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NR2B-containing NMDA receptor is required for morphine-but not stress-induced reinstatement

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Abstract

Glutamate receptors are known to be densely distributed in the forebrain rewarding circuits, and glutamatergic transmission is actively involved in the regulation of rewarding and reinstating effects of drugs of abuse. Here we investigated the possible involvement of the *N*-methyl-D-aspartate (NMDA) receptors in the reinstatement of extinguished morphine conditioned place preference (CPP) in rats. We found that previously extinguished morphine (3 mg/kg, i.p.) CPP was markedly reinstated by a priming injection of morphine (2 mg/kg, i.p.) or an acute environmental stressor (forced swim for 10 min), but not by the stress induced by a 24-h food deprivation. Parallel with this, protein levels of the NMDA receptor 2B subunit (NR2B) were elevated in the nucleus accumbens (NAc) and the hippocampus, but not the prefrontal cortex, of reinstated rats. Systemic administration of an NR2B selective antagonist ifenprodil (1, 3, 10 mg/kg, i.p.) attenuated the reinstatement induced by a priming morphine injection, although not by the forced swim. Ifenprodil (2.0 μg/rat) directly injected into the NAc shell or the CA1 region of the dorsal hippocampus produced a similar effect. These results indicate that the NR2B-containing NMDA receptors in the NAc and the dorsal hippocampus play a significant role in mediating the reinstatement of rewarding responses to morphine.

Keywords: Conditioned place preference; Morphine; Nucleus accumbens; Hippocampus; Forced swim; Food deprivation; Rats

Introduction

Relapse to drug abuse following prolonged periods of abstinence remains the most difficult challenge for drug abuse treatment (O'Brien, 1997). Relapse has been modeled in animal studies through reinstatement of responses to drug self-administration (SA) or conditioned place preference (CPP) induced by drug, cue, or stress (de Wit and Stewart, 1981; de Wit and Stewart, 1983; Kreibich and Blendy, 2004; Lu et al., 2005; Ribeiro Do et al., 2003; Shi et al., 2004). It was reported that noncontingent priming injections of drug reinstated extinguished CPP (Parker and Mcdonald, 2000; Lu et al., 2002; Itzhak and Martin, 2002). Several stressors, including footshock, induced reinstatement of previously extinguished response associated with drug reward in SA-extinct rats (Goeders, 2002). However, the efficacy of the forced swim (FS), reported to effectively reinstate cocaine CPP (Kreibich and Blendy, 2004), and food deprivation (FD), successful in reinstatement of cocaine-seeking behavior (Carroll, 1985), have not yet been evaluated in the reinstatement of morphine CPP.

Reinstatement of conditioned responses to drug reward involves the mesolimbic dopamine system: the ventral tegmental area (VTA) and the nucleus accumbens (NAc) (Placenza et al., 2004; Rompre and Wise, 1989; Ranaldi et al., 1999; Kalivas and McFarland, 2003; McFarland et al., 2004). In addition, stressors recruit additional brain structures including the hippocampus (Black et al., 2004), the medial prefrontal cortex (mPFC) (Fuchs et al., 2005; Sun and Rebec, 2005; Ventura et al., 2005), and the extended amygdala (Amy) complex (Alheid and Heimer, 1988; Heimer, 2003) to reinstate previously extinguished responses (Capriles et al., 2003; Shaham et al., 2003; McFarland et al., 2004). While dopamine plays a major role in these circuits, some evidence also indicates

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that glutamate is equally important in the regulation of reinstatement through these circuits (Wang et al., 2005). This is supported by a dense expression of glutamate receptors in these areas. Among the glutamate receptor subtypes, *N*-methyl-D-aspartate (NMDA) receptors (NMDARs) have drawn most attention. Most NMDARs in the brain are heteromeric complexes consisting of constructive NR1 and functional NR2 (A–D) subunits (Narita et al., 2000). Distributions of NR1, NR2A and NR2B, but not NR2C and NR2D, are consistently seen in the brain regions related to the reinstatement of drug seeking (Mori and Mishina, 1995). Very recently, Ribeiro Do et al. (2005) reported that the reinstatement of morphine CPP may be largely independent of dopaminergic transmission, while the activation of NMDARs seems to underlie this process. However, it is unclear how different subunits of NMDARs contribute to the aforementioned phenomenon.

The present study was therefore designed to define the subunit-specific contribution of NMDARs to the regulation of morphine CPP by examining changes in expression of individual NMDA receptor subunit proteins in the reinstatement-related brain regions. Furthermore, the effect of NMDA receptor antagonist on the reinstatement of morphine CPP was explored. Ifenprodil, an antagonist highly selective for the NR2B subunit, was used in the present study for antagonizing NR2B activities following its systemic or intracranial administration.

Materials and methods

Subjects

All experiments were performed on male Sprague-Dawley rats, obtained from the Experimental Animal Center, Peking University, weighing 180–200 g at the beginning of the experiment. The total number of rats used in the present experiments was 494; due to unsatisfied cannulae placements and natural bias to the CPP apparatus, etc., 87 rats were excluded. Animals were housed 4 per cage in a 12:12-h light/dark cycle (lights on at 07:00 h) with food and water available at all times. The room temperature was maintained at 22±1°C and relative humidity at 45%–55%. Animals were conditioned and tested during the light phase of the cycle. They were handled daily during the first week after arrival. All experimental procedures were approved by the Animal Use Committee of Peking University Health Science Center.

Apparatus

Conditioning was conducted in black colored rectangular PVC boxes (795×230×250 mm^3), containing three chambers separated by guillotine doors (Shi et al., 2004). The two large black conditioning chambers (A and C, 280×220×225 mm^3) were separated by a small gray center choice chamber B (135×220×225 mm^3). Chamber A has 4 light-emitting diodes (LEDs) forming a square on the wall and a stainless steel mesh floor (225×225 mm^2), chamber C has 4 LEDs forming a triangle on the wall and a stainless-steel rod floor (15 mm apart), whereas chamber B has a plain floor. Fourteen photobeams were placed across chambers with 47.5 mm apart. Through a computer interface, the time spent for the rat in each chamber was recorded by means of infrared beam crossings.

Place preference paradigm

The conditioned place preference paradigm was performed as follows (Table 1).

Preconditioning phase

On day 0, rats were placed in the center choice chamber with the guillotine doors open to allow access to the entire apparatus for 15 min, and time spent in each side was recorded. These data were used to assign animals into groups with approximately equal preference for each side.

Conditioning phase

Beginning on day 1, the animals were allocated to stay for a period of 45 min in the lateral chambers twice daily (09:00 and 15:00) for 4 days, with the saline group receiving 0.9% sodium chloride injection on both sides of the boxes, whereas the drug group received morphine (3 mg/kg, i.p.) on one side and saline on the other side. Drug-paired sides were counterbalanced among all groups.

Testing phase

On day 5 at 09:00, all of the animals were placed in the center choice chamber with the guillotine doors open to allow free access to the entire apparatus for 15 min, and the time spent in each side was recorded.

Table 1
Experimental paradigm for reinstatement

Day	0	1–4	5	6–67	68	69–71	72
Treatment	Pre-test	Conditioning (M-S)×4	Test	Natural extinction	Test	Ifenprodil	Reinstatement

Experimental paradigm for research on reinstatement of morphine-induced CPP Preconditioning test (Pre-test): animals are tested for initial bias. Conditioning phase (Conditioning): distinct sides of conditioning boxes are paired with either morphine (M) (3 mg/kg, i.p.) or saline (S) injections on days 1–4, a 45-min period twice daily. Test is given on day 5 to examine preference. Natural Extinction: No intervening tests are performed. Test is given on day 68 to confirm that CPP is extinguished. In order to explore the effect of ifenprodil on reinstatement of morphine CPP, ifenprodil is injected (systemic or local, once a day from the 69th day to the 71st day). Arrow represents exposure to either morphine priming or stress. Morphine prime is a morphine injection (2 mg/kg, i.p.) given 15 min before testing. One of the stressors is 10-min forced swim, followed 15 min later by exposure to the conditioning boxes. Another is 24-h food deprivation. Gray columns represent tests of place preference.

Natural extinction phase

After the expression of CPP, rats were randomly distributed into 8 groups of 10–12 rats each. Subsequent tests for CPP were performed only once for each group. At every test point of the 12th, 20th, 36th, 68th days, two corresponding groups of 10–12 rats each, trained by morphine and saline respectively, were tested in CPP chambers. The time spent in each side was recorded. CPP was found to be completely extinguished on the 68th day (Fig. 1).

Reinstatement phase

Reinstatement of CPP induced by priming injection of morphine. After the natural extinction of CPP, rats were given a priming injection of morphine (2.0 mg/kg, i.p.) 15 min before being placed in the center choice chamber with access to the entire apparatus for 15 min. The time spent in each side was recorded. Rats in the parallel control group received an injection of saline.

Reinstatement of CPP induced by forced swim. After the natural extinction of CPP, rats were given a forced swim stress and placed in the center choice chamber with access to the entire apparatus for 15 min. The procedures used for swim stress were identical to those described previously by Borsini and Meli (1988) and Roche et al. (2003). On the 72nd day at 09:00, rats were placed in a cylindrical glass tank (45 cm high × 35 cm diameter) filled with water (25 ± 1°C) to a depth of 30 cm for 10 min. The 30-cm depth of water allowed rats to swim or float without having their tails touching the bottom of the tank. Immediately after the 10-min swim, rats were removed from the tank, towel dried, and put in a warming cage (37°C) that contained a heating pad covered with towels for 15 min. Rats were then placed in the center choice chamber with access to the entire apparatus for 15 min. Rats in the non-stress group were left in the home cage until CPP testing.

Reinstatement of CPP induced by food deprivation. From 08:00 of the 71st day to 08:00 of the 72nd day, rats received a 24-h food deprivation, with water available *ad libitum*. They were then placed in the center choice chamber with access to the entire apparatus for 15 min. Rats in the non-stress group were left in the home cage with food and water available *ad libitum* until CPP testing.

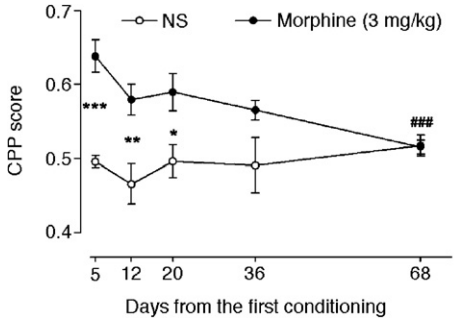

Fig. 1. Natural extinction of morphine-induced CPP. All animals used in this section of experiment experienced only one test for place preference after the completion of CPP training. Complete extinction of CPP induced by 3 mg/kg morphine occurred no later than day 68. Data are expressed as mean ± SEM, $n = 10–12$ in each group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared between morphine groups and saline groups at the same days. $^{\#\#\#}P<0.001$ compared with day 0 among the morphine groups (two-way ANOVA, Bonferroni post-test).

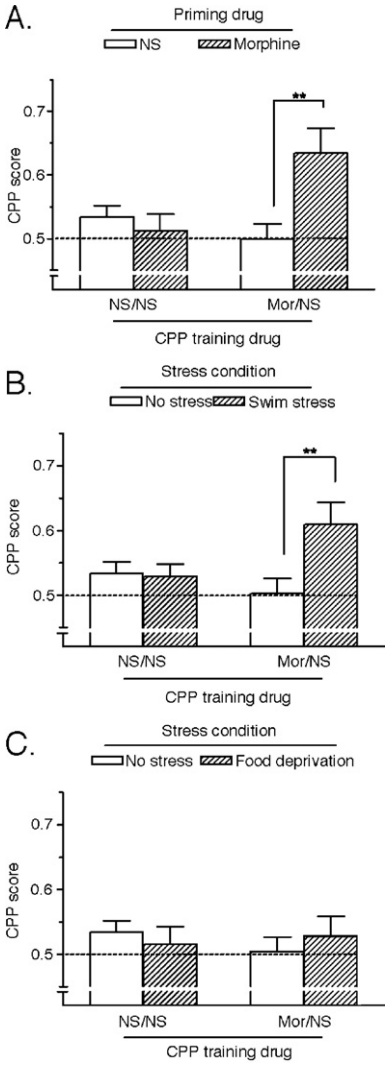

Fig. 2. Reinstatement of morphine CPP by morphine priming (A), forced swim stress (B), but not food deprivation (C). In conditioning, NS/NS means receiving injections of normal saline on both sides of the sides, Mor/NS means receiving morphine on one side in the morning and saline on the other side in the afternoon in the conditioning phase. Data are expressed as mean ± SEM, $n = 9–12$ in each group. $^{*}P<0.05$, $^{**}P<0.01$.

Implantation of microinjection cannulae

On day 61, the rats were treated with atropine methyl nitrate (0.4 mg/kg, i.p.), and penicillin (1.5×10^5 U/rat) and were anesthetized with chloral hydrate (35 mg/kg, i.p.), and mounted on a Kopf stereotaxic apparatus (Kopf Instruments, Tujunga, CA). The scalp was incised and retracted, and the head position was adjusted to place bregma and lambda in the same horizontal plane. Small burr holes (1 mm in diameter) were drilled on the skull for bilateral placement of stainless steel guide cannulas (0.8 mm in outer diameter) into the NAc shell (anteroposterior

(AP) +1.5 mm, lateral (L) ±0.9 mm, dorsoventral (DV) −6.5 mm) and the CA1 region of the dorsal hippocampus (AP −3.8 mm, L ±2.2 mm, DV −2.7 mm), 2 mm and 1 mm above the intended site of injection, respectively. Guide cannulae were anchored to the skull with sterile stainless steel screws and the dental acrylic cement. After surgery, stainless steel obturators (0.4 mm in outer diameter) were inserted into the guides in order to prevent cannula occlusion. The obturators were removed and replaced every other day during the 7-day recovery period. To habituate animals to the microinjecting

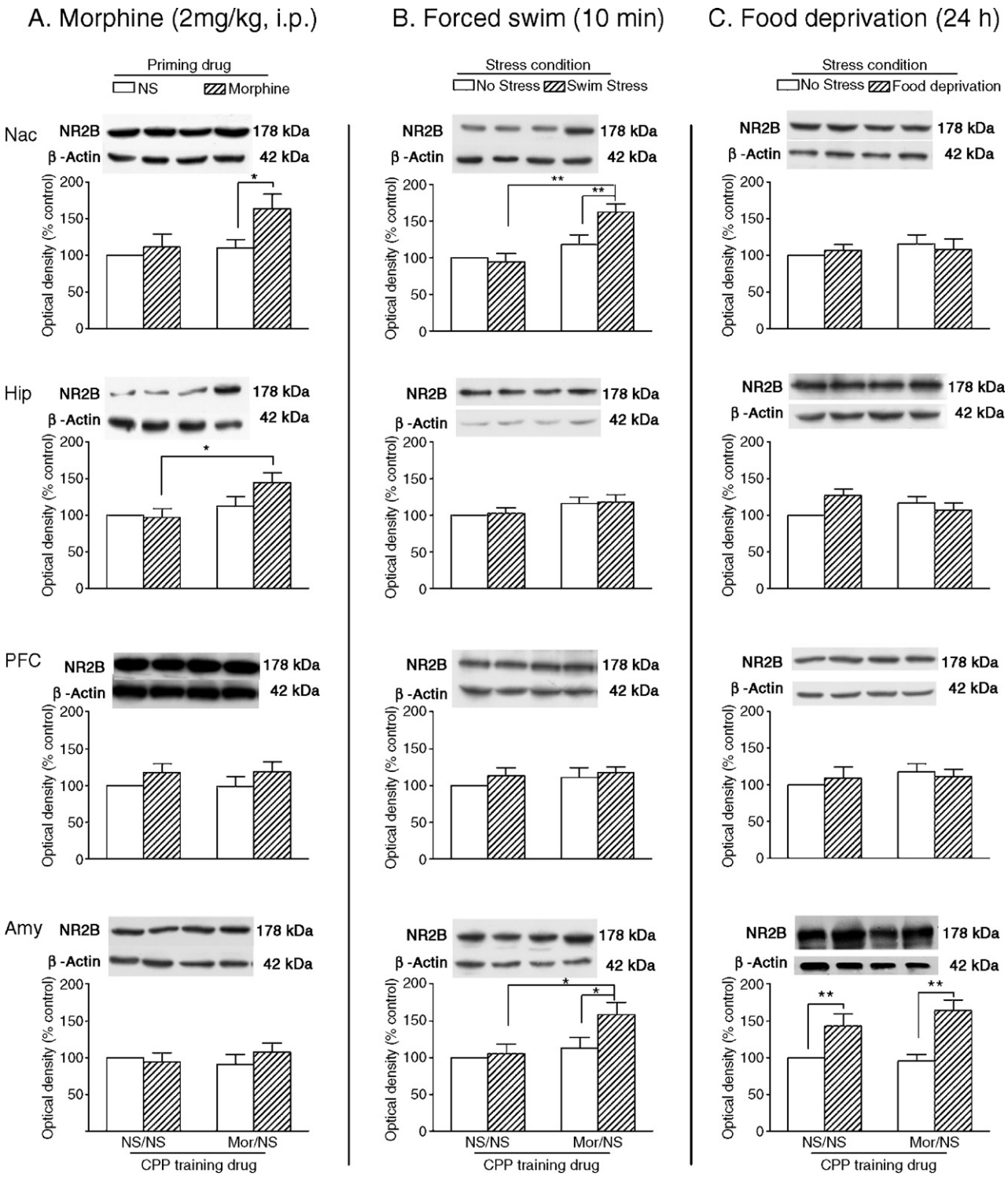

Fig. 3. Change of NR2B levels in the brain regions related to reinstatement. (A) NR2B levels in the nucleus accumbens (NAc) and the hippocampus (Hip) increased after morphine-induced reinstatement of morphine place preference. (B) NR2B levels of the NAc and the amygdala (Amy) increased after force swim-induced reinstatement of morphine place preference. (C) NR2B levels of the Amy increased after food deprivation-induced reinstatement of morphine place preference. Data are expressed as mean±SEM, $n=4$. *$P<0.05$, **$P<0.01$. The abbreviations bear the same meaning as in Fig. 2.

procedures, all rats were given daily 'mock' microinjection for 3 days, with injector placed within but did not extend beyond the guide cannula, prior to their first microinjection. The infusion pump did not engage the syringe plungers during this mock infusion procedure but was allowed to run for 3 min to habituate the rats to the weak noise of the pump.

Microinjections

Subjects were microinjected from day 69 to day 71, before reinstatement testing. Obturators were removed and bilateral infusion cannulae (0.4 mm in outer diameter) were inserted, extending 2.0 mm and 1.0 mm beyond the tip of guide cannulae in the NAc shell and the CA1, respectively. Bilateral microinjections of vehicle or ifenprodil (1.0 μg/0.5 μl per side; Sigma, St. Louis, MO) were administered into the nuclei. This dose of ifenprodil was chosen according to the estimate that the dose of the drug microinjected into brain nucleus should be around 0.1% of the dose for systematic injection. Since systematic injection of ifenprodil (10 mg/kg or 2 mg for a rat of 200 g) produced complete inhibition of morphine-induced reinstatement of CPP in rats (Fig. 4A), a dose of 2.0 μg/rat was chosen for intra-nuclear injection. The injection was performed through an infusion pump (0.1 μl/min) while the rat was gently held. The microinjectors were left in place for an additional minute to allow for drug diffusion. The obturators were then replaced, and rats were placed back to their home cages.

Histology

Histological verification of cannula location was performed after behavioral testing. Rats were anesthetized with chloral hydrate (35 mg/kg, i.p.) and perfused across the heart with 0.9% saline (200 ml) followed by 10% formalin solution (30 ml). After removal from the skull, the brains were post-fixed in 10% formalin solution for 2 d and in 10% formalin and 30% sucrose solution until sectioning. Coronal sections (50 μm thick) were cut on a cryostat (−19°C) and wet-mounted on glass microscope slides. Only those animals whose cannulae were correctly placed were used for data analysis.

Tissue dissection and preparation

Rats were decapitated immediately after testing for reinstatement. The brains were removed and placed on an ice-cooled plate for dissection of the NAc, the hippocampus, the amygdala, and the prefrontal cortex according to stereotaxin atlas of Paxinos and Watson (1997). Tissue samples were frozen in liquid nitrogen and stored at −80°C until analysis. The tissue was homogenized in 10 volumes of ice-cold buffer containing 20 mM Tris–HCl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulfonyluoride, 25 mg/ml of leupeptin, 0.1 mg/ml of aprotinin and 0.32 M sucrose using a Potter-Elvehjem tissue grinder with Teflon pestle. The homogenate was then centrifuged at 1000×*g* for 10 min and the supernatant was centrifuged at 100,000×*g* for 30 min at 4°C. The pellet was then homogenized in homogenizing buffer containing 0.2% (w/v) Triton X-100. The homogenate was kept at 4°C for 60 min with occasional stirring and then centrifuged at 100,000×*g* for 30 min at 4°C. The resulting supernatant was used as the membrane fraction. Protein concentrations were determined using a BCA assay (Pierce, Rockford, IL).

Immunoblotting of NMDA receptor subunits

Equivalent amounts of membrane preparations (50 μg) for each sample were resolved in 7.5% SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were incubated in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBST) containing 5% nonfat milk for 1 h at room temperature with agitation to block nonspecific binding. The membrane was incubated with primary antibody diluted in TBS (NMDAζ1 (NR1), NMDAε1 (NR2A) and NMDAε2 (NR2B), 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) containing 5% nonfat dried milk overnight at 4°C. The membrane was washed twice for 5 min and then twice for 10 min in TBST, followed by 1 h of incubation at room temperature with horseradish peroxidase-conjugated rabbit anti-goat IgG (Zhongshan Biotechnology, Beijing, China) diluted 1:10,000 in TBS containing 5% nonfat dried milk. After this incubation, the membranes were washed twice for 5 min and then three times for 10 min in TBST. The antigen–antibody peroxidase complex was finally detected by enhanced chemiluminescence (Zhongshan Biotechnology, Beijing, China) according to the manufacturer's instructions and visualized by exposure to Kodak film (Eastman Kodak, Kodak, NJ). The bands on the autoradiogram were quantified with the TotalLab 2.01 Analysis System (Phoretix,

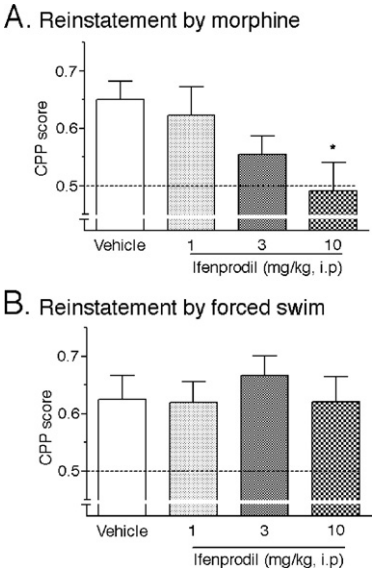

Fig. 4. Effect of ifenprodil (i.p., once a day from the 69th day to the 71st day) on reinstatement of morphine CPP. (A) Dose-dependent reduction of morphine-induced reinstatement of morphine CPP after systemic injection of ifenprodil. (B) No effect of systemic injection of ifenprodil on forced swimming-induced reinstatement of morphine CPP. Data are expressed as mean ± SEM, $n = 9–12$ in each group. *$P < 0.05$, compared with the group injected with vehicle. The abbreviations bear the same meaning as in Fig. 2.

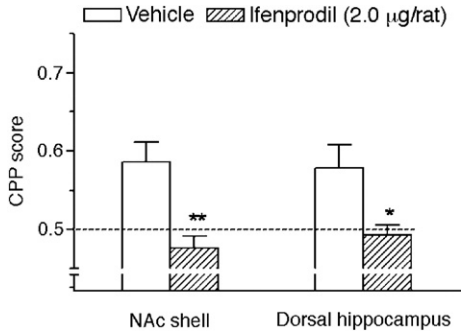

Fig. 5. Effect of microinjection of ifenprodil (once a day from the 69th day to the 71st day) into brain regions on morphine-induced reinstatement. The reinstating effect of morphine priming is inhibited by microinjection of ifenprodil into either the NAc shell (left pair of columns) or the CA1 region of dorsal hippocampus (right pair of columns). Data are expressed as mean±SEM, $n=9$–11. *$P<0.05$, **$P<0.01$, compared with group injected with vehicle. The abbreviations bear the same meaning as in Fig. 2.

UK), and the optical density of each band of the NMDA receptors (NMDARs) subunits was corrected by the optical density of the corresponding β-actin band. The values are presented as a percentage of the control.

Drugs

Morphine hydrochloride was purchased from the first pharmaceutical factory of Shenyang, China. Ifenprodil was purchased from Sigma, USA. Morphine was dissolved in saline. Rats were injected with morphine at 3 mg/kg, i.p. in the conditioning phase of CPP and morphine at 2 mg/kg, i.p. in the reinstatement phase. Ifenprodil tartrate was dissolved in DMSO (dimethylsulfoxide, from Sigma, USA) and diluted in 5% DMSO with 9% Tween 80/saline before use; the later solution was used for vehicle injections. Ifenprodil was injected intraperitoneally (1, 3, 10 mg/kg) or microinjected intracranially (2.0 μg/rat).

Statistical analysis

CPP score represents the index of place preference for each rat, calculated by dividing the time spent in the drug-paired compartment by the time spent in both conditioning compartments (Shi et al., 2004). Data were processed by commercially available software Graph Pad Prism 4.0. Results were presented as mean±SEM. Results from Figs. 1–3 were analyzed with two-way analysis of variance (ANOVA) followed by Bonferroni post-test, results from Fig. 4 were analyzed with one-way ANOVA followed by Newman-Keuls post-test, and that from Fig. 5 were analyzed with two-tailed Student's *t*-test. The accepted level of statistical significance is $P<0.05$.

Results

Reinstatement of morphine place preference

Natural extinction of morphine-induced place preference

174 rats received preconditioning test on day 0 (mean value of CPP score was 0.49), morphine/saline alternative injections (88 rats, as morphine CPP rats) or saline injections twice a day (86 rats, as CPP control rats) from day 1 to day 4, then five paired groups of 10–15 CPP rats and 10–15 control rats were tested the CPP score at day 5, 12, 20, 32, 68 respectively. Fig. 1 showed the curve for natural extinction of CPP. Complete extinction of morphine-induced place preference occurred at day 68. Two-way ANOVA indicated a significant effect of the training drug (morphine/saline or saline/saline) [$F(1,105)=31.10$, $P<0.0001$] and the interaction between training drug and day [$F(4,105)=3.06$, $P=0.0198$]. There was no significant difference among the variable days [$F(4,105)=1.57$, $P=0.1874$].

Reinstatement of the extinguished place preference

320 rats received preconditioning test on day 0 (the mean value of CPP score was 0.49), conditioning form day 1 to day 4, post-conditioning test on day 5 (mean value of score in morphine CPP rats was 0.63, and in control rats was 0.50). After natural extinction of a 64-day period, rats showed no significant preference to the morphine-paired compartment (the mean value of morphine CPP score was 0.50). Therefore, reinstatement was tested at day 72 in order to investigate the reinstating effects of morphine priming and stressors on CPP (Fig. 2) and the influence of ifenprodil [systematically injected (in Fig. 4) or microinjected into brain regions (in Fig. 5)] on their reinstating effects.

Morphine priming induces reinstatement of morphine place preference

Fig. 2A shows the effect of morphine priming on reinstatement of extinguished morphine CPP. Two-way ANOVA indicated significant effects of the priming drug [$F(1,40)=4.71$, $P=0.0360$] and the interaction between priming drug and CPP training drug [$F(1,40)=9.23$, $P=0.0042$], although no significant effect of CPP training drug [$F(1,40)=2.88$, $P=0.0976$] was observed. Bonferroni post-test showed that an extinguished morphine CPP could be robustly reinstated by a noncontingent administration of a priming dose of morphine (2 mg/kg, i.p.) ($P<0.01$ compared with the group conditioned by morphine and not subjected to morphine priming), a result in agreement with previous studies using the place preference paradigm as a model to study the mechanisms of relapse to drug addiction (Popik et al., 2006). The priming injection had no effect on saline-trained rats.

Forced swim test induces reinstatement of morphine place preference

Fig. 2B shows the effect of FS on reinstatement of extinguished morphine CPP. Two-way ANOVA indicated significant effects of the stress condition (no stress or swim stress) [$F(1,42)=4.56$, $P=0.0387$] and the interaction between stress condition and CPP training drug [$F(1,42)=5.38$, $P=0.0253$], although no significant effect of CPP training drug [$F(1,42)=1.10$, $P=0.2992$] was observed. Bonferroni post-test showed that rats previously demonstrating CPP and exposed to the swim stress display a significant preference for the side previously paired with morphine ($P<0.05$ compared

with the group conditioned by morphine and not subjected to swim stress). The swim stress exposure had no effect on saline-trained rats.

Food deprivation failed to induce reinstatement of morphine place preference

Fig. 2C shows the effect of food deprivation (FD) on reinstatement of extinguished morphine CPP. Two-way ANOVA showed no significant effects of the stress condition (FD or no stress) [$F(1,43)=0.02$, $P=0.9013$], the CPP training drug [$F(1,43)=0.14$, $P=0.7107$], and the interaction between these two factors [$F(1,43)=0.70$, $P=0.4090$], indicating that FD failed to induce reinstatement of morphine CPP.

Change of NMDARs subunits levels following reinstatement of morphine place preference

To investigate the molecular correlates of the reinstatement behavior, we assessed changes in the levels of NMDARs subunits in brain regions associated with reward and drug craving. Western blotting analysis of membrane proteins with a goat polyclonal immunoglobulin G specific to each NMDA receptor subunit revealed a significant increase in the levels of NR2B subunit in discrete brain regions among three strategies of reinstatement, but no change in the protein levels of NR1 and NR2A subunits in the NAc, the hippocampus, the prefrontal cortex, and the amygdala (data about NR1 and NR2A were not shown). The results are shown in Fig. 3.

NR2B levels in the NAc and the hippocampus increased following morphine-induced reinstatement of morphine place preference

Fig. 3A showed the effects of morphine priming on the change of NR2B subunits levels. In the data from the NAc, hippocampus, PFC, and amygdala, two-way ANOVA revealed a significant effect of priming drug in the NAc [$F(1,12)=5.21$, $P=0.0415$] and a significant effect of CPP training drug in the hippocampus [$F(1,12)=0.10$, $P=0.0206$], although no other significant effects were found after morphine priming. Bonferroni post-test revealed that in the morphine-induced CPP rats, morphine prime injection increased NR2B levels in the NAc ($P<0.05$, compared with the group subjected a saline priming); in the rats subjected to morphine priming, CPP training by morphine increased NR2B levels in the hippocampus ($P<0.05$, compared with the CPP training by saline).

NR2B levels in the NAc and the amygdala increased following FS-induced reinstatement of morphine place preference

Fig. 3B showed change of NMDARs subunits levels after swim stress. In the data from the NAc, hippocampus, PFC, and amygdala, two-way ANOVA revealed significant effects of CPP training drug [$F(1,12)=17.17$, $P=0.0014$] and the interaction of CPP training drug and stress condition (no stress or swim stress) [$F(1,12)=5.53$, $P=0.0366$] in the NAc; there was a significant effect of CPP training drug [$F(1,12)=7.15$, $P=0.0203$] in the amygdala; no other significant effect were observed in these four brain regions. Bonferroni post-test revealed increased NR2B levels in the NAc and the amygdala from morphine trained CPP rats subjected to swim stress ($P<0.01$ in the NAc and $P<0.05$ in the amygdala, compared with morphine trained CPP rats without exposure to swim stress, or rats trained by saline and subjected to swim stress).

NR2B levels in the amygdala increased after FD-induced reinstatement of morphine place preference

Fig. 3C showed change of NMDARs subunits levels after food deprivation stress. In the data from the NAc, hippocampus, PFC, and amygdala, two-way ANOVA revealed a significant effect of stress condition (FD or no stress) in the amygdala [$F(1,12)=22.87$, $P=0.0004$], but no other significant effects were observed in these four brain regions. Bonferroni post-test revealed increased NR2B levels in the amygdala from morphine trained CPP rats subjected to FD stress ($P<0.01$, compared with morphine trained CPP rats subjected to no stress, or rats trained by saline and subjected to swim stress).

Effect of systemic injection of ifenprodil on reinstatement of morphine place preference

To determine whether an active involvement of NR2B-containing NMDARs is necessary for reinstatement, rats were given systemic injection of different doses of ifenprodil (1, 3, 10 mg/kg) after the extinction of morphine CPP. Fig. 4 showed the effect of systemic injection of ifenprodil on reinstatement induced by morphine priming (Fig. 4A) and FS (Fig. 4B). One-way ANOVA indicated ifenprodil dose-dependently inhibited the reinstating effect of morphine priming [$F(3,42)=2.929$, $P<0.0446$], but had no influence on the reinstating effect of FS [$F(3,40)=0.3218$, $P=0.8095$].

Effect of microinjection of ifenprodil into brain regions on morphine-induced reinstatement

Based upon findings after systemic administration of ifenprodil we decided to identify brain regions involved in the inhibitory effect of ifenprodil on morphine-induced reinstatement. After the extinction of morphine CPP, ifenprodil was microinjected for 3 consecutive days, once a day from day 69 to day 71. Student's *t*-test indicated microinjection of ifenprodil into the NAc as well as the CA1 region of the dorsal hippocampus inhibited the reinstating effect of morphine priming [$t(19)=3.902$, $P=0.0010$, $t(16)=2.612$, $P=0.0189$ respectively] (Fig. 5).

Histological verification of injection sites

Fig. 6 shows the distribution of infusion sites in the NAc shell and the CA1 region of the dorsal hippocampus, plotted on drawings of coronal sections from the atlas of Paxinos and Watson (1997). Cannula placements in the NAc shell were medial to the anterior commissure (Fig. 6A), and that in the

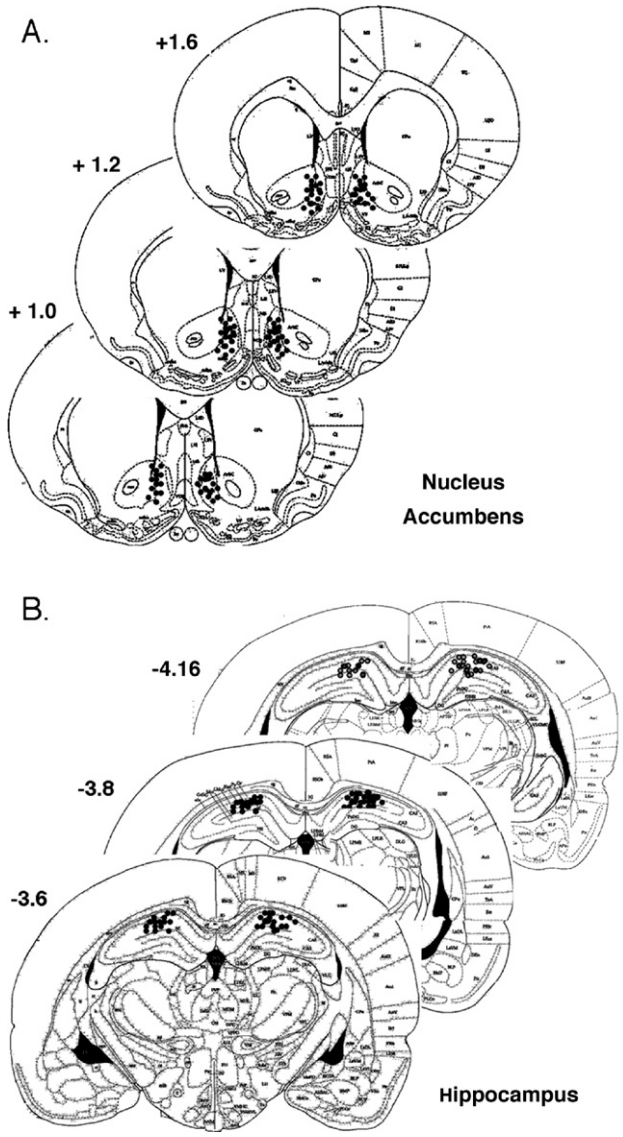

Fig. 6. Distribution of infusion sites in the nucleus accumbens shell (A) and the CA1 region of the dorsal hippocampus (B), plotted on drawings of coronal sections from the atlas of Paxinos and Watson (1997).

CA1 region were in the dorsal hippocampus (Fig. 6B). No excessive mechanical damage caused by the repeated micro-injections into any of the brain regions was observed.

Discussion

In the present study we found that previously extinguished morphine (3 mg/kg, i.p.) CPP could be markedly reinstated by a priming injection of morphine (2 mg/kg, i.p.) or an acute environmental stressor (forced swim for 10 min), but not by the stress induced by a 24-h food deprivation. Concerning the effective brain sites, NR2B subunit levels were elevated in the NAc and hippocampus, but not the prefrontal cortex, of reinstated rats. In accordance with this, microinjection of ifenprodil (2.0 μg/rat) directly into the NAc shell as well as the CA1 region of the dorsal hippocampus inhibited the reinstating effect of morphine priming. Comparing drug with natural reinforcement, it was found that systemic administration of an NR2B selective antagonist ifenprodil (1, 3, 10 mg/kg, i.p.) dose-dependently attenuated the reinstating effect of morphine priming, but had no influence on the reinstating effect of forced swim stress. These results indicate that the NR2B-containing NMDARs in the NAc and the dorsal hippocampus might play a significant role in mediating the reinstatement of rewarding responses to morphine.

There are two ways to observe the process of extinction of CPP: the natural decay and an active training of using conditioning cue without reinforcement. To avoid complicated procedure of repeated exposure to environmental cue, we have chosen a natural-extinguishing paradigm. Three different strategies were adopted to reinstate CPP. In accordance with earlier reports (Ribeiro Do et al., 2005; Shi et al., 2004; Wang et al., 2000), an extinguished morphine-induced CPP can be robustly reinstated by a noncontingent administration of a priming dose of morphine (2 mg/kg, i.p.).

Exposure to stress has been shown to dramatically increase the vulnerability to drug abuse (Shalev et al., 2001a; Stewart, 2003). Generally, to elicit stress-induced reinstatement of self-administration, the stressor must be given in the same context as the drug conditioning. For example, footshock stress given within the self-administration chambers reinstated heroin-seeking behavior (Shalev et al., 2000). The same procedure of footshock stress given outside the conditioning context was reported ineffective (Shalev et al., 2000). However, since external stressors outside of the drug-taking environment did induce relapse in humans (Shiffman, 1982; Sinha et al., 2000; Sinha et al., 1999), it seems worthwhile to test in animal models whether external stressor could lead to reinstatement of CPP.

FS stress is a common behavioral paradigm used to evaluate antidepressant efficacy. In the present study, we observed that exposure to FS reinstated morphine place preference in rats. FS was performed in an area separate from the conditioned chambers, suggesting that exposure to a stress in an environment distinct from conditioning can also induce reinstatement of place preference. Deprivation of food has also been used to elicit reinstatement (Shalev et al., 2001b; Highfield et al., 2002). This is another environmental stressor performed in the home cages, a context different from that of conditioning. On the other hand, deprivation of food may be characterized as a chronic 'internal' stressor, as contrast to FS which is an acute discrete 'external' stressor.

It is interesting to note that an experience of 24-h fasting failed to reinstate morphine CPP. However this negative result may not be conclusive. For example, several studies have shown that reinstatement of drug seeking is profoundly affected by the duration of the withdrawal period following the termination of heroin and cocaine SA. Highly responding to drug seeking was only detected in the middle-term (from the 6th day till the 25th day) of withdrawal period (Shalev et al., 2001a), whereas in the present study reinstatement was tested 64 days after the last conditioning. Further studies may help to find the time window to depict this phenomenon.

The major hypothesis that guides the current neurobiological research on drug addiction is that chronic drug exposure causes

long-lasting neuroadaptive changes in the brain at the molecular and cellular levels that may contribute to compulsive drug use and relapse (Nestler, 2004; Chao and Nestler, 2004). It is reasonable to believe that at least some of these molecular/neurochemical alterations are associated with reinstatement of drug seeking (Shaham et al., 2003). Glutamate receptors, especially NMDARs were reported to be critically involved in opiate-induced neural and behavioral plasticity (Trujillo, 2000). Ribeiro Do et al. (2005) presented the first report that NMDA – but not dopamine – receptor antagonists blocked drug-induced reinstatement of morphine CPP. Popik et al. (2006) showed that memantine treatment during extinction period may abolish the ability of drug-related cues to evoke reinstatement. Memantine was a noncompetitive low-to-moderate affinity NMDA receptor antagonist, and nonselective for NMDA receptor subunits. To our knowledge, there are no reports so far on specific NMDA receptor subunits in mediating reinstatement of drug seeking and addiction memory. In the present study, an over-expression of NR2B subunit in the NAc was observed in rats with a relapse into CPP, induced by both a priming injection of morphine and 10-min FS. An over-expression of NR2B subunit in the hippocampus, however, was only observed in reinstatement induced by morphine but not by FS. It is thus proposed that an up-regulation of NR2B subunit in the NAc is a common phenomenon in the reinstatement of CPP, while in the hippocampus it may be relatively specific for morphine-induced reinstatement. Our data also demonstrated that an increase of NR2B level in the amygdala is inducible more easily by stress than by drug. Considering that NR2B levels are increased in the amygdala on reinstatement day after an exposure to FD, regardless of whether animals were trained by saline or morphine in the conditioning paradigm, we propose that up-regulation of NR2B in the amygdala is a consequence of stress, which then mediates stress-related effects on behavior such as anxiety-like behavior and unconditioned startle (Adamec et al., 1999a, b; Maroun and Richter-Levin, 2003). Therefore, the increase of NR2B in the amygdala may not participate in reinstatement of CPP, although further research is required to elaborate this possibility.

While all functional NMDARs appear to contain NR1 subunits, we failed to observe any changes in NR1 subunits following morphine priming or stress. Existing data are not enough to clearly explain why the increase in NR2B proteins was not accompanied by a similar increase in NR1 subunits. One possibility is that other NR1 subunits that are composed of another NMDA receptor subunits such as NR3A (Ma and Sucher, 2004) may be decreased and thus the total amounts of NR1 subunits are apparently unchanged following morphine treatment.

Several reports have demonstrated that some NMDA receptor antagonists, such as dizocilpine (MK-801) and phencyclidine, produce a significant CPP. In addition, NMDA receptor antagonists, such as phencyclidine and ketamine are self-administered by monkeys and are recognized as drugs of abuse in humans. Ifenprodil, an NR2B selective antagonist, has been proved to induce neither place preference nor place aversion (Ma et al., 2006), without leading to abuse in clinical use (Suzuki et al., 1999). Our previous data demonstrated that ifenprodil selectively blocked the morphine-induced CPP rather than the natural reinforcers (food, social-interaction)-induced CPP, suggesting light side-effect of ifenprodil (Ma et al., 2006). Furthermore, it has been reported that dizocilpine but not ifenprodil produced hyperlocomotion and disrupted spatial learning (Chizh et al., 2001). The unique effect of ifenprodil may be attributed to its low affinity for NR2A containing NMDARs, which may play an important role in the rewarding effect induced by non-selective antagonists such as MK-801.

The effect of ifenprodil on reinstatement of drug craving has not been previously studied. In the present study, we observed a dose-dependent reduction of morphine- but not FS-induced reinstatement after systemic injection of ifenprodil, suggesting that the neural mechanisms mediating drug-induced reinstatement are NMDARs-dependent, which is dissociable from the molecular/neurochemical substrates mediating stress-induced reinstatement. Glutamate is responsible for most of the excitatory neurotransmission in the brain and has been shown to be clearly involved in the learning and memory processes since NMDA antagonists impair memory in a wide variety of tasks and species (Riedel et al., 2003). In accordance with this, a putative explanation for the inhibition of drug-induced reinstatement of morphine CPP observed in this study is that ifenprodil interferes with the capacity of memory to remind the animal of the associations learned during conditioning and to produce a reactivation of place preference. Further study of intracranial local injections suggested that activation of NR2B-containing NMDARs in both the NAc and the hippocampus is indispensable for morphine-induced reinstatement.

It is generally accepted that glutamatergic pathway can be activated by stress in certain brain areas, which was involved in dopamine-associated psychiatric disorders (Moghaddam, 2002). However, the abovementioned idea seems difficult to reconcile with the failure of ifenprodil in blocking stress-induced reinstatement. It could be due to that the FS-induced reinstatement of morphine CPP is largely independent of glutamatergic pathway, at least the pathway heavily dependent on NR2B-containing NMDARs. It should be mentioned that the brain corticotrophin-releasing factor (CRF), a peptide involved in stress, has been proved to be important in relapse to heroin seeking induced by stressors (Shaham et al., 1997). The effect of CRF in reinstatement might have been still active even when NR2B-containing NMDARs were blocked by ifenprodil.

Conclusions

Using the CPP paradigm, we have shown that either a priming injection of morphine or exposure to forced swim as an acute, external stressor in a distinct context can precipitate reinstatement of previously extinguished morphine-induced CPP, but food deprivation for 24 h failed to so. Furthermore, up-regulation of NR2B subunit in the NAc and the hippocampus may be important for the reinstatement of CPP. In contrast, up-regulation of NR2B subunit in the amygdala, although definitely accompanied with those two types of stress, may be less important for the reinstatement of morphine CPP. No change in NR2B expression was observed in the prefrontal

cortex. Ifenprodil, an antagonist of NMDARs highly selective for the NR2B subunit produced a marked inhibitory effect on reinstatement induced by a priming injection of morphine, but not forced swim. Microinjection studies revealed that the NAc shell and the CA1 region of the dorsal hippocampus are important brain regions involved in the inhibitory effect of ifenprodil on morphine-induced reinstatement. The results imply that NR2B selective antagonist may have a potential use for the treatment of opiate addiction.

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