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A Nonrewarding NMDA Receptor Antagonist Impairs the Acquisition, Consolidation, and Expression of Morphine Conditioned Place Preference in Mice

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Abstract *N*-methyl-D-aspartate (NMDA) receptor antagonists block morphine-induced conditioned place preference (CPP). Although polyamines are endogenous modulators of the NMDA receptor, it is not known whether polyaminergic agents induce CPP or modulate morphine-induced CPP. Here, we examined whether polyamine ligands modify morphine CPP acquisition, consolidation, and expression. Adult male albino Swiss mice received saline (0.9 % NaCl, intraperitoneally (i.p.)) or morphine (5 mg/kg, i.p.) and were respectively confined to a black or a white compartment for 30 min for four consecutive days for CPP induction. The effect of arcaine (3 mg/kg, i.p.) or spermidine (30 mg/kg, i.p.), respectively, an antagonist and an agonist of the polyamine-binding site at the NMDA receptor, on the acquisition, consolidation, and expression of morphine CPP was studied. In those experiments designed to investigate whether spermidine prevented or reversed the effect of arcaine, spermidine (30 mg/kg, i.p.) was administered 15 min before or 15 min after arcaine, respectively. Arcaine and spermidine did not induce CPP or aversion per se. Arcaine (3 mg/kg, i.p.) impaired the acquisition, consolidation, and expression of morphine CPP. Spermidine prevented the impairing effect of arcaine on the acquisition of morphine CPP but not the impairing effect of arcaine on consolidation or expression of morphine CPP. These results suggest that arcaine may impair morphine CPP acquisition by modulating the polyamine-binding site at the NMDA receptor. However, the arcaine-induced impairment of consolidation and expression of morphine CPP seems to involve other mechanisms.

Keywords Addiction · Arcaine · Conditioned place preference · Morphine · Spermidine

Introduction

Opiate addiction is a complex relapsing brain disease process that is characterized by compulsive seeking and taking an opiate and the emergence of a negative emotional state when the access to the opiate is denied [1]. Morphine is a potent opioid analgesic that is widely used for pain relief, but it produces both psychological and physical dependence [2, 3]. The reinforcing effects of opiates have long been known and demonstrated in both humans and experimental animals [4]. The conditioned place preference (CPP) paradigm has been widely used to assess the rewarding effects of a variety of drugs, including opiates [5]. The CPP paradigm is based upon the idea that contextual stimuli can acquire conditioned rewarding properties when paired with addictive drugs, reflecting their liability to be abused [6]. In fact, morphine induces CPP in both rats and mice [7, 8], and this experimental paradigm has been used to unveil some of the neurochemical mechanisms involved in rewarding and addiction development.

Carlos Fernando Mello and Maribel Antonello Rubin contributed equally to this work.

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Accumulating evidence suggests a role for the mesolimbic dopaminergic system in the rewarding effects of morphine [9–11], although there is also evidence indicating that morphine may induce reward by mechanisms that do not involve dopamine [12]. Morphine injection into the ventral tegmental area increases dopamine (DA) extracellular levels in the nucleus accumbens [13]. The mechanism by which morphine increases dopaminergic activity probably involves the inhibition of GABAergic inhibitory interneurons in the ventral tegmental area [13, 14]. However, neurotransmitters other than DA have been implicated in the development and maintenance of addiction to opiates [15–17] and a role for the glutamatergic system has been proposed [18–21]. In fact, blocking glutamatergic signaling in the VTA suppresses VTA-DA neuron excitation by morphine, indicating a permissive role of glutamate *N*-methyl-D-aspartate (NMDA)/AMPA receptors in this effect of morphine [22]. Both competitive and uncompetitive NMDA receptor antagonists inhibit morphine-, amphetamine-, and cocaine-induced CPP [23–29], suggesting a role for NMDA receptor in drug addiction [30–35]. However, most of NMDA receptor antagonists produce severe stimulant and psychotomimetic-like effects in animals [36–38] and humans [39, 40]. In addition, NMDA antagonists induce CPP per se [30, 41, 42]. Such a rewarding effect has been shown for ketamine [43], dizocilpine [27, 43], phencyclidine [44–46], and dextromethorphan [46]. In fact, it is fairly known that these compounds have abuse potential [47, 48].

Polyamines, naturally occurring polycations that allosterically activate NMDA receptors containing the GluN2B subunit, bind at a dimer interface between GluN1 and GluN2B subunit [49]. It has been shown that arcaine, a competitive antagonist of the NMDA receptor polyamine-binding site, and morphine induce cross-state dependency in rats [50], suggesting that polyamine antagonists may interfere with behavioral responses previously associated with morphine. Therefore, one might suppose that polyamine-binding site antagonists, as other NMDA receptor antagonists, could also modify the rewarding effects of opioids. As such, these drugs could be potentially useful to decrease morphine-driven behavior, particularly if they did not cause rewarding effects per se. Considering that (1) NMDA receptor agonists facilitate morphine CPP [30, 35, 51, 52]; (2) glutamate receptor antagonists block the development of CPP induced by rewarding drugs [53]; (3) polyamines allosterically activate NMDA receptor [49]; and (4) the polyamine antagonist arcaine interfere with a behavioral response previously associated with morphine [50]; in the current study, we investigated whether arcaine and spermidine have rewarding or aversive properties per se and if they alter the acquisition, consolidation, and expression of morphine CPP.

Methods

Animals

Adult male Swiss mice (25–30 g), bred in the animal house of the Federal University of Santa Maria, housed six to a cage, and maintained in a day/night cycle at temperature of 21 °C with access to water and food ad libitum were used. All experiments were carried out in the light phase and are in accordance with Brazilian law no. 11.794/2008, which is in agreement with the Policies on the Use of Animals and Humans in Neuroscience Research and with the Institutional and National Regulations for Animal Research (process 2340010415).

Drugs

1,4-Diguanidinobutane sulfate (arcaine) was obtained from Pfaltz & Bauer (Waterbury, CT, USA), *N*-(3-aminopropyl)-1, 4-butanediamine trihydrochloride (spermidine) was obtained from Sigma-Aldrich Co (St. Louis, MO, USA), and morphine sulfate was obtained from Cristália (Itapira, São Paulo, Brazil). All drugs solutions were prepared in saline (0.9 % NaCl), and the injections were performed intraperitoneally (i.p.) in a 10-ml/kg injection volume.

Apparatus

The conditioned place preference apparatus consisted of three wooden compartments separated by guillotine doors. Two of the compartments (A and B) were identical in size (18 cm length × 16 cm width × 40 cm height) but had different walls and floor colors and floor texture. Compartment A had black walls and a white floor covered by a wire mesh grid. Compartment B had white walls and a smooth black floor. The small center compartment (10 × 10 × 40 cm) was gray. During the conditioning phases, the compartments were separated by guillotine door.

Conditioned Place Preference

Conditioned place preference (CPP) consisted of a 7-day schedule with three distinct phases: pre-conditioning, conditioning, and post-conditioning (test) and was carried out according to Fukushiro and colleagues [54], with minor modifications.

Pre-conditioning In these sessions, each mouse was injected with saline (0.9 % NaCl, 10 ml/kg, i.p.) and placed in the apparatus for 15 min. During this time, the animal was

allowed to freely explore the three compartments. In the second day of pre-conditioning, the time spent in each compartment was recorded. Placement in each compartment was considered as placement of the front paws and the head. Since mice preferred the black compartment (A) during the pre-conditioning session, the white compartment was chosen as the drug-paired compartment (mean permanence time in the black (A): 567 ± 101 s and white (B): 204 ± 65 s in the pre-conditioning session, $t(27) = 11.99$; $p < 0.001$). Moreover, animals were distributed among groups according to place preference, in such a way that all groups had similar baseline place preference.

Conditioning Place conditioning was established in four consecutive days. In each day, two 30 min conditioning sessions were carried out, spaced 5 h and 30 min apart. On the first and the third days of conditioning, between 8:00 and 12:00, each animal was injected with saline and immediately confined to the black compartment (A, non drug-paired compartment) of the apparatus for 30 min. Immediately after the session, the animal was returned to its home cage. Between 14:00 and 18:00, each animal was injected with morphine, arcaine, or spermidine and confined to the white compartment (B, drug-paired compartment). On the second and the fourth days of conditioning, the procedure was performed in the reverse order, that is, from 8:00 to 12:00, the animals were injected with morphine, arcaine, or spermidine and confined to the white compartment; between 14:00 and 18:00, mice were injected with saline and confined to the black compartment.

Post-conditioning or Testing This phase was carried out 24 h after the last conditioning session. Animals were allowed to freely explore the compartments of the apparatus for 15 min. The time spent in each compartment during the 15-min session was recorded. Drug-associated place preference was calculated as the difference (in seconds) of time spent in the drug-paired compartment during the post- and pre-conditioning phases.

Measurement of Locomotor Activity

Locomotor activity in the drug-paired compartment during the test session was measured according to Tahsili-Fahadan [55]. The ground area of the compartment was divided into two equal segments by a transverse line, and locomotion was measured as the number of crossings from one half to the other over 15 min of testing, corrected for the total time spent in the respective compartment.

Experimental Design

Experiment 1: Effect of Morphine, Arcaine and Spermidine on Place Preference

In this experiment, we determined whether increasing doses of morphine, arcaine, and spermidine caused CPP or aversion. Mice received different doses of morphine sulfate (0, 1.25, 2.5, 5, or 10 mg/kg, i.p.), arcaine (0, 0.3, 1, or 3 mg/kg, i.p.), or spermidine (0, 3, 10, or 30 mg/kg, i.p.) immediately before the confinement to the drug-paired compartment in the conditioning sessions. The mice were subjected to the post-conditioning session in a drug-free state. The initial doses of arcaine, spermidine, and morphine (used in the dose-effect curves) were selected based on previous studies that have shown that 5–10 mg/kg morphine induces CPP [7, 8] and the studies that have shown that while 3–30 mg/kg spermidine improves [56], 1–10 mg/kg arcaine impairs memory [57].

In order to address the possibility that arcaine and spermidine produced late effects on conditioning and expression of morphine CPP, we performed an additional experiment using spermidine (at the dose of 30 mg/kg) and arcaine (at the dose of 3 mg/kg) on conditioning place preference. In this experiment, the animals were subjected to the same experimental CPP protocol described above, except that two 60 min conditioning sessions were carried out each day. We aimed, by increasing the duration of the conditioning session, (1) to allow more time for spermidine and arcaine absorption and (2) to increase the duration of drug/context pairing. We hypothesized that if arcaine and spermidine respectively caused place preference and aversion, a longer period of exposure to the apparatus (increasing contingency) could reveal some unnoticed difference between groups.

Experiment 2: Effects of Arcaine on Acquisition, Consolidation and Expression of Morphine CPP

In order to investigate the effect of arcaine on the acquisition of morphine CPP, mice were injected with saline or arcaine (3 mg/kg, i.p.), and 15 min thereafter, they were injected with saline or morphine (5 mg/kg, i.p.). Immediately after saline or morphine injection, the animals were confined to the respective drug-paired compartment (black or white) in the conditioning sessions. The test was carried out 24 h after the last conditioning session, in a drug-free state.

To investigate the effect of arcaine on the consolidation of morphine CPP, mice were injected with saline or morphine (5 mg/kg, i.p.) immediately before the confinement to the drug-paired compartment in the conditioning sessions and with saline or arcaine (3 mg/kg, i.p.) immediately after each

conditioning session. The test was carried out 24 h after the last conditioning session, in a drug-free state.

To investigate whether arcaine altered the expression of morphine CPP, mice were injected with saline or morphine (5 mg/kg, i.p.) immediately before the confinement to the drug-paired compartment in the conditioning sessions and saline or arcaine (3 mg/kg, i.p.) 30 min prior to testing session.

Experiment 3: Effect of Spermidine and a Noneffective Dose of Morphine on CPP

The experiment shown in Fig. 2 revealed that arcaine, a reputed NMDAr antagonist, decreased CPP induced by a fully effective dose of morphine. Therefore, we supposed that spermidine, the agonist of the polyamine-binding site at the NMDAr, should facilitate the place preference induced by morphine. Therefore, we investigated whether combining spermidine and morphine, at a subeffective dose, induced CPP. The subeffective dose of morphine was chosen from the dose-response curve shown in Fig. 1a. Mice were injected with saline or spermidine, and 15 min thereafter, they were injected with saline or morphine (1.25 mg/kg, i.p.) immediately before the confinement to the drug-paired compartment in the conditioning sessions. Animals were tested on the test day in a drug-free state.

Experiment 4: Effects of Spermidine and Arcaine on the Acquisition, Consolidation, and Expression of Morphine CPP

In order to investigate the involvement of the polyamine-binding site at the NMDA receptor in the deleterious effect of arcaine on CPP acquisition, we administered the polyaminergic agonist spermidine before arcaine, and subjected the animals to morphine CPP. Animals were initially injected with saline or spermidine (30 mg/kg, i.p.) and placed in a clean waiting cage (with the same dimensions of the home cage). Fifteen minutes thereafter, they were injected with saline or arcaine (3 mg/kg, i.p.). Fifteen minutes after saline or arcaine injection, the animals were injected with saline or morphine (5 mg/kg, i.p.) and subjected to the conditioning sessions. Animals were tested on the day of testing in a drug-free state.

We also investigated whether spermidine reverses arcaine-induced impairment of CPP consolidation. Animals received saline or morphine (5 mg/kg, i.p.) immediately before the confinement to the drug-paired compartment in the conditioning sessions. Saline or arcaine (3 mg/kg, i.p.) were injected immediately after the confinement to the drug-paired compartment in the conditioning sessions. The animals were transferred to a clean waiting cage and, 15 min thereafter, the animals were injected with saline or spermidine (30 mg/kg, i.p.) and returned to their home cages. Animals were tested on the day of testing in a drug-free state. Since spermidine did not reverse the deleterious effect of arcaine on CPP consolidation, we decided to inject spermidine before arcaine, aiming to prevent the effects of the antagonist. The experimental protocol was essentially the same protocol described above, except that saline or spermidine (30 mg/kg, i.p.) were injected immediately after the confinement to the drug-paired compartment in the conditioning sessions and, 15 min thereafter, the

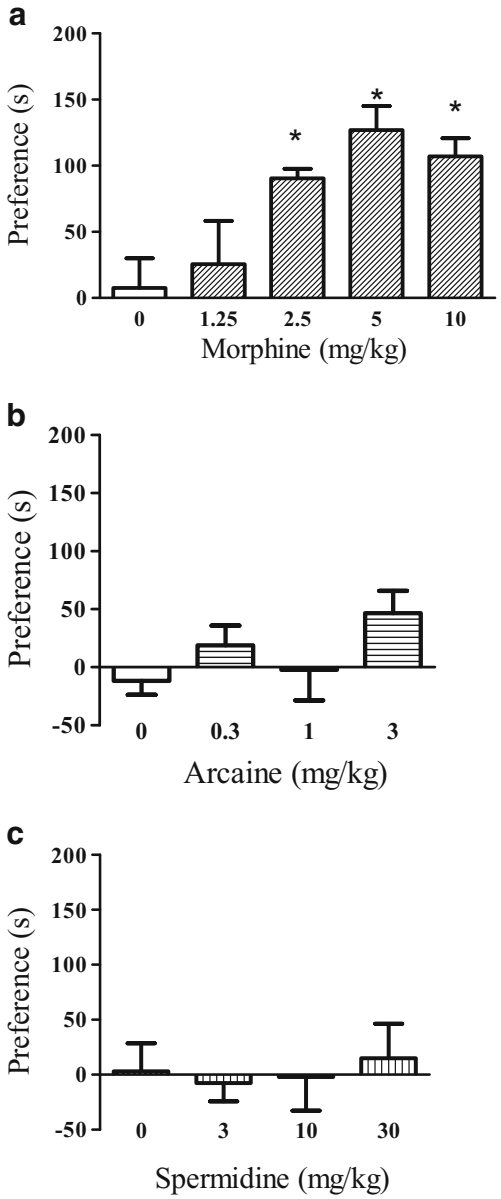

Fig. 1 Effect of intraperitoneal administration of morphine (**a**), arcaine (**b**), and spermidine (**c**) on CPP in mice. Animals received saline, morphine, arcaine, or spermidine immediately before confinement to the white compartment in the 1st, 2nd, 3rd, and 4th days of conditioning. The change of preference was assessed as the difference between the time spent in the drug-paired compartment on the day of testing and the time spent in the drug-paired compartment on the second pre-conditioning session. Data are expressed as mean ± SEM of five to seven animals per group. *$p < 0.05$ compared with control group

animals were injected with saline or arcaine (3 mg/kg, i.p.) and returned to their home cages.

We also investigated whether spermidine reversed arcaine-induced impairment of CPP expression. Animals received saline or morphine (5 mg/kg, i.p.) immediately before the confinement to the drug-paired compartment in the conditioning sessions and returned to their home cages. Saline or arcaine (3 mg/kg, i.p.) were injected 30 min before testing and saline or spermidine (30 mg/kg, i.p.) 15 min before testing. Since spermidine did not reverse the deleterious effect of arcaine on CPP expression, we injected spermidine before arcaine, aiming to prevent the effect of the antagonist. The experimental protocol was essentially the same protocol described above, except that saline or spermidine (30 mg/kg, i.p.) were injected 45 min before testing and arcaine or saline 30 min before testing.

Statistical Analysis

Data analysis was performed using one, two or three-way analysis of variance (ANOVA), depending on the experimental design. Post hoc analyses were carried out by the Student-Newman-Keuls test, when indicated. A $p < 0.05$ was considered significant.

Results

Experiment 1

Figure 1a shows the effect of morphine (1.25–10 mg/kg, i.p.) on the time spent in the white (drug-paired) compartment in mice. Statistical analysis (one-way ANOVA) revealed that morphine-induced place preference ($F(4, 25) = 6.30$; $p < 0.05$). Post hoc analysis revealed that morphine at doses of 2.5, 5, and 10 mg/kg increased the time spent in the drug-paired compartment, compared with saline control group.

Figure 1b, c respectively shows that arcaine (0.3–3 mg/kg, i.p.) and spermidine (3–30 mg/kg, i.p.) did not alter the time spent in the drug-paired compartment. Statistical analysis (one-way ANOVA) showed that neither arcaine ($F(3, 24) = 1.78$; $p > 0.05$, Fig. 1b) nor spermidine ($F(3, 19) = 0.11$; $p > 0.05$, Fig. 1c) induced CPP or aversion. Statistical analysis (one-way ANOVA) revealed that spermidine (30 mg/kg) and arcaine (3 mg/kg) did not alter the time spent in the drug-paired compartment when duration of conditioning sessions was increased to 60 min ($F(2, 18) = 0.91$; $p > 0.05$, Supplementary Fig. S1). Therefore, it seems unlikely that a longer timeframe between drug injections would alter the results.

Experiment 2

Figure 2a shows the effect of arcaine on the acquisition of morphine CPP. Statistical analysis (two-way ANOVA) revealed a significant pre-treatment (saline or arcaine) by treatment (saline or morphine) interaction ($F(1, 32) = 6.40$; $p < 0.05$), indicating that arcaine impaired morphine CPP.

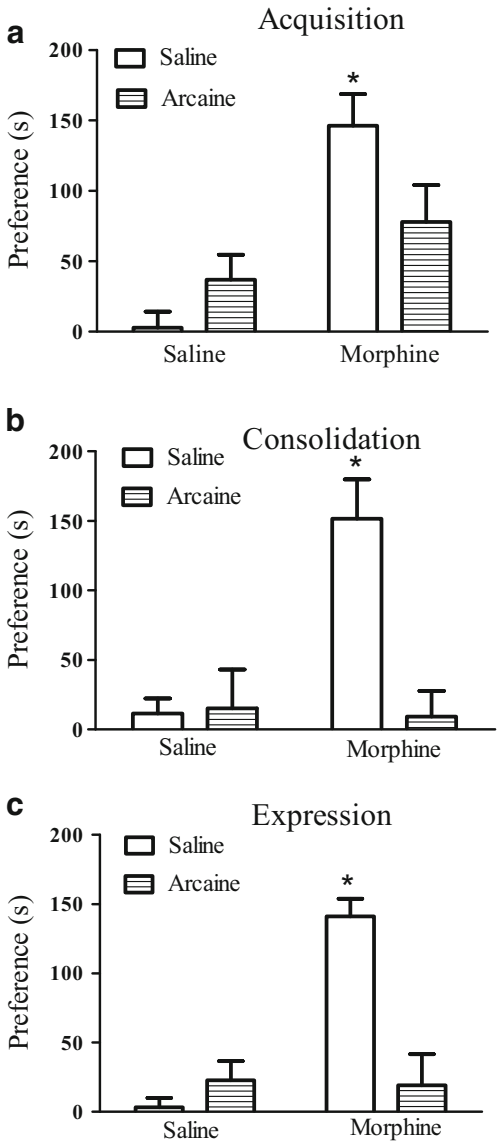

Fig. 2 Effects of intraperitoneal administration of arcaine on acquisition (**a**), consolidation (**b**), and expression (**c**) of morphine CPP in mice. CPP was induced by injecting morphine (*Mor*; 5 mg/kg) immediately before conditioning sessions. Animals received saline or arcaine (3 mg/kg) 15 min before (**a**), immediately after each conditioning session with morphine (**b**) or 30 min before testing (**c**). The change of preference was assessed as the difference between the time spent in the drug-paired compartment on the day of testing and the time spent in the drug-paired compartment on the second pre-conditioning session. Data are expressed as mean ± SEM of seven to nine animals per group. *$p < 0.05$ compared with the other groups

Figure 2b shows the effect of arcaine on the consolidation of morphine CPP. Statistical analysis (two-way ANOVA) revealed a significant treatment (saline or morphine) by post-treatment (saline or arcaine) interaction ($F(1, 24) = 10.48$; $p < 0.05$), indicating that arcaine administration after conditioning completely blocked morphine CPP.

Figure 2c shows the effect of arcaine on the expression of morphine CPP. Statistical analysis (two-way ANOVA) revealed a significant treatment (saline or morphine) by post-treatment (saline or arcaine) interaction ($F(1, 24) = 21.99$; $p < 0.05$), indicating that arcaine administration before testing completely blocked morphine CPP.

Experiment 3

Figure 3 shows the lack of effect of the combination of morphine and spermidine at noneffective doses (1.25 and 30 mg/kg, respectively) on the time spent in the drug-paired compartment. Statistical analysis (two-way ANOVA) showed no significant effects, indicating that spermidine and morphine had no additive effect.

Experiment 4

Figure 4 shows the effect of spermidine on arcaine-induced impairment of morphine CPP acquisition. Statistical analysis (three-way ANOVA) revealed a significant pre-treatment 1 (saline or spermidine) by pre-treatment 2 (saline or arcaine) by treatment (saline or morphine) interaction ($F(1, 69) = 20.92$; $p < 0.05$), indicating that spermidine prevented the deleterious effect of arcaine on the acquisition of morphine CPP.

Figure 5 shows that spermidine does not reverse arcaine-induced impairment of morphine CPP consolidation. Statistical analysis (three-way ANOVA) revealed only a significant

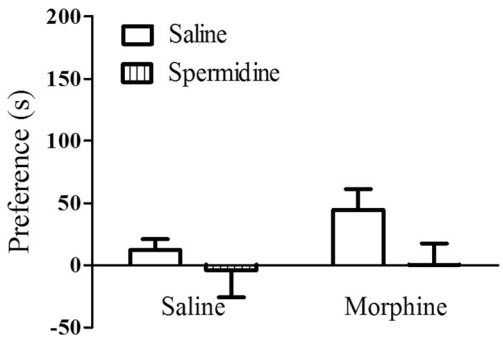

Fig. 3 Effect of intraperitoneal administration of spermidine and a noneffective dose of morphine on CPP. Animals received saline or spermidine (30 mg/kg) 15 min before saline or morphine (1.25 mg/kg) and were placed in the white compartment in the conditioning sessions. The change of preference was assessed as the difference between the time spent in the drug-paired compartment on the day of testing and the time spent in the drug-paired compartment on the second pre-conditioning session. Data are expressed as mean ± SEM of seven animals per group

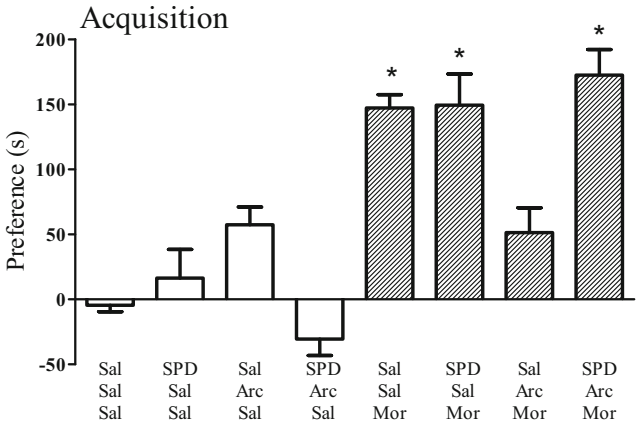

Fig. 4 Effect of intraperitoneal administration of spermidine and arcaine on the acquisition of morphine CPP. Animals received saline or spermidine (*SPD*; 30 mg/kg), and 15 min thereafter they were injected with saline or arcaine (*Arc*; 3 mg/kg). Fifteen minutes after saline or arcaine injection, the animals were injected with saline or morphine (*Mor*; 5 mg/kg) and subjected to the conditioning sessions. The change of preference was assessed as the difference between the time spent in the drug-paired compartment on the day of testing and the time spent in the drug-paired compartment on the second pre-conditioning session. Data are expressed as mean ± SEM of 9–11 animals per group. *$p < 0.05$ compared with control (Sal/Sal/Sal) group

treatment (saline or morphine) by post-treatment (saline or arcaine) interaction ($F(1, 56) = 51.51$; $p < 0.001$), indicating that the administration of arcaine during the consolidation phase blocked CPP. The administration of spermidine before arcaine did not prevent the deleterious effect of arcaine on the consolidation of morphine CPP (Supplementary Fig. S2). Statistical analysis (three-way ANOVA) revealed only a

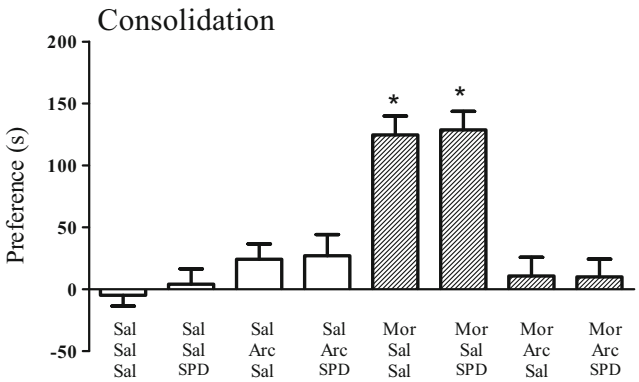

Fig. 5 Effect of intraperitoneal administration of arcaine and spermidine on the consolidation of morphine CPP. CPP was induced by injecting morphine (*Mor*; 5 mg/kg) immediately before conditioning sessions. Saline or arcaine (*Arc*; 3 mg/kg) and saline or spermidine (*SPD*; 30 mg/kg) were administered immediately after conditioning sessions and 15 min after conditioning sessions, respectively. Preference was assessed as the difference between the time spent in the drug-paired compartment on the day of testing and the time spent in the drug-paired compartment on the second pre-conditioning session. Data are expressed as mean ± SEM of eight animals per group. *$p < 0.05$ compared with control (Sal/Sal/Sal) group

significant treatment (saline or morphine) by post-treatment (saline or arcaine) interaction ($F(1, 84) = 7.18$; $p < 0.010$).

Figure 6 shows the lack of effect of spermidine on arcaine-induced impairment of the expression of morphine CPP. Statistical analysis (three-way ANOVA) showed only a significant treatment (saline or morphine) by post-treatment (saline or arcaine) interaction ($F(1, 56) = 30.24$; $p < 0.001$), indicating that, also in this experiment, arcaine impaired the expression of morphine CPP. The administration of spermidine before arcaine did not prevent the deleterious effect of arcaine on the expression of morphine CPP (Supplementary Fig. S3). Statistical analysis (three-way ANOVA) showed only a significant treatment (saline or morphine) by post-treatment (saline or arcaine) interaction ($F(1, 50) = 11.88$; $p < 0.002$). None of the pharmacological treatments of this study altered locomotor activity of the animals in the test session (data not shown).

Discussion

In the current study, we showed that the pre-training, post-training and pre-test administration of arcaine impairs morphine CPP. The deleterious effect of pre-training arcaine on morphine CPP was prevented by spermidine. Notwithstanding, the impairment of morphine CPP induced by the post-conditioning and pre-test administration of arcaine was not reversed by spermidine. Moreover, spermidine did not alter morphine CPP.

Our findings are in agreement with previous studies that have shown that morphine induces CPP [8, 55, 58, 59], an animal model to assess the rewarding effect of different drugs, including opioids [60]. It has been proposed that opioids induce CPP by activating the reward circuit [61]. According to this view, morphine binds to μ-opioid receptors present in GABAergic neurons located in the ventral tegmental area and decreases GABAergic release. By these means it would indirectly stimulate the ascending mesocorticolimbic dopamine system [62, 63]. Such a disinhibition of dopaminergic neurons in the ventral tegmental area increases DA release in nucleus accumbens [14, 23], which has been reputed to play a central role in reward. In addition, there are μ-opioid receptors expressed by nucleus accumbens and dorsal striatal neurons. Opiates can directly stimulate these receptors and produce reward in a DA-independent manner [21, 61].

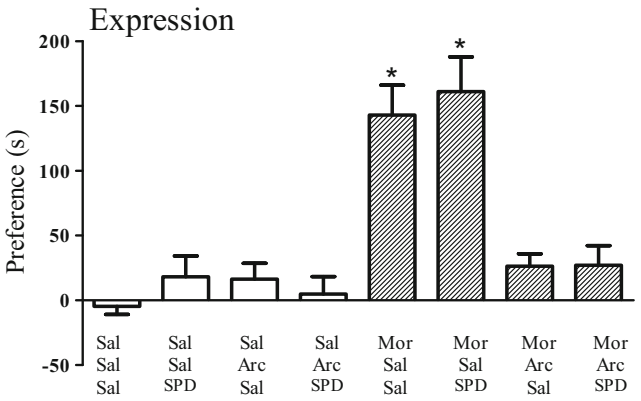

Fig. 6 Effect of intraperitoneal administration of arcaine and spermidine on the expression of morphine CPP. CPP was induced by injecting morphine (*Mor*; 5 mg/kg) immediately before conditioning sessions. Saline or arcaine (*Arc*; 3 mg/kg) and saline or spermidine (*SPD*; 30 mg/kg) were administered 30 and 15 min before test, respectively. Preference was assessed as the difference between the time spent in the drug-paired compartment on the day of testing and the time spent in the drug-paired compartment on the second pre-conditioning session. Data are expressed as mean ± SEM of eight animals per group. $^*p < 0.05$ compared with control (Sal/Sal/Sal) group

Interestingly, the acquisition, consolidation and expression of morphine CPP is attenuated or blocked by NMDA glutamate receptor antagonists, in both rats and mice [64, 65]. Accordingly, MK-801 [16, 27, 30, 43, 53, 66–69], memantine [70–72], ketamine [43, 73], ifenprodil [3, 32, 74], NPC 17742 [75], AP5 [35, 76], dextromethorphan [29, 34], agmatine [77], and CGP37849 [30] decrease morphine CPP.

Zhu and colleagues [78] have reported that the expression of the GluN1 subunit mRNA is increased in the locus ceruleus and in the hypothalamic paraventricular nucleus following 3 days of intracerebroventricular morphine infusion, suggesting that it is involved in the development of morphine dependence. Accordingly, the pre-treatment with a NMDA receptor GluN1 subunit antisense oligonucleotide attenuates morphine withdrawal syndrome [79]. However, since the GluN1 subunit is an obligatory component of functional NMDA receptors [80], GluN1 suppression itself does not tell much about the role of NMDA receptor subtypes in morphine dependence or reward-related effects. In this regard, it is important that NMDA receptor subunits GluN1, GluN2A, and GluN2B, but not GluN2C and GluN2D, are consistently expressed in the reward circuitry [81]. In fact, GluN2A receptors have been suggested to play a role in morphine dependence. Although both wild-type and GluN2A knockout mice repeatedly treated with morphine show withdrawal signs after treatment with naloxone [82], the signs of naloxone-precipitated morphine withdrawal symptoms are significantly attenuated in GluN2A knockout, compared with wild-type mice. These findings suggest that adaptive changes mediated by GluN2A subunit-containing NMDA receptors play a role in the development of morphine physical dependence [82]. Interestingly, enhancement of GluN2A protein expression is observed in the nucleus accumbens of wild-type mice after development of dependence by chronic morphine treatment [83], and the rescue of GluN2A protein, by electroporation into the nucleus accumbens of GluN2A knockout mice, significantly reverses the loss of abstinence behaviors. These findings suggest a locus-specific role for GluN2A in the development of morphine physical dependence [83]. On the other hand,

pharmacological evidence supports that GluN2B-containing NMDA receptors may be more importantly involved in morphine addiction than GluN2A-containing NMDA receptors. Accordingly, the administration of ifenprodil, a selective antagonist of GluN2B-containing NMDA receptor, suppresses morphine rewarding effects in mice [3] and rats [32, 74]. Moreover, anti-NR2B antibody administration abolishes morphine rewarding effects in mice, whereas antibodies against GluN1 and GluN2A subunits do not [84]. At last, gentiopicroside- [8] and small interference RNA-induced [59] GluN2B downregulation decreases CPP, further supporting a role for GluN2B in the rewarding effects of morphine. Since arcaine may displace polyamines from the polyamine-binding site at a dimer interface between GluN1 and GluN2B subunit [49], decreasing their function, the currently reported attenuation of morphine CPP by arcaine before conditioning session could be interpreted as additional evidence that GluN2B receptors are involved in the acquisition of morphine CPP. Moreover, one might also suggest that arcaine impairs context/drug association (acquisition) or reduces the rewarding effect of morphine. The possibility that arcaine causes place aversion, however, was refuted in the experiments that revealed that arcaine (Fig. 1b) and spermidine (Fig. 1c) do not cause place preference or aversion *per se*. As expected, from the pharmacological point of view, this effect of arcaine was prevented by the injection of spermidine (Fig. 4). These results agree with the previous finding that pre-training intra-amygdalar arcaine may impair learning [85] and that this effect is prevented by spermidine [85]. However, the administration of a combination of a non-effective dose of morphine and spermidine before conditioning did not induce CPP or aversion (Fig. 3), suggesting that the modulatory effect of polyaminergic agents on morphine-induced CPP depends on a full rewarding dose of morphine, that is, on morphine-elicited CPP establishing mechanisms.

Interestingly, the injection of arcaine immediately after the conditioning sessions abolished morphine place preference at testing. Since arcaine was injected immediately after each conditioning session, one might reasonably conclude that this effect of arcaine is not due to an alteration in the motivational state of the animal during conditioning, i.e., arcaine neither altered morphine reward nor impaired the association between context and drug state because it was injected after conditioning. Although arcaine did not induce manifested preference or aversion, due to the fact that arcaine injection was contingent upon morphine injection, it is possible that a second, maybe less rewarding association (a morphine + arcaine interoceptive state) has emerged immediately after (but behaviorally associated to) the conditioning sessions, resulting in decreased preference scores at testing. It is also possible that arcaine injection impaired the consolidation of the memory of the conditioning sessions, since arcaine impairs memory consolidation [85–89] and reconsolidation [57]. Interestingly, Zarrindast and colleagues [52] have shown that NMDA potentiates the rewarding effect of morphine, a finding that is in agreement with the view that NMDA receptor activation is involved in this effect of morphine. However, spermidine did not prevent or reverse this effect of arcaine (Fig. 5; Supplementary Fig. S2). This unexpected result suggests that mechanisms other than blocking the polyamine-binding site at the NMDAr may be involved in this effect of arcaine.

The injection of arcaine before testing blocked the expression of morphine CPP. It has been repeatedly shown that arcaine injection before testing does not alter the performance in memory tasks [50, 86], except if it is also administered immediately after training [86]. Interestingly, it has also been reported that the effect of arcaine pre-test depends on the activation of opioid receptors, since naloxone blocks the facilitatory effect of pre-test arcaine in animals injected post-training with morphine or arcaine [90]. Given that morphine-induced state dependency transfers to arcaine [50] in the inhibitory avoidance paradigm, we expected that arcaine would analogously facilitate the expression of morphine CPP. However, we found the opposite, which is in agreement with the various studies that have shown that NMDA receptor antagonists block the expression of CPP [32, 67, 68, 71, 75–77, 91]. This discrepancy may have occurred because of adaptive changes caused by daily injections of morphine (5 mg/kg) for 4 days. In fact, this injection protocol doubles the ED_{50} for morphine in the tail flick paradigm [92]. Moreover, a 4-day 10-mg/kg morphine CPP protocol has been associated with an increase of phosphorylated CREB immunoreactivity in the hippocampus, nucleus accumbens, ventral tegmental area, striatum, and prefrontal cortex, an adaptive change in the opposite direction of that caused by acutely administered morphine [93]. Therefore, it is reasonable that these adaptive changes in the opioid system may have modified the behavioral response to arcaine. At last, spermidine did not reverse the deleterious effect of arcaine on the expression of morphine CPP (Fig. 6; Supplementary Fig. S3). Such a lack of effect of spermidine on arcaine-induced impairment of morphine CPP expression is also unexpected and, again, indicates that mechanisms other than the NMDAr antagonism may be involved in this effect of arcaine. Therefore, one might argue that the effects of arcaine on the consolidation and expression of morphine CPP are not related to the antagonism of the polyamine-binding site at the NMDAr. In fact, there are reports that arcaine inhibits neuronal nitric oxide synthase and agmatinase activities [94]. By inhibiting agmatinase, the main agmatine metabolizing enzyme [95], arcaine could increase brain agmatine content and alter morphine-induced CPP, probably by mechanisms involving imidazoline and alpha$_2$-adrenoceptors.

However, the effects of agmatine on morphine CPP are conflicting, since both inhibition [77] and facilitation [55] have been reported. In what concerns nNOS, there is compelling evidence supporting the involvement of the NO/sGC/PKG signaling pathway in the acquisition of morphine CPP [96–104]. Therefore, it is possible that NOS inhibition, or other unforeseen mechanism, such as epigenetic [105], underlies the currently described inhibitory effect of arcaine on morphine CPP.

In conclusion, in this study, we show that a putative antagonist of the polyamine-binding site at the NMDA receptor and NOS inhibitor blocks morphine CPP. Whether the currently described effects of arcaine on morphine CPP are due to an anti-reward effect of the compound or a disruption of a cognitive component of the addiction process remains to be determined.

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Compliance with Ethical Standards

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

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