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NMDA receptors in the midbrain play a critical role in dopamine-mediated hippocampal synaptic potentiation caused by morphine

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ABSTRACT

A single exposure to drugs of abuse produces an NMDAR (N-methyl-D-aspartate receptor)-dependent synaptic potentiation at excitatory synapses of dopamine (DA) neurons in the ventral tegmental area (VTA) of the midbrain. All addictive drugs can increase DA concentrations in projection areas of the midbrain, including the hippocampus. Hippocampal DA release subsequently modulates hippocampal plasticity and drug-associated memories. Using *in vivo* electrophysiological recording techniques in anesthetized rats, we show that systemic injection of morphine induced hippocampal synaptic potentiation in a dose-dependent manner. Intra-VTA but not intra-hippocampus injection of morphine evoked this potentiation. Local hippocampal dopamine D1 receptors (D1R) are required in the morphine-induced synaptic potentiation and conditioned place preference (CPP). Moreover, both NMDAR activation in the VTA and VTA/hippocampus dopaminergic connections are essential for the morphine-evoked potentiation and CPP. These findings suggest that NMDAR signalings in the midbrain play a key role in regulating dopamine-mediated hippocampal synaptic plasticity underlying drug-induced associative memory.

Keywords Dopamine, hippocampus, morphine, NMDA receptors (NMDAR), synaptic plasticity, ventral tegmental area (VTA).

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INTRODUCTION

The dopaminergic pathways originating from the VTA dopamine (DA) neurons seem to be critically involved in the neuroadaptations underlying drug addiction (Wise 1996; Koob, Sanna & Bloom 1998; Everitt & Wolf 2002; Kelley & Berridge 2002). The increased DA release in the VTA projection areas is triggered in response to acute exposure to nearly all addictive drugs (Wise 1996; Koob *et al.* 1998). Hippocampus is anatomically innervated by dopaminergic projections from the VTA (Swanson 1982; Gasbarri *et al.* 1994). A large number of investigations provided evidence that DA release in the hippocampus could modulate hippocampal plasticity (Frey, Schroeder & Matthies 1990; Otmakhova & Lisman 1996; Morris *et al.* 2003) and impact on drug-associated memories (Lisman & Grace 2005).

Previous reports revealed that glutamatergic inputs to VTA modulate the development of dopamine-mediated long-term changes in the brain reward circuitry, regulate the firing rates and burst firing of VTA DA neurons and control the establishment of addictive behaviors (Chergui *et al.* 1993; Mereu *et al.* 1997; Carr & Kalivas 2008). For example, NMDAR activation in the VTA is required for drug-evoked plasticity at excitatory synapses of VTA DA neurons (Ungless *et al.* 2001; Saal *et al.* 2003). Genetic inactivation of NMDAR signaling in VTA DA neurons impairs burst firing (Zweifel *et al.* 2009) and the acquisition of cocaine- or food-induced conditioned place preference (CPP) (Zweifel *et al.* 2008, 2009). Pharmacological studies have demonstrated that intra-VTA injections of NMDA receptor antagonists blocked the acquisition of cocaine or morphine CPP (Harris & Aston-Jones 2003; Harris *et al.* 2004). Therefore, we proposed that NMDAR activation in the VTA might play a critical role in dopamine-mediated hippocampal plasticity induced by addictive drugs. Our findings provide evidence for a role of both NMDAR signalings in the midbrain and midbrain/hippocampus dopaminergic connections in morphine-induced hippocampal synaptic plasticity and CPP.

MATERIALS AND METHODS

All experiments were performed on male Sprague–Dawley rats (250–350 g) obtained from the Peking Center of Experimental Animals. Animals were housed four per cage in a 12-hour light/12-hour dark normal cycle with food and water available at all times. The room temperature was maintained at 24 ± 1°C and relative humidity at 50%. All experimental procedures were approved by the Animal use Committee of Peking University Health Science Center and carefully designed to minimize the number of animals used and their suffering.

Electrophysiological procedures

Surgery

Animals were anesthetized with 20% urethane (1.3 mg/kg, i.p.), cannulated in jugular vein for i.v. administration of drugs and then mounted on a stereotaxic apparatus (SN-3N, Narishige, Japan). Body temperature was maintained at 36.5–37.5°C via a feedback-controlled under-body heating pad. A parylene-coated tungsten microelectrode (FHC, Bowdoin, ME, USA) driven by a micro-stepping motor (PC-5N, Narishige, Japan) was lowered into the dentate gyrus (DG) granule cell layer (coordinates: AP, −3.5 mm; ML, 2.0 mm; DV, 3.0–3.5 mm from the dura) and a bipolar tungsten electrode (FHC) were implanted in the medial perforant path (MPP) (coordinates: AP, −8.1 mm; ML, 4.1 mm; DV, 3.0 mm from the dura).

Stimulation and recording

Single test stimulus (0.1 ms, delivered at 30-second intervals) was applied to the MPP at an intensity that evoked 40–50% of the maximal amplitude of the population spike (assessed by the input/output curve). The field potentials were filtered (1–3 kHz band pass) by a bioelectric amplifier (AVB-10, Nihon Kohden, Japan) and digitized by a CED 1401 interface for off-line analysis using the Spike 2 software (Cambridge Electronic Design, Cambridge, UK). Population spikes (PS) of the field potentials in the DG induced by stimulating the MPP were recorded before and after morphine injection. The PS amplitude was measured from its negative-going apex to the tangent line joining the first two positive peaks. The baseline amplitude of PS was examined for stability for 30 minutes. The responses were monitored for 3 hours following morphine administration.

Drug and drug administration

Drugs

Morphine hydrochloride was purchased from the First Pharmaceutical Factory of Shenyang (Shenyang, China). *R*(+)-SCH-23390 hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO, USA). SKF38393, NMDA and D-AP5 were purchased from Tocris Biosciences (Bristol, UK). All drugs were dissolved in saline.

Drug administration

Drugs were administered by either i.v. injections or local brain microinjections. The control group received equal volume of saline. For local brain microinjections, needle (30-gauge) connected by polyethylene tubing to a 1-μl Hamilton syringe was introduced, protruding 0.5 mm from the tip of the guide cannula (23-gauge). To avoid the mechanical effect of the microinjection needle on electrophysiological recordings, a guide cannula–electrode assembly would be used for intra-DG microinjections (Messaoudi *et al.* 2002). The DG or VTA was infused with 1 μl solution (1 μl per rat) (infusion rate: 1 μl/minute) ipisilaterally to the recording site. After drug infusion, the needle was left in the place for an additional 60 seconds to allow diffusion.

Behavioral procedures

Surgery

Animals were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and positioned in a Kopf stereotaxic instrument. The 23-gauge stainless steel guide cannula were bilaterally implanted 1 mm above the DG (coordinates: AP, −3.5 mm; ML, 2.0 mm; DV, 3.5 mm from the skull surface) or VTA (coordinates: AP −5.8 mm; ML, 0.8 mm; DV, 8.5 mm from the skull surface) according to the atlas of Paxinos & Watson (1998). Cannulas were secured to the skull by jeweler's screws and acrylic dental cement. To prevent clogging, stainless steel stylets (30-gauge) were placed in the guide cannula until the animals were given intracerebral injections. The animals were allowed 7 days to recover before place conditioning processes.

Drug administration

The animals were gently restrained by hand; the stylets were removed from the guide cannula and replaced by 30-gauge injection needles, terminating 1 mm below the tip of the guides. Each injection unit was connected by polyethylene tubing to 1-μl Hamilton syringe. The left and right DG or VTA were infused with a 0.5-μl solution on each side (1 μl per rat) over a 60-second period. The needle was left in the place for an additional 60 seconds to allow diffusion, and then the stylet was reinserted into the guide cannula.

CPP procedures

Three phases (pre-test, conditioning and test) were included: (1) Pre-test was conducted on day 0. All groups

of rats were placed in the central compartment and allowed them to freely explore the entire apparatus for 15 minutes. Rats that spent more time (over 100 seconds) in one of the outer chambers than the other were excluded from the study. (2) Conditioning was performed on day 1–4. Rats that received morphine (i.p.) were immediately confined to one outer chamber for 45 minutes in the morning and then injected with saline and confined to the alternative outer chamber for the same time period in the afternoon. Saline control groups received saline on both sides of the apparatus. (3) Test was done on day 5. Rats were also placed in the central compartment, allowed to freely explore the entire apparatus for 15 minutes to determine which outer chamber they preferred.

Experiment 1

Effect of morphine training dose on CPP. We assessed morphine CPP in four different groups of rats that were injected with saline or morphine (0.1, 1 or 3 mg/kg, i.p.).

Experiment 2

Effect of D1R activation in the DG on the acquisition of morphine CPP. SCH23390 (1 μg per rat) or SKF38393 (1 μg per rat) was injected into the DG immediately before each morphine injection during the conditioning phase. To determine the reversal effect of SCH23390 on the response induced by SKF38393, SCH23390 (1 μg per rat) was injected into the DG 5 minutes prior to the administration of SKF38393 (1 μg per rat) in the same experiment.

Experiment 3

Effects of NMDAR activation in the VTA and the VTA/DG dopaminergic connections on the acquisition of morphine CPP. NMDA (0.03 μg per rat) or AP5 (1 μg per rat) was injected into the VTA immediately before each morphine injection during the conditioning phase. To determine the reversal effect of AP5 on the response induced by NMDA, AP5 (1 μg per rat) was injected into the VTA 5 minutes prior to the administration of NMDA (0.03 μg per rat) in the same experiment. To further detect whether the D1Rs in the DG play a role in the response evoked by NMDAR activation in the VTA, SCH23390 (1 μg per rat) or SKF38393 (1 μg per rat) administered into the DG 5 minutes prior to the administration of NMDA (0.03 μg per rat) or AP5 (1 μg per rat) in the same experiment separately.

Histology

At the end of each microinfusion experiment, 4% methylene blue in phosphate-buffered saline (0.5 ml) was injected into the infusion site. Electrode placements were marked by electrolytic lesion (20 μA positive depolarizating DC current for 20 seconds). The animal was then euthanized by an overdose of pentobarbital sodium and perfused intracardially with saline (0.9% NaCl, 150 ml), followed by 300 ml of 4% paraformaldehde. The brains were removed and fixed at 4°C for at least 24 hours. The tissue was then sectioned into 20-μm-thick coronal slices on a cryostat and stained with cresyl violet.

Data analysis

For electrophysiological experiments, amplitudes of PS were averaged every 5 minutes and normalized for each animal as a percentage of the mean baseline values obtained during the 30 minutes immediately before drug treatment. PS amplitudes in the last 1 hour of recordings (120–180 minutes after morphine or saline injection) were averaged in one animal and then across animals to give an average magnitude of PS for the group. Data were shown as mean ± SEM. Data sets were analyzed with either paired *t*-test, one-way or two-way repeated-measures analysis of variance (ANOVA) followed by *post hoc* Dunnett, Newman–Keuls or Bonferroni tests. For behavioral experiments, CPP score was calculated by dividing the time spent in the drug-paired compartment by the time spent in both conditioning compartments. Values are expressed as mean ± SEM and analyzed with two-way ANOVA followed by Bonferroni post test. The accepted level of statistical significance was $P < 0.05$.

RESULTS

Systemic injection of morphine induced synaptic potentiation in the DG

We recorded the PS in the DG evoked by stimulating the MPP in anesthetized rats. Saline or different doses of morphine (0.1, 0.5, 1 or 5 mg/kg) was i.v. applied to induce the synaptic response at MPP-DG synapses, respectively. The time course of morphine-induced synaptic plasticity is shown in Fig. 1a. We found that neither saline (open circles, $n = 4$; 101.0 ± 0.5%, paired *t* test, $P > 0.05$ versus baseline) nor 0.1 mg/kg morphine (blue circles, $n = 6$; 100.6 ± 5.8%, paired *t* test, $P > 0.05$ versus baseline) affected synaptic transmission, but 5 mg/kg morphine (red squares, $n = 6$; 148.3 ± 2.2%, paired *t* test, $P < 0.05$ versus baseline) induced synaptic potentiation which lasted at least for 3 hours. Fig. 1b revealed that relatively higher doses of morphine (0.5, 1 and 5 mg/kg) induced synaptic potentiation ($F_{4,23} = 34.11$, $P < 0.0001$).

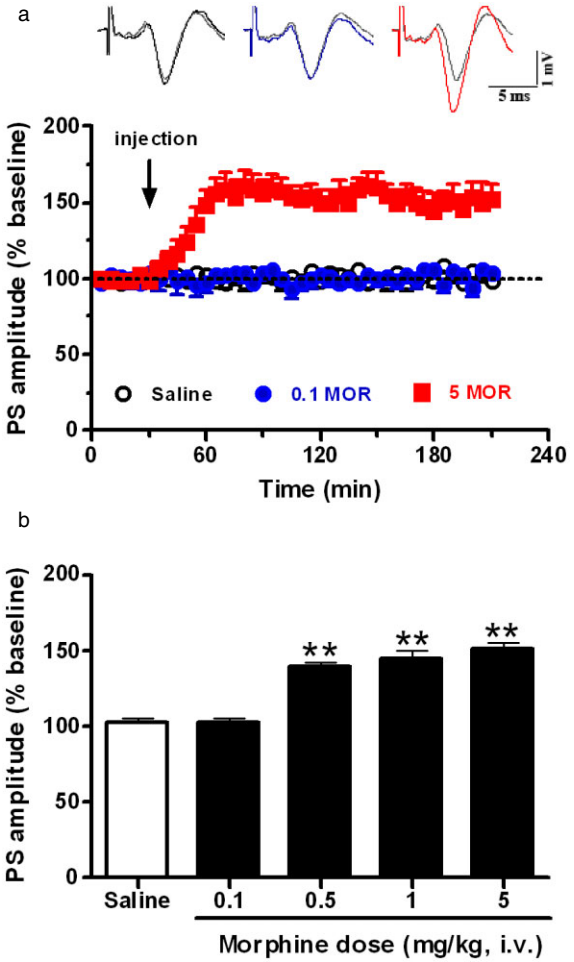

Figure 1 Morphine-induced synaptic potentiation at the MPP-DG synapse. (a) Time course of changes in the population spike (PS) amplitudes. Saline (open circles, $n = 4$), 0.1 mg/kg morphine (blue circles, $n = 6$) or 5 mg/kg morphine (red squares, $n = 6$) was injected (i.v.) at 30 minutes time (downward arrow). The amplitude of PS was expressed as a percentage of baseline value. Representative traces of PS amplitude are plotted above the curve to indicate potentiation of synaptic transmission compared to the baselines (gray traces). Two-way analysis of variance (ANOVA) with repeated measures showed significant effects of groups ($F_{2,13} = 53.42$, $P < 0.0001$) and group × time interactions ($F_{82,533} = 5.18$, $P < 0.0001$). The PS amplitude increased significantly over baseline and stayed potentiated for at least 3 hours after 5 mg/kg morphine ($F_{41,205} = 11.94$, one-way repeated-measures ANOVA, $P < 0.0001$). (b) Summary of the PS magnitudes after injection of saline or different doses of morphine ($n = 4$–6 per group, one-way ANOVA followed by *post hoc* Dunnett tests, $^{**}P < 0.01$ versus saline)

Intra-VTA but not intra-DG administrations of morphine evoked hippocampal synaptic potentiaton

Subsequently examined question was raised: whether local morphine application in hippocampus or other key brain areas could affect synaptic plasticity? To answer this question, different doses of morphine (0.1 or 1 μg) or saline was ipsilaterally microinfused into the DG, respectively. As shown in Fig. 2a, saline (open circles, $n = 4$; 104.6 ± 0.7%, paired t test, $P > 0.05$ versus baseline), 0.1 μg morphine (blue circles, $n = 6$; 101.9 ± 0.7%, paired t test, $P > 0.05$ versus baseline) or 1 μg morphine (red squares, $n = 6$; 101.4 ± 0.6%, paired t test, $P > 0.05$ versus baseline) did not induce synaptic potentiation.

Because VTA is an important primary target for addictive drugs including morphine, it is possible that morphine could influence hippocampal plasticity by local injection into the VTA. We thus ipsilaterally microinfuse different doses of morphine (0.1 or 1 μg) or saline into the VTA, respectively. The time course of morphine-induced synaptic plasticity is shown in Fig. 2b. We found that neither saline (open circles, $n = 4$; 101.7 ± 0.6%, paired t test, $P > 0.05$ versus baseline) nor 0.1 μg morphine (blue circles, $n = 6$; 103.6 ± 0.8%, paired t test, $P > 0.05$ versus baseline) affected synaptic transmission, but 1 μg morphine (red squares, $n = 6$; 155.2 ± 3.5%, paired t test, $P < 0.05$ versus baseline) induced synaptic potentiation.

Morphine-induced hippocampal synaptic potentiation requires dopamine D1R activation in the DG

Since hippocampal dentate gyrus receives dopaminergic innervations from the VTA (Verney *et al.* 1985), we next want to know if the D1R in the DG was involved in regulating this plasticity. When SCH23390 (1 μg), a D1R antagonist, was ipsilaterally microinfused alone into the DG, there was no significant change in synaptic transmission (Fig. 3a, $n = 4$; 101.5 ± 0.6%, paired t test, $P > 0.05$ versus baseline). However, microinfusion of SCH23390 into the DG preceding intra-VTA infusion of a higher dose of morphine (1 μg), completely prevented the morphine-induced synaptic potentiation (Fig. 3b, black circles, $n = 6$; 103.3 ± 0.7%, paired t test, $P > 0.05$ versus baseline). In paired control experiments that saline microinfusion preceded the morphine (1 μg) administration, morphine (1 μg) induced a significant potentiation over the baseline (Fig. 3b, gray circles, $n = 5$; 147.4 ± 3.8%, paired t test, $P < 0.05$ versus baseline).

When SKF38393 (1 μg), a D1R agonist, was ipsilaterally microinfused alone into the DG, there was no significant change in synaptic transmission (Fig. 3c, $n = 4$; 100.7 ± 0.5%, paired t test, $P > 0.05$ versus baseline). Microinfusion of SKF38393 into the DG preceding intra-VTA infusion of a lower dose of morphine (0.1 μg), however, enabled the morphine (0.1 μg) to induce synaptic potentiation (Fig. 3d, gray circles, $n = 6$; 150.4 ± 3.6%, paired t test, $P < 0.05$ versus baseline). In paired control experiments that saline microinfusion preceded the morphine (0.1 μg) administration, morphine (0.1 μg) failed to affect synaptic transmission (Fig. 3d, black circles, $n = 5$; 104.5 ± 0.6%, paired t test, $P > 0.05$ versus baseline).

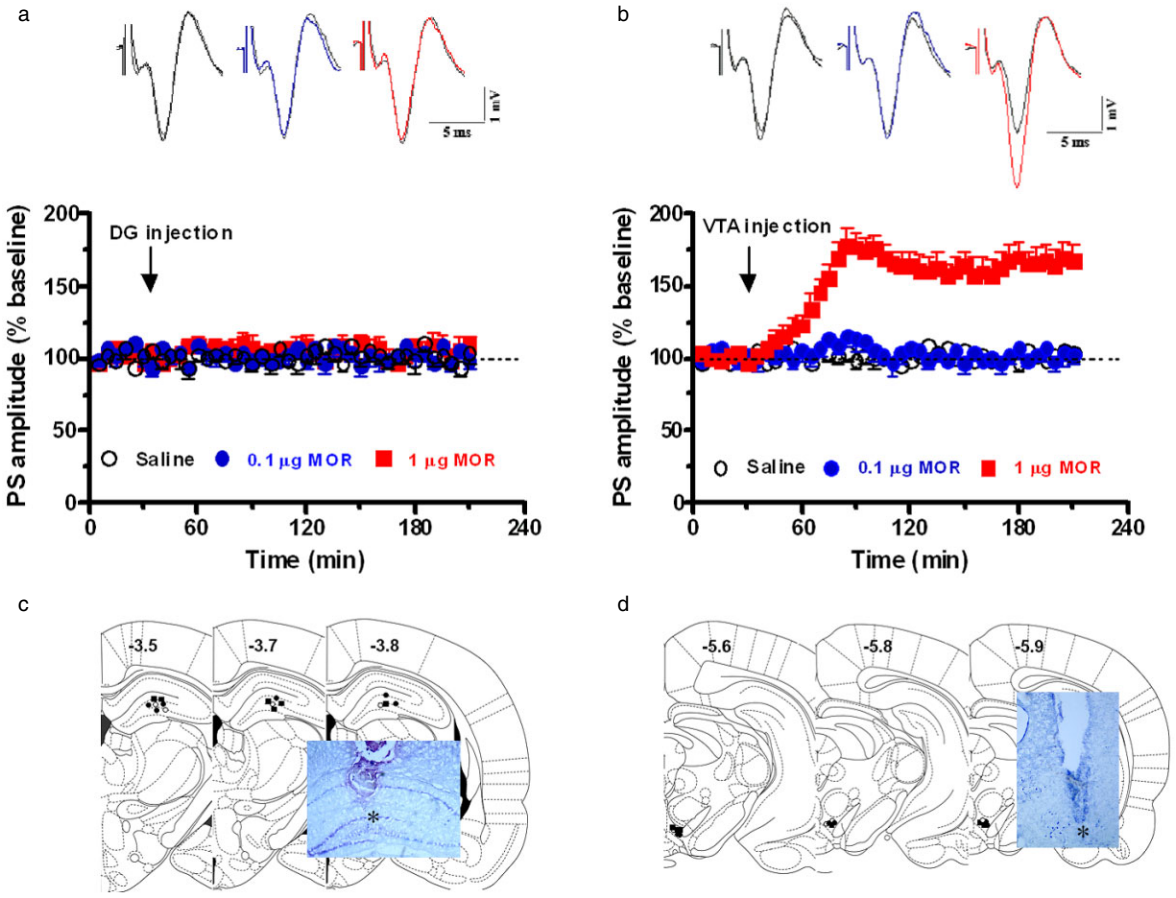

Figure 2 Intra-VTA but not intra-DG injection of morphine evoked synaptic potentiaton at the MPP-DG synapse. Saline (open circles; DG, $n = 4$; VTA, $n = 4$), 0.1 μg morphine (blue circles; DG, $n = 6$; VTA, $n = 6$) or 1 μg morphine (red squares; DG, $n = 6$; VTA, $n = 6$) was separately microinjected into the DG (a) or VTA (b) at 30 minutes time (downward arrow). Representative traces of PS amplitude are plotted above curves to indicate potentiation of synaptic transmission compared to the baselines (gray traces). (a) Two-way ANOVA with repeated measures showed no significant effects of groups ($F_{2,13} = 0.09$, $P > 0.05$) and group × time interactions ($F_{82,533} = 0.99$, $P > 0.05$). (b) Two-way repeated-measures ANOVA showed significant effects of groups ($F_{2,13} = 58.12$, $P < 0.0001$) and group × time interactions ($F_{82,533} = 7.33$, $P < 0.0001$). The PS amplitude increased significantly over baseline and stayed potentiated for at least 3 hours after 1 μg morphine ($F_{41,205} = 12.01$, one-way repeated-measures ANOVA, $P < 0.0001$). (c, d) Representative and schematic representation of injection sites in the DG (c) and the VTA (d) for rats used in the experiments (○, Saline; ●, 0.1 μg MOR, ■, 1 μg MOR). The numbers represent the coordinate posterior from bregma. The asterisk indicates the location of the injection site

Morphine-induced hippocampal synaptic potentiation requires NMDAR activation in the VTA and VTA/hippocampus dopaminergic connections

Glutamate inputs to the VTA play a critical role in the regulation of the firing activity of VTA DA neurons (Murase *et al.* 1993). The hippocampal dentate gyrus is innervated by dopaminergic terminals from the VTA (Verney *et al.* 1985). Therefore, we examined whether morphine-induced hippocampal synaptic plasticity requires NMDAR activation in the VTA and VTA/hippocampus dopaminergic connections. When AP5 (1 μg), a NMDAR antagonist, was ipsilaterally microinfused alone into the VTA, there was no significant change in synaptic transmission (Fig. 4a, $n = 4$; 101.9 ± 5.9%, paired t test, $P > 0.05$ versus baseline). However, microinfusion of AP5 into the VTA preceding intra-VTA infusion of a higher dose of morphine (1 μg) completely prevented the morphine-induced synaptic potentiation (Fig. 4b, black circles, $n = 6$; 102.6 ± 0.54%, paired t test, $P > 0.05$ versus baseline). In paired control experiments that saline microinfusion preceded the morphine (1 μg) administration, morphine (1 μg) induced a significant potentiation over the baseline (Fig. 4b, gray circles, $n = 5$; 147.3 ± 3.4%, paired t test, $P < 0.05$ versus baseline). Moreover, the inhibition of this synaptic potentiation caused by AP5 in the VTA is reversed by intra-DG infusion of SKF38393 ($F_{2,14} = 48.41$, $P < 0.0001$, Fig. 4e).

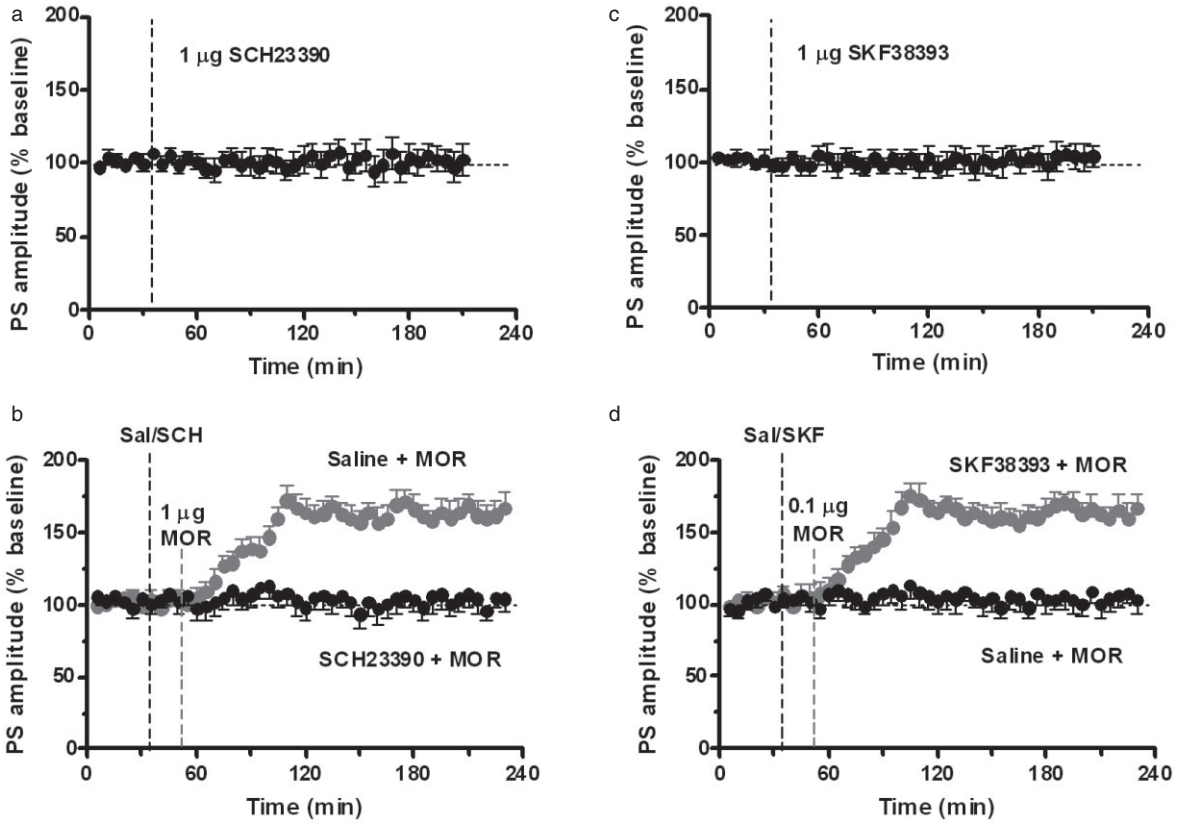

Figure 3 Synaptic potentiation induced by intra-VTA injection of morphine requires dopamine D1R activation in the DG. (a) Intra-DG injection of SCH23390 (1 μg, $n = 4$) had no effect on the basic synaptic transmission at the MPP-DG synapse ($F_{41,123} = 1.23$, $P > 0.05$, one-way repeated-measures ANOVA). (b) Intra-DG injection of SCH23390 (1 μg, black circles, $n = 6$) 15 minutes before morphine (1 μg) inhibited morphine-induced potentiation. When saline (1 μl) was microinjected before morphine (gray circles, $n = 5$), morphine-induced potentiation was observed. Two-way repeated-measures ANOVA showed significant effects of groups ($F_{1,9} = 17.53$, $P < 0.01$) and group × time ($F_{45,405} = 40.24$, $P < 0.001$). (c) Intra-DG injection of SKF38393 (1 μg, $n = 4$) had no effect on the basic synaptic transmission at the MPP-DG synapse ($F_{41,123} = 1.47$, $P > 0.05$, one-way repeated-measures ANOVA). (d) When saline (1 μl) was microinjected before morphine (0.1 μg, black circles, $n = 5$), morphine failed to induce synaptic potentiation. Intra-DG injection of SKF38393 (1 μg, gray circles, $n = 6$) 15 minutes before morphine, however, enabled morphine to evoke potentiation. Two-way repeated-measures ANOVA showed significant effects of groups ($F_{1,9} = 21.86$, $P < 0.01$) and group × time ($F_{45,405} = 43.76$, $P < 0.001$)

When NMDA (0.03 μg), a NMDAR agonist, was ipsilaterally microinfused alone into the VTA, there was no significant change in synaptic transmission (Fig. 4c, $n = 4$; 101.3 ± 0.5%, paired t test, $P > 0.05$ versus baseline). Microinfusion of NMDA into the VTA preceding intra-VTA infusion of a lower dose of morphine (0.1 μg), however, enabled the morphine (0.1 μg) to induce synaptic potentiation (Fig. 4d, gray circles, $n = 6$; 151.4 ± 4.0%, paired t test, $P < 0.05$ versus baseline). In paired control experiments that saline microinfusion preceded the morphine (0.1 μg) administration, morphine (0.1 μg) failed to affect synaptic transmission (Fig. 4d, black circles, $n = 5$; 102.9 ± 0.6%, paired t test, $P > 0.05$ versus baseline). Moreover, the increase of this synaptic transmission caused by NMDA in the VTA is blocked by intra-DG infusion of SCH23390 ($F_{2,14} = 49.65$, $P < 0.0001$, Fig. 4f).

Morphine-induced CPP requires NMDAR activation in the VTA and VTA/hippocampus dopaminergic connections

We next examined whether the morphine-induced synaptic potentiation is correlated with morphine-induced behavioral change using the CPP model. As shown in Fig. 5a, relatively higher doses of morphine (1 and 3 mg/kg) caused significant CPP, compared to saline (0 mg/kg) (Fig. 5a, $F_{3,70} = 4.15$, $P < 0.01$), but no preference was observed at dose of 0.1 mg/kg. The doses of morphine used in the present study did not affect the locomotor activity in the testing phase (data not shown).

Then we identified whether D1R activation in the DG is also required for morphine-induced CPP. Immediately before each morphine training in rats, SCH23390 (1 μg per rat), SKF38393 (1 μg per rat) or saline (1 μl per rat),

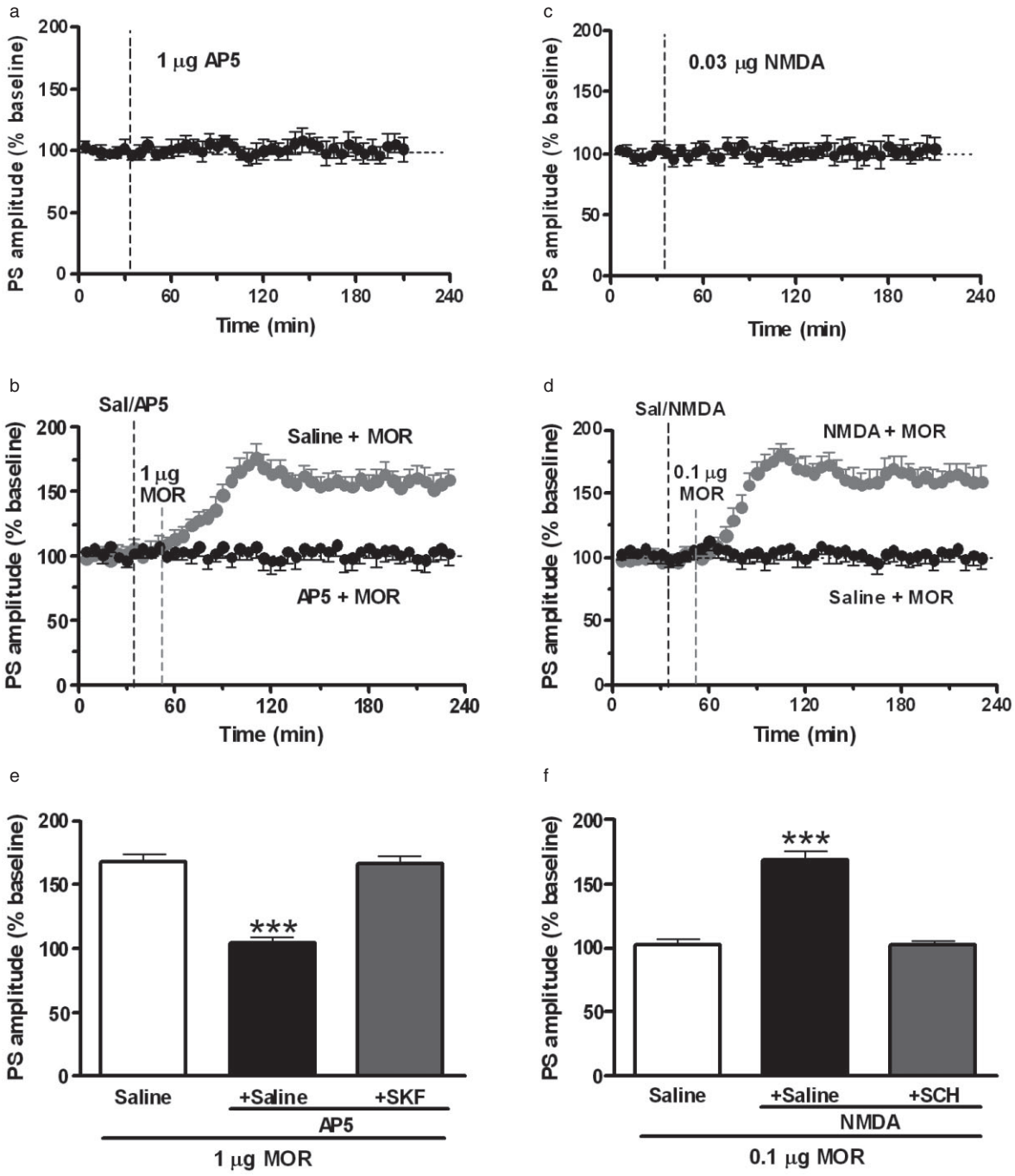

Figure 4 Synaptic potentiation induced by intra-VTA injection of morphine requires NMDAR activation in the VTA and VTA/hippocampal dopaminergic connections. (a) Intra-VTA injection of AP5 (1 μg, $n = 4$) had no effect on the basic synaptic transmission at the MPP-DG synapse ($F_{41,123} = 1.32$, $P > 0.05$, one-way repeated-measures ANOVA). (b) Intra-VTA injection of AP5 (1 μg, black circles, $n = 6$) 15 minutes before morphine (1 μg) inhibited morphine-induced potentiation. When saline (1 μl) was microinjected before morphine (gray circles, $n = 5$), morphine-induced potentiation was observed. Two-way repeated-measures ANOVA showed significant effects of groups ($F_{1,9} = 13.26$, $P < 0.01$) and group × time ($F_{45,405} = 36.93$, $P < 0.0001$). (c) Intra-VTA injection of NMDA (0.03 μg, $n = 4$) had no effect on the basic synaptic transmission at the MPP-DG synapse ($F_{41,123} = 1.39$, $P > 0.05$, one-way repeated-measures ANOVA). (d) When saline (1 μl) was microinjected before morphine (0.1 μg, black circles, $n = 5$), morphine failed to induce synaptic potentiation. Intra-VTA injection of NMDA (0.03 μg, gray circles, $n = 6$) 15 minutes before morphine, however, enabled morphine to evoke potentiation. Two-way repeated-measures ANOVA showed significant effects of groups ($F_{1,9} = 16.38$, $P < 0.01$) and group × time ($F_{45,405} = 38.82$, $P < 0.001$). (e) The inhibition of morphine (1 μg)-induced synaptic potentiation caused by AP5 in the VTA is reversed by intra-DG injection of SKF38393 ($n = 5$–6 rats per group, one-way ANOVA followed by *post hoc* Newman–Keuls tests, ***$P < 0.001$ versus saline control group). (f) The increase of morphine (0.1 μg)-induced synaptic transmission caused by NMDA in the VTA is blocked by intra-DG injection of SCH23390 ($n = 5$–6 rats per group, one-way ANOVA followed by *post hoc* Newman–Keuls tests, ***$P < 0.001$ versus saline control group)

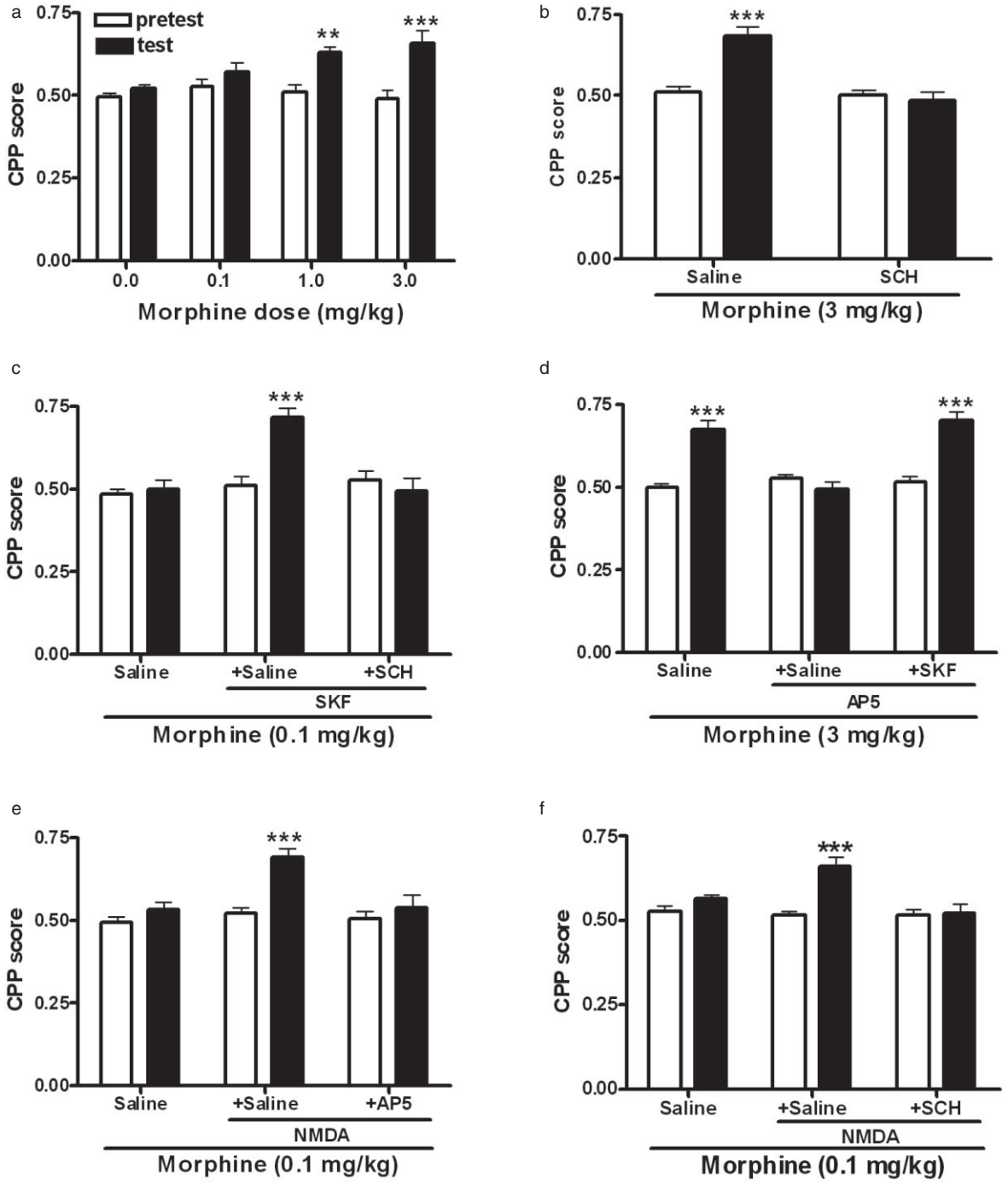

Figure 5 Morphine-induced CPP requires NMDAR activation in the VTA and VTA/hippocampus dopaminergic connections. (a) Place preference produced by saline or different doses of morphine. (b, c) Effect of intra-DG injections of saline, SCH23390 or SKF38393 on morphine CPP. (d–f) Effect of intra-VTA injections of saline, AP5 or NMDA on morphine CPP, and the modulatory effect of intra-DG injections of dopaminergic drugs upon the NMDAR response. Blank and black columns represent data from pre- and post-conditioning test, respectively. Data are expressed as mean ± SEM, 9–10 rats per group. Two-way ANOVA followed by *post hoc* Bonferroni tests, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the pre-test value of the same group

was bilaterally microinfused into the DG separately. Although saline injection before training with a higher dose of morphine (3 mg/kg) still resulted in the acquisition of CPP, CPP was not induced when the D1 inhibitor SCH23390 was administered (Fig. 5b, $F_{1,34} = 17.16$, $P < 0.001$). Conversely, although saline injection before training with a lower dose of morphine (0.1 mg/kg) failed to induce CPP, CPP was able to be acquired when

the D1R agonist SKF38393 was administered, and this effect could be blocked by co-injection SCH 23390 in the DG (Fig. 5c, $F_{2,\ 52} = 11.16$, $P < 0.0001$). The drugs also did not change locomotor activity (data not shown).

To examine whether morphine-induced CPP requires NMDAR activity in the VTA and VTA/hippocampus dopaminergic connections, AP5 (1 μg per rat), NMDA (0.03 μg per rat) or saline (1 μl per rat), was bilaterally microinfused into the VTA immediately before each morphine training in rats separately. Although saline injection before training with morphine (3 mg/kg) still resulted in the acquisition of CPP, CPP was not induced when the NMDAR antagonist AP5 was administered, and this effect could also be reversed by co-injection of SKF38393 in the DG (Fig. 5d, $F_{2,\ 52} = 16.35$, $P < 0.0001$). Conversely, although saline injection before training with morphine (0.1 mg/kg) failed to induce CPP, CPP was able to be induced when NMDA was administered. In addition, co-injection of AP5 in the VTA (Fig. 5e, $F_{2,\ 54} = 5.06$, $P < 0.01$) or co-injection of SCH 23390 in the DG (Fig. 5f, $F_{2,\ 54} = 6.64$, $P < 0.01$) could block this effect. The drugs also did not change locomotor activity (data not shown).

DISCUSSION

In the present study, we found that systemic morphine dose-dependently evoked synaptic potentiation in the DG. Here we also demonstrated for the first time that local injections of morphine into the VTA but not into the DG induced synaptic potentiation in a dose-dependent manner, although there have been *in vivo* and *in vitro* evidence showing that morphine can induce this potentiation (Akaishi *et al.* 2000; Zhu & Zhou 2003). Moreover, our results showed that the synaptic potentiation induced by intra-VTA injection of morphine requires dopamine D1R activation in the DG, consistent with a recent report showing that *in vivo* nicotine-induced synaptic potentiation requires D1R activation in the DG (Tang & Dani 2009). Since hippocampus also receives dopaminergic innervations arising from the substantia nigra (SN) (Scatton *et al.* 1980), and reward-related fMRI of SN activation is associated with enhanced hippocampus-dependent long-term memory formation (Wittmann *et al.* 2005), we cannot exclude the possibility that the intra-SN administration of morphine may induce this potentiation.

More importantly, we found that the synaptic potentiation induced by intra-VTA injection of morphine required both NMDAR activation in the VTA and VTA/DG dopaminergic connections. A number of evidence has indicated that morphine can increase the firing rate and bursting activity of VTA DA neurons (Gysling & Wang 1983; Matthews & German 1984; Georges, Le & Aston-Jones 2006) by suppressing GABA inhibitory input to DA neurons in the VTA (Johnson & North 1992). However, glutamatergic inputs to VTA regulate the firing activity of VTA DA neurons and modulate its local plasticity. For example, glutamate injected into the prefrontal cortex increased burst firing of VTA DA neurons (Murase *et al.* 1993). Genetic inactivation of NMDAR in VTA DA neurons impairs burst firing (Zweifel *et al.* 2009). NMDAR activation is also necessary for drug-evoked strength at excitatory synapses of VTA DA neurons (Ungless *et al.* 2001; Saal *et al.* 2003), and further studies showed that selectively inactivation of functional NMDAR in VTA DA neurons eliminated the induction of drug-evoked synaptic strengthening (Engblom *et al.* 2008; Zweifel *et al.* 2008). Thus, we propose that glutamate-mediated excitatory inputs to VTA are likely to involve the activation of DA neurons during acute morphine administration. Moreover, since DG is innervated by dopaminergic projections from the VTA (Verney *et al.* 1985; Gasbarri *et al.* 1994; Leranth & Hajszan 2007), and dopamine release in the DG modulates plasticity in the medial perforant path synaptic inputs (Tang & Dani 2009; Hamilton *et al.* 2010), it is possible that morphine acts through NMDAR in the VTA to increase DA release in the DG. Here we identified that NMDAR activation in the VTA is essential for dopamine-mediated hippocampal synaptic potentiation induced by morphine.

Linking morphine-evoked synaptic plasticity and morphine-associated behavior, we confirm that dopamine D1R activation in the DG is also necessary for morphine-induced CPP. Several previous studies have shown that drug-induced CPP requires the dorsal hippocampus and its local DA signaling. For example, functional inactivation of the dorsal hippocampus by muscimol (Meyers, Zavala & Neisewander 2003; Meyers *et al.* 2006) or neurotoxic lesions of the dorsal DG by colchicine (Hernandez-Rabaza *et al.* 2008) prevented cocaine-induced CPP. Systemic administration of D1R antagonist blocked nicotine-induced CPP (Tang & Dani 2009). Pharmacological manipulation of hippocampal dopamine D1R indicated that morphine-induced CPP requires D1R activation in the hippocampal CA1 area (Rezayof *et al.* 2003). Our data also suggest that the dopamine D1R signal in the DG may play a critical role in the formation of morphine-related learning and memory.

A number of recent reports have demonstrated that drug-induced CPP requires the VTA and its local NMDAR signaling. For example, reversible inactivation of the VTA by lidocaine decreased the acquisition of morphine-induced CPP (Moaddab, Haghparast & Hassanpour-Ezatti 2009). Pharmacological studies showed that intra-VTA injections of NMDA receptor antagonists blocked the acquisition of cocaine or morphine CPP (Harris & Aston-Jones 2003; Harris *et al.* 2004). However, there still exist

disparities regarding the role of NR1 subunit of the NMDARs in VTA DA neurons in developing CPP. Loss of NR1 expression in DA neurons completely blocked cocaine CPP (Zweifel *et al.* 2008), while another two publications supporting a role of NMDARs on non-dopaminergic neurons reported intact CPP with inactivated NR1 in DA neurons (Engblom *et al.* 2008; Luo *et al.* 2010). In our present study, except for dopamine DR1 activation in the DG, we also found that morphine-induced CPP requires both NMDAR activation in the VTA and VTA/DG dopaminergic connections. Although either DR1 activation in the DG or NMDAR activation in the VTA alone is necessary for morphine-induced CPP, it is noticeable that VTA/DG loop is also very important. Our behavioral results that the inhibition of morphine CPP caused by AP5 in the VTA is reversed by intra-DG injection of SKF38393 indicated that the conditioned behavioral response to morphine depends on a hierarchic process involving VTA/DG dopaminergic connections.

In this study, we focused on the MPP-DG pathway, because the MPP conveys contextual and spacial information from the entorhinal cortex to the DG (Ferbinteanu, Holsinger & McDonald 1999; Tang & Dani 2009). Furthermore, the DG is mostly involved in sustaining a primary representation of space during learning (Rolls 1996; Rolls & Kesner 2006). It is not difficult to understand why the formation of associations between drug use and environmental stimuli requires dopamine D1R activation in the DG. Synaptic adaptations within the mesocorticolimbic DA system that occur in response to addictive drugs may contribute to addiction-related behaviors (Kauer & Malenka 2007; Thomas, Kalivas & Shaham 2008; Kalivas *et al.* 2009; Bowers, Chen & Bonci 2010; Russo *et al.* 2010; Schmidt & Pierce 2010; Wolf & Ferrario 2010). The recent finding that reversal of cocaine-evoked potentiation abolished locomotor sensitization to cocaine provided further evidence supporting the view (Pascoli, Turiault & Luscher 2012). In combination with the behavioral findings from our present study, the electrophysiological results showing the preventive effect of local inhibition of the dopamine D1R in the DG on morphine-induced synaptic potentiation seems to support this notion.

In conclusion, the present study demonstrated that intra-VTA but not intra-DG injection of morphine induced synaptic potentiaton at the MPP-DG synapse, and the drug-evoked synaptic potentiation or CPP required both NMDAR activation in the VTA and VTA/DG dopaminergic connections. These findings suggest that NMDAR signals in the VTA play a key role in regulating dopamine-mediated hippocampal synaptic plasticity underlying the conditioned behavioral response to morphine.

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Authors Contribution

LH conducted the experiments, analyzed the data and drafted the manuscript. XHJ provided critical revision of the manuscript for important intellectual content. CLC and GGX helped on the technical supports. BZ guided the project and finalized the manuscript. All authors have critically reviewed content and approved final version submitted for publication.

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