates that the mesocorticolimbic dopamine system is implicated in the acute rewarding effects of opiates (Olmstead and Franklin, 1997a,b). Morphine is known to excite dopamine neurons in the ventral tegmental area through the inhibition of gamma-aminobutyric acid (GABA) ergic inhibitory interneurons and thereby increase dopamine transmission to the nucleus accumbens (Tzschentke, 1998). However, it seems that this dopamine pathway may not be the only one responsible for opioid reward. The ventral tegmental area receives cholinergic projections from the laterodorsal and pedunculopontine tegmental nuclei that directly and indirectly influence the activity of dopamine neurons (Miller and Blaha, 2005; Miller et al., 2005). In agreement with this, several studies have demonstrated that the regulation of dopamine release by acetylcholine is needed for rewarding brain stimulation (Yeomans and Baptista, 1997; Yeomans et al., 1993, 2001).

Acetylcholine has been suggested to have an important role in controlling reward related behavioral, feeding and motor performances (Grillner et al., 1999; Di Chiara, 2000). This neurotransmitter exerts its action by binding to specific membrane receptors that are divided into two major subclasses: muscarinic (M_1–M_5) and nicotinic (are formed from combinations of five subunits arising from α2–α10 and β2–β4)

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(McGehee and Role, 1995; Jones et al., 1999). In the ventral tegmental area, the majority of dopamine neurons, GABA neurons and glutamatergic terminals express nicotinic acetylcholine receptors (Klink et al., 2001; Wooltorton et al., 2003). Less attention has been paid to muscarinic acetylcholine receptors in the reward system, although their presence in the ventral tegmental area has also been demonstrated (Vilaro et al., 1990; Weiner et al., 1990). It is well known that the muscarinic (Gronier and Rasmussen, 1998; Gronier et al., 2000) and/or nicotinic (Calabresi et al., 1989; Dani et al., 2001) excitation of the ventral tegmental area dopaminergic neurons cause release of dopamine in the nucleus accumbens which play an important role in the activation of reward systems (Yeomans and Baptista, 1997; Yeomans et al., 1993). It has also been shown that the ventral tegmental area has high concentrations of the acetylcholine synthesizing enzyme, choline acetyltransferase, and its catabolic enzyme, acetylcholinesterase (Kobayashi et al., 1975; Greenfield, 1991).

Conditioned place preference has been used extensively in investigation of the neurobiological bases of the rewarding effective properties of various drugs (Hsu et al., 2002). Conditioned place reference consists of an acquisition phase during which the rats receive the drug in one distinctive environment and a test or expression phase in which drug-free animals are tested for their preference of the environment previously paired with the drug (Cervo and Samanin, 1995). Our previous studies indicate that morphine produces a conditioned preference for the place in which it has been administered in rats (Rezayof et al., 2002, 2003, 2006, 2007; Zarrindast et al., 2003b, 2005, 2006) and suggest that several neurotransmitter systems may be necessary for the acquisition of morphine-induced place preference. Considering that the release of acetylcholine within the ventral tegmental area can depolarize dopaminergic neurons through nicotinic and muscarinic receptors (Calabresi et al., 1989; Pidoplichko et al., 1997; Gronier and Rasmussen, 1998) and that there is an interaction between opioids and cholinergic system (Walker et al., 1991; Zarrindast and Jamshidzadeh, 1992), the main aim of the present study was to clarify the roles of muscarinic and nicotinic acetylcholine receptors of the ventral tegmental area on morphine-induced place preference.

2. Materials and methods

2.1. Animals

Male Wistar rats (240–300 g, Pasteur institute, Iran) were housed in groups of four at an ambient temperature of 20–22 °C. A 12-h light–dark cycle was imposed with lights on at 06:00 h. The rats had free access to food pellets and tap water. Each experimental group was consisted of 8 animals. Each animal was used once only. The experiments were performed between 8:00 a.m. and 5:00 p.m. All procedures were carried out in accordance with institutional guidelines for animal care and use.

2.2. Drugs

The drugs used in the study were morphine sulfate (Temad Co., Tehran, Iran), physostigmine, atropine, mecamylamine and nicotine bitartrate (Sigma, St. Louis, CA, USA). All drugs were dissolved in sterile 0.9% saline except for nicotine that was dissolved in saline and the pH was adjusted to 7.2±0.1 with NaOH (0.1 N). Physostigmine, atropine, mecamylamine and nicotine were administered into the ventral tegmental area and morphine was injected subcutaneously. Control animals received either saline or vehicle.

2.3. Surgical and infusion procedures

All surgical procedures were conducted under ketamine–xylazine (50 mg/kg ketamine-4 mg/kg xylazine) anaesthesia. Stainless steel, 23-gauge guide cannulas were implanted (bilaterally) 1.5 mm above the intended site of injection according to the atlas of Paxinos and Watson (1986). Stereotaxic coordinates for the ventral tegmental area were incisor bar (−3.3 mm), −5.8 mm posterior to the bregma, ±0.9 mm lateral to the sagittal suture and 8 mm from the top of the skull. Cannulas were secured to anchor jewellers screws with dental acrylic. To prevent clogging, stainless steel stylets (30 gauge) were placed in the guide cannulas until the animals were given the ventral tegmental area injection. All animals were allowed 1 week to recover from surgery and clear anesthetic.

For drug infusion, the animals were gently restrained by hand; the stylets were removed from the guide cannulas and replaced by 30-gauge injection needles (1.5 mm below the tip of the guide cannula). Each injection unit was connected by polyethylene tubing to 1 μl Hamilton syringe. The left and right ventral tegmental area was infused with a 0.5 μl solution on each side (1 μl/rat) over a 60-s period. The injection needles were left in place for an additional 60 s to allow diffusion and then the stylets were reinserted into the guide cannulas. During the infusion procedure, the experimenter loosely held the animals.

2.4. Apparatus

The testing apparatus for the conditioned place preference paradigm was similar to that used by Carr and White (1983). It consisted of three wooden compartments. Two of the compartments (A and B) were identical in size (40×30×30 cm) but differed in shading and texture. Compartment A was white with black horizontal stripes 2 cm wide on the walls and also had a textured floor. The other compartment (B) was black with vertical white stripes 2 cm wide and also had a smooth floor. The third compartment (C) was a red tunnel (40×15×30 cm). It protruded from the rear of the two large compartments and connected the entrances to them.

2.5. Behavioral testing

Conditioned place preference consisted of a 5-day schedule with three distinct phases: pre-conditioning, conditioning and testing. This method (Unbiased design) was similar to that used in our previous experiments (Rezayof et al., 2006, 2007; Zarrindast et al., 2005).

2.5.1. Pre-conditioning

During this phase (day 1), each animal was placed in the third compartment (C) with the guillotine doors removed to allow access to the entire apparatus for 15 min. The amount of time spent in each compartment was measured to assess unconditioned preference (the position of the rat was defined by the position of its front paws). In the particular experimental setup used in this study, the animals did not show an unconditioned preference for either of the compartments. Animals were then randomly assigned to one of two groups for place conditioning and a total of eight animals were used for each subsequent experiments.

2.5.2. Conditioning

Place conditioning phase started 1 day after pre-conditioning phase. This phase consisted of six, 45-min sessions (three saline and three drug pairing). These sessions were conducted twice each day (from day 2 to day 4) with a 6-h interval. On each of these days, separate groups of animals received one conditioning session with morphine and one with saline. During these sessions, the animals were confined to one compartment by closing the removable wall. Animals of each group were injected with morphine and were immediately confined to one compartment of the apparatus for 45 min. Following administration of saline, the animals were confined to the other compartment for 45 min. Treatment compartment and order of presentation of morphine and saline were counterbalanced for either group.

2.5.3. Post-conditioning or testing

This phase was carried out on day 5, 1 day after the last conditioning session, in a morphine-free state. Each animal was tested only once. For testing, the removable wall was raised, and the animals had a free choice in the apparatus for 15 min. The time spent in drug-paired compartment was recorded for each animal and the change of preference was calculated as the difference (in seconds) between the time spent in the drug-paired compartment on the testing day, and the time spent in this compartment in the pre-conditioning day (De Fonseca et al., 1995).

2.6. Locomotor testing

Locomotor activity was measured, based on a method used our previously (Tzschentke and Schmidt, 1997; Rezayof et al., 2006, 2007), during the post-conditioning phase (Belzung and Barreau, 2000; Zarrindast et al., 2000), in a morphine-free state. For this purpose the ground area of A and B compartments was divided into 4 equal sized squares. Locomotion was measured as the number of crossings from one square to another during 15 min.

2.7. Experimental designs

2.7.1. Experiment 1 indicates the dose-response effect of place conditioning produced by morphine

Four doses of morphine sulfate (0.5, 2.5, 5 and 7.5 mg/kg, s.c.) were tested for producing place preference. A separate group of animals received saline (1 ml/kg, s.c.) in the two compartments (A and B) in order to confirm that the injection and conditioning schedule were not affecting the time allotment in the apparatus. This group was used as control. Locomotor activity was also measured in the testing phase (Fig. 1).

2.7.2. Experiment 2 shows the effect of physostigmine on the acquisition of conditioned place preference in the absence or presence of morphine

In a group of animals, three doses of a cholinesterase inhibitor, physostigmine (0.5, 2.5 and 5 μg/rat) were given just before the administration of saline (1 ml/kg, s.c.), during the conditioning phase. One additional group received saline (1 μl/rat), just before saline (1 ml/kg, s.c.) during the conditioning phase and served as a control. All groups were tested 24 h after the last conditioning session, with no preceding injection. Locomotor activity was also measured during testing.

In another group, animals received saline (1 μl/rat) or physostigmine (0.5, 2.5 and 5 μg/rat), immediately before the administration of morphine (0.5 mg/kg) during the conditioning sessions. The animals were tested 24 h after the last conditioning session, with no preceding injection. Locomotor activity was also evaluated during testing (Fig. 2).

2.7.3. Experiment 3 demonstrates the effect of atropine on the acquisition of conditioned place preference in the absence or presence of morphine

In a group of animals, the effect of the muscarinic acetylcholine receptor antagonist, atropine (1, 2 and 4 μg/rat)

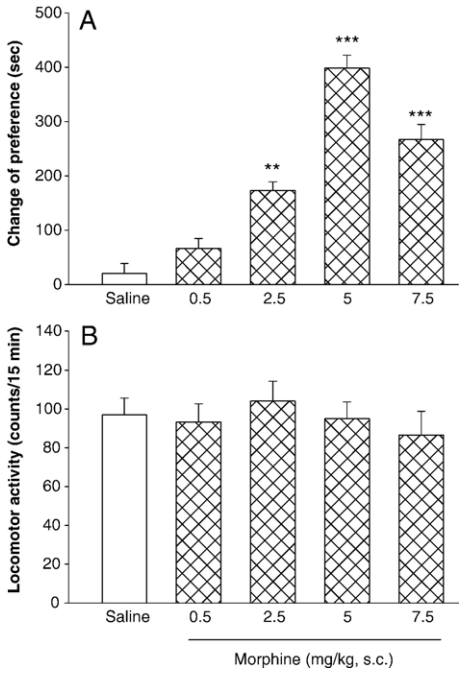

Fig. 1. Place conditioning produced by morphine. Four doses of morphine sulfate (0.5–7.5 mg/kg, s.c.) and saline (1 ml/kg, s.c.) were given in a 3-day schedule of conditioning. On the test day, animals were observed for 15 min and change of preference was assessed as the difference between the time spent on the day of testing and the time spent on the day of pre-conditioning session (Graph A). Locomotor activity in test session has been recorded by the number of crossing from one square to another in the drug-paired compartment for 15 min during the testing of place preference (Graph B). Values are the mean ± S.E.M. of eight rats per group. $^{**}P<0.01$; $^{***}P<0.001$, compared with the saline control group.

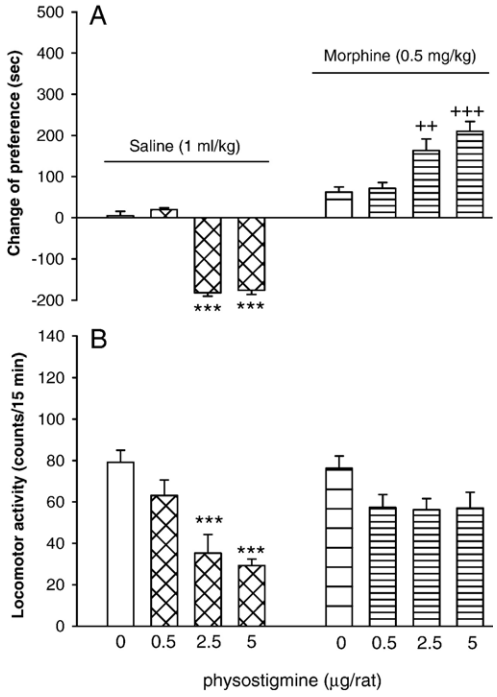

Fig. 2. The effects of bilateral intra-ventral tegmental area injection of physostigmine on the acquisition of a conditioned place preference in the absence or presence of morphine. Four groups of animals received the injection of different doses of physostigmine (0–5 μg/rat), just prior to saline (1 ml/kg, s.c.) and another four groups received physostigmine (0–5 μg/rat) just prior to morphine (0.5 mg/kg, s.c.) in a 3-day schedule of conditioning. Change of preference (Graph A) and locomotor activity (Graph B) for all of the groups were tested 24 h after the last conditioning session. Values are the mean±S.E.M. of eight rats per group. ***$P<0.001$, compared with the saline control group. ++$P<0.01$, +++$P<0.001$, compared with the morphine (0.5 mg/kg) control group.

on place conditioning under the 3-day schedule was tested. An additional group received saline (1 μl/rat) plus saline (1 ml/kg, s.c.) during the conditioning phase and was used as control. Locomotor activity was also evaluated during testing.

In a second group of animals, the effect of atropine on the acquisition of morphine-induced place preference was evaluated. Animals were injected with atropine (1, 2 and 4 μg/rat) or saline (1 μl/rat), just before morphine administration (5 mg/kg) during the conditioning sessions and were tested on the fifth day of the schedule with no preceding injection. Locomotor activity was also measured in the testing phase (Fig. 3).

2.7.4. Experiment 4 indicates the effect of atropine on the physostigmine-induced potentiation of the morphine response

In this experiment, the animals received an intra-ventral tegmental area injection of saline (1 μl/rat) or atropine (2, 4 and 8 μg/rat). After 5 min, they were injected by saline (1 μl/rat) or physostigmine (5 μg/rat). Finally, they received morphine (0.5 mg/kg) or saline (1 ml/kg, s.c.) during the conditioning phase. All animals were tested 24 h after the last conditioning session, with no preceding injection. During testing, locomotor activity of the animals was measured (Fig. 4).

2.7.5. Experiment 5 shows the effect of nicotine on the acquisition of conditioned place preference in the absence or presence of morphine

In a group of animals, three doses of nicotine (0.25, 0.5 and 1 μg/rat) were given just before the administration of saline (1 ml/kg, s.c.), during the conditioning phase. One additional group received vehicle (1 μl/rat), just before saline (1 ml/kg, s.c.) during the conditioning phase and served as a control. All groups were tested 24 h after the last conditioning session, with no preceding injection. Locomotor activity was also measured during testing.

In another group, animals received vehicle (1 μl/rat) or nicotine (0.2, 0.5 and 1 μg/rat), immediately before the administration of morphine (0.5 mg/kg) during the conditioning sessions. The animals were tested 24 h after the last conditioning session, with no preceding injection. Locomotor activity was also evaluated during testing (Fig. 5).

2.7.6. Experiment 6 shows the effect of mecamylamine on the acquisition of conditioned place preference in the absence or presence of morphine

In a group of animals, the effect of the nicotinic acetylcholine receptor antagonist, mecamylamine (2.5, 5 and 7.5 μg/rat) on place conditioning under the 3-day schedule was tested. An additional group received saline (1 μl/rat) plus saline (1 ml/kg,

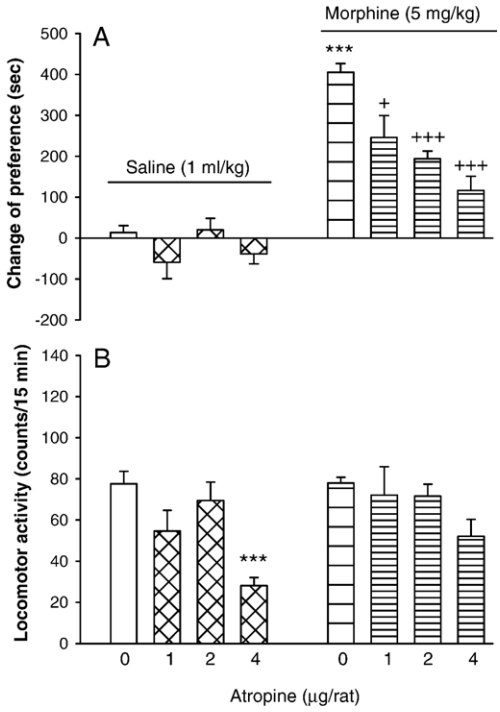

Fig. 3. The effects of bilateral intra-ventral tegmental area injection of atropine on the acquisition of a conditioned place preference in the absence or presence of morphine. Four groups of animals received atropine (0–4 μg/rat), just prior to saline (1 ml/kg, s.c.) and another four groups received atropine (0–4 μg/rat) just prior to morphine (5 mg/kg, s.c.) in a 3-day schedule of conditioning. Change of preference (Graph A) and locomotor activity (Graph B) for all of the groups was tested 24 h after the last conditioning session. Values are the mean±S.E.M. of eight rats per group. ***$P<0.001$, compared with the saline control group. +$P<0.05$, +++$P<0.001$, compared with the morphine (5 mg/kg) control group.

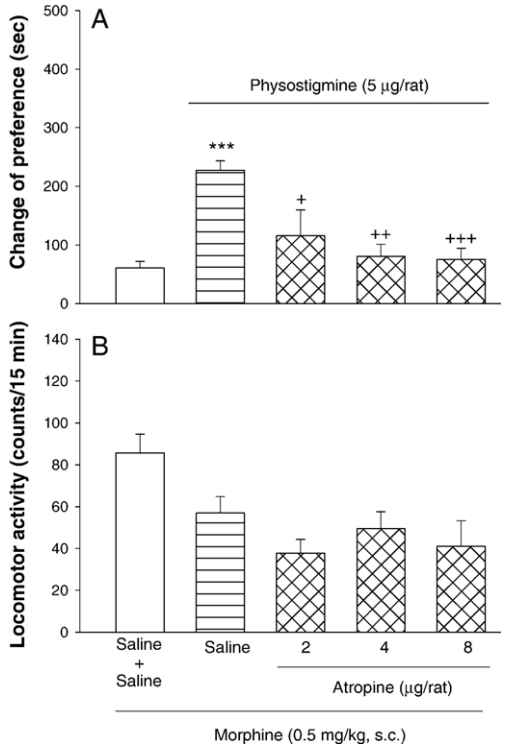

Fig. 4. The effects of bilateral intra-ventral tegmental area pretreatment with atropine on physostigmine-induced changes in the acquisition of place preference. On the conditioning sessions, five groups of animals received saline (1 μl/rat) or atropine (2–8 μg/rat) 5 min prior to intra-ventral tegmental area injection of either saline (1 μl/rat) or physostigmine (5 μg/rat) and immediately were given morphine (0.5 mg/kg, s.c.). Change of preference (Graph A) and locomotor activity (Graph B) for all of the groups was tested 24 h after the last conditioning session. Values are the mean±S.E.M. of eight rats per group. ***$P<0.001$, compared with the saline/saline/morphine control group. + $P<0.05$, ++$P<0.01$, +++$P<0.001$, compared with the saline/physostigmine/morphine control group.

s.c.) during the conditioning phase and was used as control. Locomotor activity was also evaluated during testing.

In a second group of animals, the effect of mecamylamine on the acquisition of morphine-induced place preference was evaluated. The animals were injected with mecamylamine (2.5, 5 and 7.5 μg/rat) or saline (1 μl/rat), just before morphine administration (5 mg/kg) during the conditioning sessions and were tested on the fifth day of the schedule with no preceding injection. Locomotor activity was also measured in the testing phase (Fig. 6).

2.7.7. Experiment 7 indicates the effect of mecamylamine on the nicotine-induced potentiation of the morphine response

Five groups of animals received an intra-ventral tegmental area injection of saline (1 μl/rat) or mecamylamine (5, 7.5 and 10 μg/rat). After 5 min, they were injected by either vehicle (1 μl/rat) or nicotine (1 μg/rat). Finally, they received morphine (0.5 mg/kg) or saline (1 ml/kg, s.c.) during the conditioning phase. All animals were tested 24 h after the last conditioning session, with no preceding injection. During testing, locomotor activity of the animals was measured (Fig. 7).

2.8. Verification of cannulae placements

After completion of the experimental sessions, each animal was killed with an overdose of chloroform. Subsequently, 1.0 μl of ink was injected into the ventral tegmental area by a 30-gauge injection cannula, which projected a further 1.5 mm ventral to the tip of the guide to aid in histological verification. The brains were removed and perfused with a 10% formalin solution 10 days before sectioning. Sections were examined to determine the location of the cannulas aimed for the ventral tegmental area. The cannula placements were verified using the atlas of Paxinos and Watson (1986). Data from rats with cannula placements outside the ventral tegmental area were excluded from the analyses.

2.9. Statistics

The data are expressed as means±S.E.M. The statistical analyses were performed using one- and two-way analysis of variance (ANOVA) with score (i.e., the differences between post-conditioning and pre-conditioning time spent in the drug-associated compartment) as the dependent factor. Post-hoc comparison of means was carried out with the Tukey test for multiple comparisons, when appropriate. The level of statistical significance was set at $P<0.05$. Calculations were performed using the SPSS statistical package.

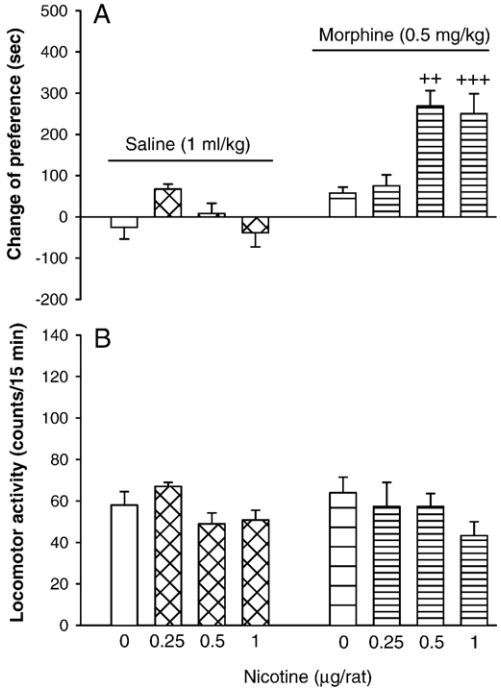

Fig. 5. The effects of bilateral intra-ventral tegmental area injection of nicotine on the acquisition of a conditioned place preference in the absence or presence of morphine. Four groups of animals received nicotine (0–1 μg/rat), just prior to saline (1 ml/kg, s.c.) and another four groups received nicotine (0–1 μg/rat) just prior to morphine (0.5 mg/kg, s.c.) in a 3-day schedule of conditioning. Change of preference (Graph A) and locomotor activity (Graph B) for all of the groups was tested 24 h after the last conditioning session. Values are the mean±S.E.M. of eight rats per group. ++$P<0.01$, +++$P<0.001$, compared with the morphine (0.5 mg/kg) control group.

3. Results

3.1. Experiment 1: morphine-conditioned place preference

The conditioning treatments with morphine induced a conditioned place preference for the drug-associated place (Fig. 1A). One-way ANOVA revealed that morphine caused a significant dose related preference [F (4,35)=51.7, P<0.0001]. Significant conditioning was observed at doses of 2.5, 5 and 7.5 mg/kg. The maximum response was obtained with 5 mg/kg of morphine. No significant effect was observed for locomotor activity in the testing phase [F(4,35)=0.4, P>0.05] (Fig. 1B).

3.2. Experiment 2: the effect of physostigmine on the acquisition of conditioned place preference in the absence or presence of morphine

Fig. 2A shows the effect of bilateral intra-ventral tegmental area injection of physostigmine with or without morphine on the acquisition of conditioned place preference. Data were analyzed by a two-way ANOVA. The results indicated an interaction between morphine and physostigmine in the acquisition of place preference [within-group comparison: treatment effect: F (1,56)=176.9, P<0.0001, dose effect: (3,56)=2.8, P<0.05, treatment×dose interaction: F (3,56)=32.7, P<0.0001]. In addition, one-way ANOVA revealed that intra-ventral tegmental area injection of physostigmine (2.5 and 5 μg/rat) alone produced conditioned place aversion [F(3,28)=31.3, P<0.0001]. Furthermore, the lower dose of morphine (0.5 mg/kg) alone did not induce a significant place preference, but co-administration of the higher doses of physostigmine (2.5 and 5 μg/rat) with 0.5 mg/kg of morphine significantly induced conditioned place preference [F(3,28)=12.2, P<0.0001].

Fig. 2B illustrates the effects of the drugs on the locomotor activity in the testing phase. Two-way ANOVA also revealed a significant effect of treatment [F(1,56)=4.4, P<0.05], dose [F(3,56)=11.7, P<0.0001] and treatment×dose interaction [F(3,56)=3.2, P<0.05]. One-way ANOVA also revealed that the higher doses of physostigmine (2.5 and 5 μg/rat) alone decreased the locomotor activity [F(3,28)=12.6, P<0.0001], but in combination with morphine (0.5 mg/kg) had no effect on locomotor activity [F(3,28)=2.1, P>0.05].

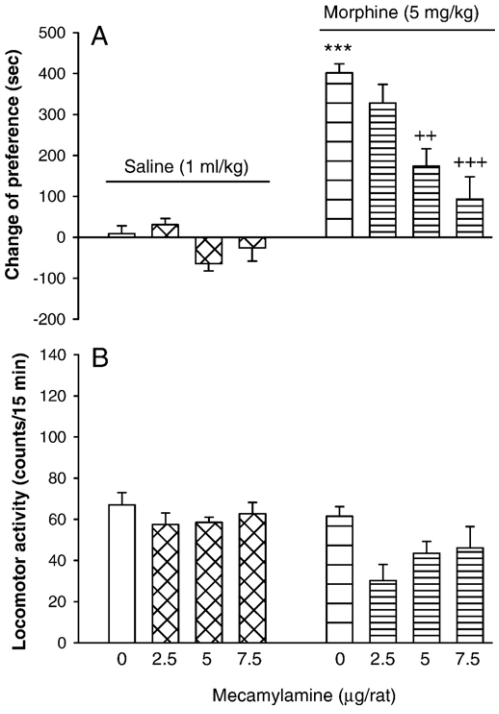

Fig. 6. The effects of bilateral intra-ventral tegmental area injection of mecamylamine on the acquisition of a conditioned place preference in the absence or presence of morphine. Four groups of animals received mecamylamine (0–7.5 μg/rat), just prior to saline (1 ml/kg, s.c.) and another four groups received mecamylamine (0–7.5 μg/rat) just prior to morphine (5 mg/kg, s.c.) in a 3-day schedule of conditioning. Change of preference (Graph A) and locomotor activity (Graph B) for all of the groups was tested 24 h after the last conditioning session. Values are the mean±S.E.M. of eight rats per group. ***P<0.001, compared with the saline control group. ++P<0.01, +++P<0.001, compared with the morphine (5 mg/kg) control group.

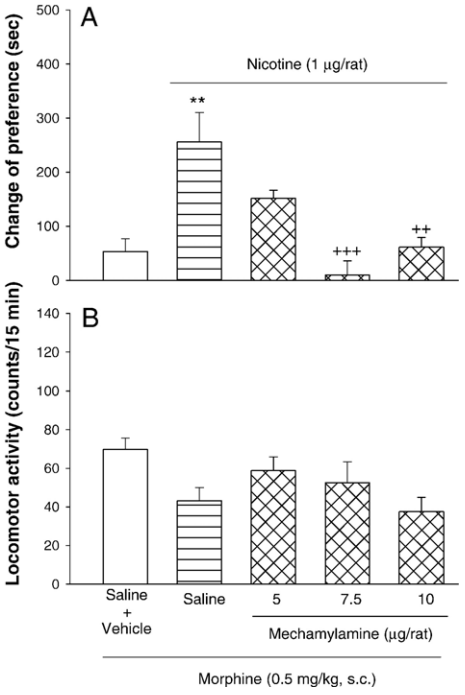

Fig. 7. The effects of bilateral intra-ventral tegmental area pretreatment with mecamylamine on nicotine-induced changes in the acquisition of place preference. On the conditioning sessions, five groups of animals received saline (1 μl/rat) or mecamylamine (5–10 μg/rat) 5 min prior to the injection of either saline (1 μl/rat) or nicotine (1 μg/rat) and immediately were given morphine (0.5 mg/kg, s.c.). Change of preference (Graph A) and locomotor activity (Graph B) for all of the groups was tested 24 h after the last conditioning session. Values are the mean±S.E.M. of eight rats per group. **P<0.01, compared with the saline/saline/morphine control group. ++P<0.01, +++P<0.001, compared with the saline/nicotine/morphine control group.

3.3. Experiment 3: the effect of atropine on the acquisition of conditioned place preference in the absence or presence of morphine

Fig. 3A indicates the effects of bilateral intra-ventral tegmental area injection of the muscarinic acetylcholine receptor

antagonist, atropine in the absence or presence of morphine on the acquisition of conditioned place preference. Data were analyzed by a two-way ANOVA. The results indicated an interaction between morphine and atropine in the acquisition of place preference [within-group comparison: treatment effect: $F(1,56)=143.8$, $P<0.001$, dose effect: $(3,56)=10.9$, $P<0.001$, treatment × dose interaction: $F(3,56)=4.9$, $P<0.01$]. In addition, one-way ANOVA revealed that the morphine (5 mg/kg) but not atropine (1, 2 and 4 μg/rat) alone induced a significant place preference [$F(4,35)=53.9$, $P<0.0001$]. Furthermore, intra-ventral tegmental area administration of atropine (1, 2 and 4 μg/rat) dose-dependently inhibited the morphine-induced place preference [$F(3,28)=13.8$, $P<0.0001$].

Fig. 3B illustrates the effects of the drugs on the locomotor activity in the testing phase. Two-way ANOVA also revealed a significant effect of dose [$F(3,56)=7.4$, $P<0.001$]. The results indicated no significant effect of treatment [$F(1,56)=3.6$, $P>0.05$] nor the treatment × dose interaction [$F(3,56)=1.1$, $P>0.05$]. One-way ANOVA also revealed that the higher doses of atropine (4 μg/rat) alone decreased the locomotor activity [$F(3,28)=7.4$, $P<0.001$], but in combination with morphine had no effect on locomotor activity [$F(3,28)=1.6$, $P>0.05$].

3.4. Experiment 4: the effect of atropine on physostigmine response during morphine conditioning

Fig. 4A shows the effect of intra-ventral tegmental area administration of atropine on the induced changes by morphine (0.5 mg/kg) in combination with physostigmine. One-way ANOVA indicated that atropine (2, 4 and 8 μg/rat) altered the response induced by the injection of physostigmine (5 μg/rat) plus morphine (0.5 mg/kg) [One-way ANOVA: $F(4, 35)=8.6$, $P<0.0001$]. Post-hoc analysis showed that atropine reversed the effect of physostigmine response.

Fig. 4B illustrates the effects of the drugs on the locomotor activity in the testing phase. One-way ANOVA revealed that co-administration of atropine and physostigmine plus morphine had no effect on the locomotor activity [$F(3,28)=1.0$, $P>0.05$].

3.5. Experiment 5: the effect of nicotine on the acquisition of conditioned place preference in the absence or presence of morphine

Fig. 5A shows the effect of bilateral intra-ventral tegmental area injection of nicotine with or without morphine on the acquisition of conditioned place preference. Data were analyzed by a two-way ANOVA. The results indicated an interaction between morphine and nicotine in the acquisition of place preference [within-group comparison: treatment effect: $F(1,56)=45.5$, $P<0.001$, dose effect: $(3,56)=4.8$, $P<0.01$, treatment × dose interaction: $F(3,56)=7.0$, $P<0.001$]. In addition, one-way ANOVA revealed that the lower dose of morphine (0.5 mg/kg) and nicotine (0.25, 0.5 and 1 μg/rat) alone did not induce a significant place preference [$F(4,35)=2.8$, $P>0.05$]. Furthermore, the higher doses of nicotine (0.5 and 1 μg/rat) potentiated the morphine-induced place preference [$F(3,28)=9.8$, $P<0.0001$].

Fig. 5B indicates the effects of the drugs on locomotor activity in the testing phase. Two-way ANOVA also revealed no significant effect of dose [$F(3,56)=2.1$, $P>0.05$], treatment [$F(1,56)=0.3$, $P>0.05$] as well as the treatment × dose interaction [$F(3,56)=1.5$, $P>0.05$]. One-way ANOVA also revealed that nicotine alone [$F(3,28)=3.0$, $P>0.05$] or in combination with morphine [$F(3,28)=1.3$, $P>0.05$] had no effect on the locomotor activity.

3.6. Experiment 6: the effect of mecamylamine on the acquisition of conditioned place preference in the absence or presence of morphine

Fig. 6A indicates the effects of bilateral intra-ventral tegmental area injection of the nicotinic acetylcholine receptor antagonist, mecamylamine in the absence or presence of morphine on the acquisition of conditioned place preference. Data were analyzed by a two-way ANOVA. The results indicated an interaction between morphine and mecamylamine in the acquisition of place preference [within-group comparison: treatment effect: $F(1,56)=113.2$, $P<0.001$, dose effect: $(3,56)=11.79$, $P<0.001$, treatment × dose interaction: $F(3,56)=6.9$, $P<0.01$]. In addition, one-way ANOVA revealed that morphine (5 mg/kg) but not mecamylamine (2.5, 5 and 7.5 μg/rat) alone induced a significant place preference [$F(4,35)=78.5$, $P<0.0001$]. Furthermore, the administration of mecamylamine (5 and 7.5 μg/rat) dose-dependently inhibited the morphine-induced place preference [$F(3,28)=10.9$, $P<0.001$].

Fig. 6B shows the effect of the drugs on locomotor activity in the testing phase. Mecamylamine alone [one-way ANOVA; $F(3,28)=2.0$, $P>0.05$] or in combination with morphine [one-way ANOVA; $F(3,28)=2.9$, $P>0.05$] had no effect on locomotor activity.

3.7. Experiment 7: the effect of mecamylamine on nicotine response during morphine conditioning

Fig. 7A shows the effect of intra-ventral tegmental area administration of mecamylamine on the induced changes by morphine (0.5 mg/kg) in combination with nicotine. One-way ANOVA indicated that different doses of mecamylamine (7.5 and 10 μg/rat) altered the response induced by nicotine (1 μg/rat) plus morphine (0.5 mg/kg) [One-way ANOVA: $F(4, 35)=8.1$, $P<0.001$]. Post-hoc analysis showed that mecamylamine reversed the effect of nicotine response.

Fig. 7B indicates the effects of the drugs on the locomotor activity in the testing phase. One-way ANOVA revealed that co-administration of a higher dose of mecamylamine (10 μg/rat) and nicotine (1 μg/rat) plus morphine (0.5 mg/kg) had no effect on locomotor activity during the testing phase [$F(3, 28)=1.2$, $P>0.05$].

4. Discussion

Our data indicate that in the dose range of 0.5–7.5 mg/kg, morphine produces a significant conditioned place preference for the drug-associated place. These findings supported

previous studies (De Fonseca et al., 1995; Olmstead and Franklin, 1997a,b) and demonstrated that morphine induces rewarding effects which, through a mechanism of associative learning, becomes connected to the environment in which these effects occurred (Tzschentke and Schmidt, 1995). Morphine at the doses used in our experiments did not alter locomotor activity in comparison with that to the control group. This is in agreement with other evidence indicating that the conditioned stimulus is a critical determinant of the form of conditioned locomotor response in a morphine conditioning setup (Sukhotina, 2001; Lu et al., 2002).

It is well known that an opiate-induced place preference depends on activation of the mesolimbic dopamine system (Tzschentke, 1998; Manzanedo et al., 2001). Although, other neural sites and neurotransmitter systems may be involved, because they mediate processes that are necessary for the development of a conditioned place preference, yet they do not process the rewarding effect of opiates (Olmstead and Franklin, 1997a,b). Thus, the present study examined the role of muscarinic and nicotinic receptors of the ventral tegmental area on morphine-induced place preference.

The current study shows that bilateral intra-ventral tegmental area injection of an anticholinesterase, physostigmine with an ineffective dose of morphine (0.5 mg/kg) significantly induced conditioned place preference in a dose-dependent manner. This is consistent with the findings that indicate co-administration of physostigmine and morphine significantly increases the antinociceptive effect of the opiate (Beilin et al., 1997). Furthermore it has been demonstrated that the rewarding effects of morphine, as measured in the conditioned place preference paradigm, are reduced in muscarinic M_5 receptor-deficient mice (Basile et al., 2002). Gronier et al. (2000) showed that the stimulation of the ventral tegmental area muscarinic receptors evokes dopaminergic burst firing and dopamine release in the ventral tegmental area, nucleus accumbens and frontal cortex. Thus, there may be a possibility that in our data, the injection of physostigmine into the ventral tegmental area potentiates the dopamine properties of morphine in the nucleus accumbens through such a pathway. The present data also indicate that intra-ventral tegmental area administration of atropine dose-dependently inhibited the morphine (5 mg/kg)-induced place preference. Moreover, it is interesting to note that pretreatment with atropine during conditioning, attenuated the increase of morphine reward by physostigmine. The results are in agreement with other data showing that infusion of atropine into the ventral tegmental area inhibited self-stimulation and reduced intake of food (Rada et al., 2000). Previous studies have also indicated that injections of atropine into the ventral tegmental area inhibited brain-stimulation reward (Yeomans and Baptista, 1997). The blockade of ventral tegmental area muscarinic acetylcholine receptors attenuates the excitatory effects of morphine on mesoaccumbens and nigrostriatal dopaminergic transmission (Miller et al., 2005). Our results obtained by atropine injection may further support the hypothesis that the ventral tegmental area muscarinic acetylcholine receptor mechanism may be involved in morphine-induced place preference.

In this study, intra-ventral tegmental area injection of the higher doses of physostigmine, but not atropine, alone produced conditioned place aversion. Grillner et al. (1999) demonstrated that muscarine, carbacol and physostigmine reduced the excitatory synaptic potentials in the dopaminergic neurons of the ventral tegmental area and substantia nigra pars compacta. They suggested that this inhibitory effect is mediated by the activation of presynaptic muscarinic M_3 receptors. Moreover, several investigations indicated that the muscarinic receptors have direct postsynaptic actions on neurons and also modulate synaptic transmission by acting on different types of receptors via presynaptic inhibition in a number of brain regions (Hsu et al., 1995; Auerbach and Segal, 1996). Moreover, intra-ventral tegmental area administration of the higher doses of physostigmine or atropine alone decreased the locomotor activity. Therefore, the effect of the higher dose (but not the lower dose) of physostigmine on the production of conditioned place aversion may be due to the influence of the drug on locomotion. Thus, the possibility should be considered that the decreased locomotor activity in the test session induced by the drugs might interfere with the expression of morphine-induced place preference.

In a set of experiments, the effects of bilateral microinjections of the nicotinic acetylcholine receptor agonist and/or antagonist into the ventral tegmental area on morphine-induced place preference have been evaluated. Our results show that bilateral intra-ventral tegmental area injection of nicotine or mecamylamine alone did not induce place preference or aversion. Many reports have demonstrated that systemic or intra-ventral tegmental area nicotine elicits drug-seeking behavior in animals (Corrigall, 1999; David et al., 2006). However, nicotine exerts opposite motivational effects that has made it difficult to demonstrate robust place preference of the kind shown by other drugs of abuse like morphine and amphetamine (Di Chiara, 2000). Laviolette and Van der Kooy (2003a,b) indicated that direct infusions of nicotine into the ventral tegmental area can produce both rewarding and aversive motivational effects by using the conditioned place preference procedure. Some studies showed that systemic administration of nicotine could not produce conditioned place preference or conditioned place aversion (Clarke and Fibiger, 1987; Parker, 1992), while others have reported that nicotine could induce conditioned place aversion (Jorenby et al., 1990) or conditioned place preference (Dewey et al., 1999; Zarrindast et al., 2003a). This controversy may be due to doses of nicotine, the kind of injection or the different methods used.

Moreover, the bilateral injection of nicotine into the ventral tegmental area potentiated the morphine (0.5 mg/kg) response and elicited a significant conditioned place preference. The existence of post-synaptic nicotinic acetylcholine receptors mediating the acute stimulant effect of nicotine on dopamine neurons of ventral tegmental area has been suggested (Di Chiara, 2000). Nicotine acts within the ventral tegmental area region to initiate processes which are critical to the reinforcing properties of the drug (Corrigall et al., 1994). Considerable evidence suggests that systemic, intra-nucleus accumbens or intra-ventral tegmental area infusion of nicotine increases

dopamine release in the nucleus accumbens (Ferrari et al., 2002; Wonnacott et al., 2005). In addition, it is well known that the stimulation of the mesolimbic dopamine system is of critical importance for the reinforcing and stimulatory properties of morphine (Olmstead and Franklin, 1997a,b; McBride et al., 1999). Therefore, it seems likely that the potentiation of morphine response by intra-ventral tegmental area injection of nicotine may be mediated through dopaminergic mechanism. The present data also indicate that intra-ventral tegmental area administration of mecamylamine dose-dependently inhibited the morphine (5 mg/kg)-induced place preference. Furthermore, the antagonist decreased the nicotine-induced potentiation of the morphine response. The results are in agreement with previous investigations showing that blockade of nicotinic receptors with mecamylamine inhibited cocaine-induced place preference (Zachariou et al., 2001). Further support may be obtained from data showing that peripheral or intra-ventral tegmental area injection of mecamylamine reduces alcohol's effects on the mesolimbic dopamine system (Blomqvist et al., 1993; Ericson et al., 1998). Some investigations have indicated that the nicotinic acetylcholine receptors of the ventral tegmental area dopamine and GABA neurons can be blocked by mecamylamine (Mansvelder et al., 2002; Wooltorton et al., 2003). On the other hand, it should be noted that learning and memory play an important role in drug addiction (Hyman and Malenka, 2001; Wickegren, 1998). Drug-induced place preference behavior requires memory for the association between environmental cues and the affective state produced by the drug treatment (White and Carr, 1985; Hsu et al., 2002). Considerable evidence suggests that the central cholinergic system and cholinergic receptor activation are involved in learning and memory processes (Zarrindast et al., 1998; Power et al., 2003; Dani et al., 2001). The involvement of nicotinic innervation of the ventral tegmental area in memory function has been suggested (Levin et al., 1994). In addition, several studies show that central or systemic administration of atropine or mecamylamine and lesions of the cholinergic system cause memory impairments while the acetylcholine receptor agonists improves memory (Sara, 2000; Boccia et al., 2001). Levin et al. (1994) reported that intra-ventral tegmental area injection of mecamylamine significantly impaired radial arm maze working memory performance. Therefore it is likely that the injections of cholinergic receptor agonists or antagonists into the ventral tegmental area facilitate or inhibit learning and memory, which in turn elicits or inhibits morphine-induced place preference, respectively.

It has been shown that nicotinic acetylcholine receptor agents also produce a locomotor stimulating effect (Chintoh et al., 2003). Nicotine elicits biphasic inhibitory-stimulatory effects on locomotion in a baseline-dependent fashion (for a review see Di Chiara, 2000). However, an acute effect of the drug on motor behavior may be ruled out, as the post-conditioning test was carried out 24 h after the last drug administration (Gholami et al., 2002). Our results showed that the administration of nicotine or mecamylamine alone and/or with morphine in conditioning sessions did not produce a significant change in locomotor activity in the test session.

In conclusion, the intra-ventral tegmental area injections of cholinergic receptor agonists potentiate and antagonists block morphine-induced place preference, therefore muscarinic and nicotinic acetylcholine receptors of ventral tegmental area are involved in mediating the rewarding effects of morphine. It appears that the stimulation of dopaminergic neuronal pathway in the ventral tegmental area by muscarinic and/or nicotinic receptor agonists causes release of dopamine in the nucleus accumbens which similar to morphine may play an important role in the acquisition of morphine-induced place preference.

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