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Re-exposure to morphine-associated context facilitated long-term potentiation in the vSUB-NAc glutamatergic pathway via GluN2B-containing receptor activation

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ABSTRACT

The glutamatergic projection from the ventral subiculum of the hippocampus (vSUB) to the nucleus accumbens (NAc) shell has been reported to play a key role in drug-related behavior. The GluN2B subunit of N-methyl-D-aspartate receptors (NMDARs) in the NAc can be selectively elevated after the retrieval of drug-conditioned memory. However, whether the increased GluN2B-containing NMDARs (GluN2B-NMDARs) are able to alter the synaptic plasticity of the vSUB-NAc glutamatergic pathway remains unclear. Here, we found that the long-term potentiation (LTP) in the vSUB-NAc pathway was facilitated and the GluN2B subunit protein level was elevated in synaptoneurosomes of the NAc shell, but not in the core, following morphine-induced conditioned place preference (CPP) expression in rats. The facilitated LTP was prevented by the GluN2B-NMDAR antagonist RO25-6981. Also, a neurochemical disconnection following microinjection of RO25-6981 into the NAc shell, plus microinfusion of GABA agonist baclofen and muscimol into the contralateral vSUB prevented the expression of morphine-induced CPP. These findings suggest that the retrieval of drug-associated memory potentiated synaptic plasticity in the vSUB-NAc pathway, which was dependent on GluN2B-NMDAR activation in the NAc shell. These findings provide a new explanation for the mechanisms that underlie the morphine-associated-context memory. The GluN2B-NMDARs may be regarded as a potential target for erasing morphine-related memory.

Keywords Conditioned place preference, hippocampus, long-term potentiation, morphine, NMDA receptors, nucleus accumbens.

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INTRODUCTION

Drug addiction has been considered to be a pathological form of learning and memory that shares some synaptic and molecular mechanisms with normal learning and memory. Modifications in glutamatergic synaptic transmission have also been suggested to play important roles in drug-associated learning and memory (Kauer & Malenka 2007; Luscher 2013; Nestler 2013; van Huijstee & Mansvelder 2014).

Rapid synaptic potentiation in the nucleus accumbens (NAc) was found after cue-induced reinstatement of cocaine and nicotine self-administration (Gipson *et al.* 2013a, 2013b). It is well known that the NAc is an integral part of brain limbic circuits that is critical in mediating the reward or addictive properties of commonly abused drugs (Wise 1996). Anatomical evidence has indicated that the NAc receives glutamatergic inputs from the ventral subiculum of the hippocampus (vSUB) (Groenewegen *et al.* 1987). Contextual information that is essential for the drug reward property could be transferred from the hippocampus to the NAc to mediate the reinstatement of drug seeking or consumption (Taepavarapruk & Phillips 2003). Chemical or electrical stimulation of the vSUB elevated the NAc dopamine (DA) extracellular levels and reinstated drug-seeking behavior after an extinction period (Legault & Wise 1999; Legault *et al.* 2000; Vorel *et al.* 2001; Taepavarapruk & Phillips 2003). Additionally, recent studies have found that

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the ventral hippocampal afferents to the NAc shell are selectively potentiated following repeated cocaine treatment (Britt *et al*. 2012).

Our previous studies showed that a dynamic change of the GluN2B-containing *N*-methyl-D-aspartate receptor (NMDAR) level in the NAc and hippocampus was synchronous following the expression, extinction and reinstatement of morphine-induced conditioned place preference (CPP). The GluN2B-NMDAR protein level in these two regions was augmented following the expression of morphine CPP and resumed to the saline-conditioned level after the extinction of morphine CPP, and then it was elevated again by morphine priming (Ma *et al*. 2006, 2007). Other studies found that GluN2B-NMDAR antagonists could abolish relapse-associated synaptic modulation (Shen *et al*. 2011) and prevent the reinstatement of drug-seeking behaviors (Gipson *et al*. 2013b; Portugal *et al*. 2014). GluN2B-NMDARs appear to be critical in controlling these drug-induced changes of synaptic strength and the expression of drug-related behaviors. Because the over-expression of GluN2B-NMDARs promoted long-term potentiation (LTP) (Tang *et al*. 1999; Wang *et al*. 2009), we hypothesized that the retrieval of morphine-associated-context memory could induce changes in plasticity of the vSUB-NAc pathway, which is associated with the modulated GluN2B subunit level in the NAc following morphine CPP expression.

In the current study, we first recorded a change in the LTP in the vSUB-NAc pathway and measured the GluN2B level in synaptoneurosomes of the NAc after morphine CPP expression. Then, we observed the effect of the neurochemical disconnection of the vSUB-NAc pathway on morphine CPP expression.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Grade II) were purchased from the Animal Center of Peking University (Beijing), and they weighed 250–280 g at the beginning of the experiment. Four rats per cage were housed in a thermo-regulated room (22 ± 1 °C) with a 12: 12 h light-dark cycle, and had access to food and water *ad libitum*. All of the experimental procedures were approved by the Animal Use Committee of Peking University Health Science Center and were carefully designed to minimize the number of animals used and their suffering.

CPP conditioning procedure

Apparatus

Place conditioning was conducted in an unbiased designed, three-chambered CPP apparatus. The two black conditioning chambers with distinct visual and tactile cues (A and C, $280 \times 220 \times 225\,mm^3$) were separated by a small gray center choice chamber B ($135 \times 220 \times 225\,mm^3$) accessed through guillotine doors. Chamber A had 4 light-emitting diodes (LEDs) forming a square on the wall and a stainless-steel mesh floor ($130 \times 130\,mm^2$), while chamber C had 4 LEDs forming a triangle on the wall and a stainless-steel rod floor (130 mm apart). Chamber B had a gray wall and a plain floor. Fourteen photobeams were placed across the chambers and were 47.5 mm apart. Using a computer interface, the time spent in each chamber was recorded by infrared beam crossings.

Pre-conditioning test. On day 0, the rats were placed in chamber B with the guillotine doors removed to allow the rats to explore the entire apparatus freely for 15 min. Rats that had a bias for either of the lateral chambers (preference score > 0.6) were excluded from the experiments.

Conditioning with morphine. Animals were allowed two training sessions per day (at 8:30 a.m. and 3:30 p.m.) for 4 days (Days 1–4). During conditioning, rats of the morphine CPP group (morphine-paired group) received an intraperitoneal (i.p.) injection of saline (2 ml/kg) and were confined in one lateral chamber for 45 min. Seven hours later, 4 mg/kg morphine (Pharmaceutical Factory of Qinghai, Qinghai, China) was given before the morphine CPP rats were confined in the other chamber for 45 min. Animals in the saline CPP group (saline-paired group) were injected with saline before being confined in the alternate lateral chamber. The saline CPP group served as a control group that retrieves a non-drug memory. Morphine or saline-unpaired rats received two morphine or saline injections in home cages at the same time as the CPP rats. The purpose of these two control groups was to detect the effects of drug exposure without the associative memory of the drug and context.

Post-conditioning test. On testing day, all of the animals were placed in chamber B with the guillotine doors removed to allow access to all of the compartments for 15 min. The CPP score was defined as the time spent in the morphine-paired chamber divided by the total time spent in both the morphine and saline-paired chamber during the CPP testing.

Electrophysiology in vivo

After the expression of morphine CPP, the rats were anesthetized with urethane (1.2-1.5 g/kg, i.p.) and mounted in a stereotaxic apparatus (Narishige, Tokyo, Japan).

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Concentric bipolar stimulating electrodes (Friedrick Haer & Co., Bowdoinham, ME, USA) were placed in the vSUB [AP, −5.8-6.3 mm; ML, +5.2-5.6 mm; DV, −6.0-7.0 mm from brain surface] at a 10°-angle. Parylene-coated tungsten microelectrodes (FHC) were aimed at the NAc shell (AP, +1.6-1.8 mm; ML, +0.8-1.0 mm; DV, −6.0-7.0 mm).

A field potential was evoked at 0.033 Hz, with a 0.2 ms pulse width, and the stimulus intensity was adjusted to evoke approximately 50% of the maximum field response. LTP was induced by a 10-Hz (900 pulses for 1.5 min, the same intensity as the test stimulus) stimulation. The field potential was amplified by an AC pre-amplifier (AVB-10, Nihon Kohden, Japan) , which was band-pass filtered at 0.3-300 Hz and digitized by a CED 1401 interface for off-line analysis using the Spike 2 software (Cambridge Electronic Design, Cambridge, UK). The field potential amplitude was averaged for every 3 min and normalized for each animal as a percentage of the baseline field potential amplitude. LTP was defined as 20% increase in the baseline field potential that was maintained for at least 30 min after the stimulation.

The sites of stimulating and recording electrodes were marked by electrolytic lesion (20.0 μA positive depolarizing DC current for 20 s) immediately after the electrophysiological studies. Then rats were subjected to routine histological verification (see details in the histological part). The data were excluded in analysis if the electrodes were not correctly positioned.

To investigate the role of GluN2B-NMDAR in the LTP, NMDAR antagonist AP-5 (10 mM, 1 μl, dissolved in saline; Sigma Aldrich, St. Louis, MO, USA) and the GluN2B-NMDAR antagonist RO25-6981 (5 μM, 1 μl, dissolved in saline; Tocris, London, UK) were infused locally into the NAc 30 min before the LTP induction. A microinjection needle (OD 0.30 mm) was inserted at a 10° angle into the NAc shell (approximately 200 μm rostral to the tip of the recording electrodes) or core (AP, +1.6 mm; ML, +2.0 mm; DV, −7.0 mm) slowly (10 μm/30 s) to avoid maiming the local anatomical structure. After the field potentials were stable for at least 15 min, the drug was infused through a microinjection pump (0.5 μL/min). Then, the baseline field potential was recorded for 30 min before the LTP induction.

Neurochemical disconnection of the vSUB-NAc pathway

Surgery. Rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and secured in a Kopf stereotaxic apparatus. A stainless-steel guide cannula (OD 0.64 mm) was placed into the NAc shell (AP, +1.6 mm; ML, ± 0.9 mm; DV, −7 mm from skull) and vSUB (AP, −5.8 mm; ML, ±5.5 mm; DV, −7 mm). The bilateral group had the cannula inserted on one side of the NAc shell and on the other side of the vSUB. The ipsilateral group had the cannula on the same side of the NAc shell and vSUB. All of the animals were allowed to recover for 5 days before morphine training.

Neurochemical disconnection. One day before the CPP testing day, obdurators were removed and a sham infusion procedure (injector was placed in the guide cannulae for 2 min, but no infusion was administered) was conducted to habituate the rats to the routine of infusions. During drug microinfusion, infusion needles (OD 0.30 mm) were extended 1.0 mm below the guide cannula. The injection was performed through an infusion pump (0.2 μl/min, Harvard apparatus, Holliston, MA, USA). The injector was left in place for 2 min to ensure drug diffusion. The animals' behaviors were tested 15 min later. The neurochemical disconnection group had a unilateral infusion of GluN2B-NMDAR antagonist RO25-6981 (2 μg/0.5 μl, 0.5 μl) into the NAc shell combined with a contralateral injection of the GABA agonists baclofen and muscimol (12.5 μg/0.5 μl, 0.5 μl, dissolved in saline, Sigma Aldrich) into the vSUB. The sham group received injections of saline into the bilateral NAc shell and vSUB. The unilateral vSUB group received baclofen and muscimol on one side of the vSUB and saline on the other side of the NAc shell. Similarly, the unilateral NAc group received RO25-6981 in the NAc shell but saline in the vSUB. The ipsilateral group had an infusion of RO25-6981, baclofen and muscimol into the NAc shell and vSUB on the same side of the brain.

Tissue dissection and synaptosome preparation

The method of synaptosome preparation was modified from that described by Li *et al.* (2010). The rats were decapitated immediately after testing. The brains were removed and frozen in N-hexane (−70 °C) for approximately 25 s. The brains were then stored at −80 °C until further use. The NAc core and shell were obtained from 60 μm thick sections taken on a sliding freezing microtome using 16 or 12 gauge needles. The tissue punches were dissected and homogenized in ice-chilled lysis buffer containing 320 mM sucrose, 20 mM HEPES, 1 mM EDTA and 1 × protease inhibitor cocktail (Cat No. P8340; Sigma Aldrich). The homogenate was centrifuged at 2800 rpm for 10 min. Then the supernatant was further centrifuged at 12000 rpm for 10 min. The supernatant (cytosolic fraction) was discarded. The pellet (crude synaptosomal fraction) was suspended in RIPA buffer (Cat No. R0278; Sigma Aldrich) containing 50 mM Tri-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1% TritonX-100

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and 1 × protease inhibitor cocktail. Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL, USA). Sample buffer was immediately added to the homogenates.

Immunoblotting of NMDA receptor subunits

Equivalent amounts of synaptosome fractions (20 μg) for each sample were resolved on a 10% SDS-PAGE gel. After electrophoresis, proteins were transferred to polyvinylidene fluoride (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 that (GluN2A and GluN2B, 1: 2000, Millipore, Billerica, MA, USA; β-actin, 1:4000, Sigma Aldrich) contained 5% nonfat dried milk overnight at 4 °C. The membrane was then washed thrice for 5 min in TBST followed by 1 h of incubation at room temperature with horseradish peroxidase-conjugated rabbit anti-mouse IgG or anti-rabbit IgG (Zhongshan Biotechnology, Beijing, China) diluted 1: 2000 in TBS containing 5% nonfat dried milk. After this incubation, the membranes were washed twice for 5 min and then twice for 10 min in TBST. The antigen-antibody peroxidase complex was finally detected by enhanced chemiluminescence (Applygen Technologies Inc, 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 Quantity One 1-D Analysis Software (Bio-Rad, California, USA), the optical density of each band of the NMDAR subunits were normalized with the relative optical density of the β-actin band.

Histology

Rats were anesthetized with chloral hydrate (40 mg/kg, i.p.) and perfused intracardially with 0.9% saline (300 ml) followed by 4% paraformaldehyde solution (350 ml). The brains were removed and post-fixed in 4% paraformaldehyde solution for 24 h, then immersed in 20% sucrose solution followed by 30% sucrose solution until sectioning. Coronal sections (30 μm thick) were cut on a cryostat (−22 °C, Leica) and wet-mounted on glass microscope slides. Cannula placements were assessed by Nissl staining using light microscopy.

Statistical analysis

All of the statistical analyses and graphics were processed with GraphPad PRISM 5.0. Data are presented as the mean ± standard error of the mean (SEM) and analyzed with appropriate test methods (two-way ANOVA followed by Bonferroni post hoc test, repeated measures ANOVA or t-test). The statistical significance was set at $P \leqq 0.05$.

RESULTS

Facilitation of LTP in the vSUB-NAc pathway after morphine CPP expression

As shown in Fig. 1, a 10-Hz stimulation, which could not induce LTP in the saline-unpaired group, morphine-unpaired group or saline CPP group, still induced a significant LTP in the morphine CPP rats (Fig. 1b and c). Two-way ANOVA demonstrated the significant effect from the drug × treatment interaction [$F_{(1,\ 17)} = 6.13$, $p = 0.0241$] and treatment [$F_{(1,17)} = 13.73$, $p = 0.0018$]. The effect of the drug [$F_{(1,\ 17)} = 4.28$, $p = 0.0541$] did not reach statistical significance. After the 10-Hz stimulation, the field potential magnitude in the morphine CPP rats increased by 30.31 ± 5.391%, whereas the field potential magnitude in the other three control groups was similar to their baseline levels. A significant difference was found by the Bonferroni *post hoc* test (n = 5–6; $p < 0.05$, Mor-CPP *vs* Sal-CPP; $p < 0.01$, Mor-CPP *vs* Mor-unpaired). These data suggest that the LTP induction in the vSUB-NAc pathway is facilitated after the morphine CPP expression. In order to test whether other plasticity in the control groups is intact, 60 min after the 10-Hz simulation, a 200-Hz stimulation was delivered to the vSUB of all of the groups. This manipulation resulted in a reliable LTP in the three control groups (Fig. 1d, the field potential magnitude was increased by 46.25 ± 7.756% in the Sal-unpaired group, 29.16 ± 12.53% in the Mor-unpaired group, and 43.62 ± 7.781 in the Sal-CPP group). A significant effect of treatment (10 Hz *vs* 200 Hz stimulation) was found by the two-way ANOVA [$F_{(1,\ 32)} = 32.10$, $p < 0.0001$]

GluN2B subunits play a key role in the facilitation of LTP in the vSUB-NAc pathway after morphine CPP expression

A change in the LTP induction probably reflects an increase of the transmitter that was released in the presynaptic membrane or an enhancement of the receptor function or number in the postsynaptic membrane. To detect whether changes in the presynaptic transmitter release occurred in morphine CPP rats, we first compared the magnitude of the facilitation that occurred in response to paired-pulse stimulation (at intervals of 50 ms) in the vSUB-NAc pathway of morphine CPP rats. Repeated measures ANOVA showed that the paired-pulse ratio (PPR) gradually increased after the 10-Hz stimulation (Fig. 2a, n = 5; $p < 0.05$, 15 *vs* 30, 60 min, $p < 0.01$, 15 *vs* 45 min,), but it did not significantly change compared with that before the 10 Hz stimulation (0 min). This result suggests that there

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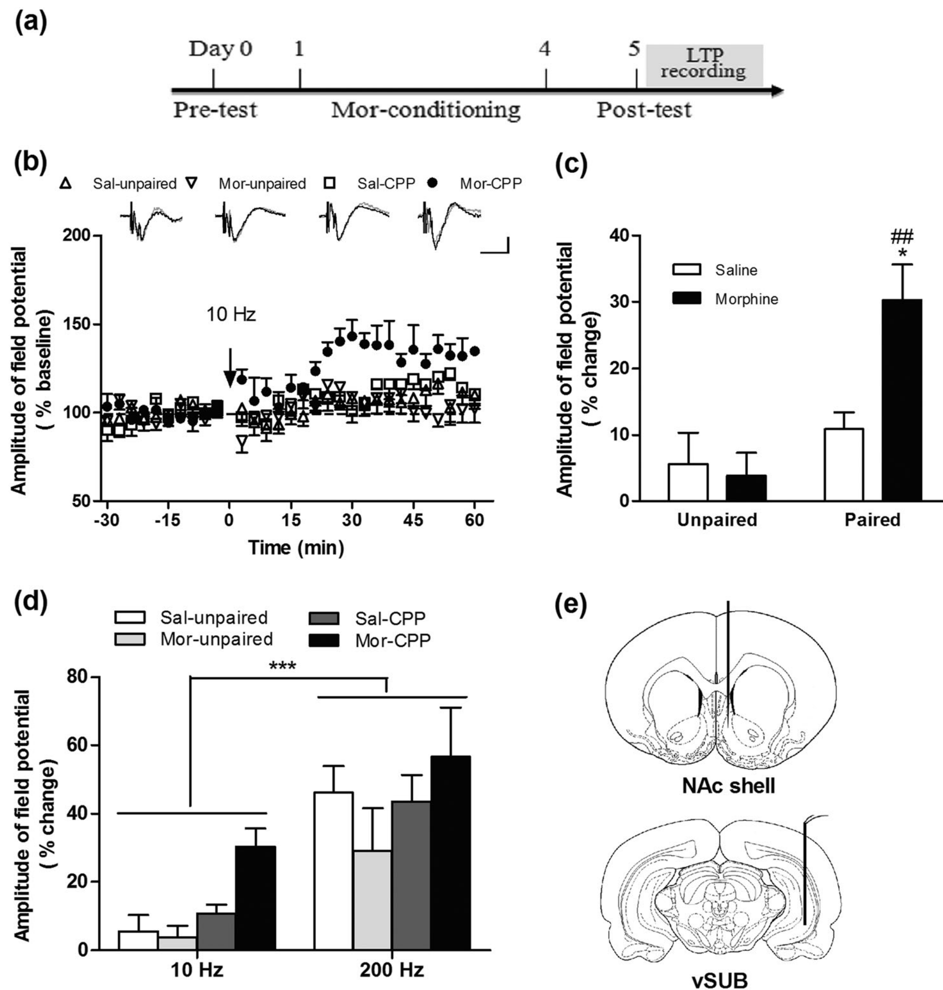

Figure 1 Enhancement of 10-Hz-induced LTP in the vSUB-NAc pathway following morphine CPP expression. (a) A schematic presentation of the experimental procedures. (b) A 10-Hz stimulation (arrow) could not induce LTP in the Sal-unpaired rats, Mor-unpaired rats and Sal-CPP rats, but could induce a reliable LTP in Mor-CPP rats (n = 5–6). The LTP 60 min after the 10-Hz stimulation plotted as a percent of the baseline (−30 min to 0 min). Representative traces at the baseline and after the 10-Hz stimulation are shown. Calibration: vertical scale bar = 1 mV, horizontal scale bar = 50 ms. (c) Summary of the magnitude of the LTP in all of the groups. Field potential amplitude 60 min after a 10-Hz stimulation represented as a percent change from the baseline. The data points shown are the mean ± SEM, $*P < 0.05$, compared with the Sal-CPP group; ## $P < 0.01$, compared with the Mor-unpaired group by two-way ANOVA and the Bonferroni *post hoc* test. (d) 200-Hz stimulation induced LTP in the vSUB-NAc pathway of the control groups. (e) Schematic diagram represents the placement of the recording electrodes (upper panel) in the NAc shell and the stimulus electrodes (lower panel) in the vSUB. Mor-CPP/morphine-paired indicates morphine CPP, and Mor-unpaired indicates that the rats received morphine injection in the homecage. The same logic applies for Sal-CPP and Sal-unpaired

was no gross increase in the presynaptic transmitter release. Furthermore, the increase in field potential magnitude was not caused by an enhancement of axonal excitability because the amplitude of the fiber volley was not significantly changed after 10 Hz stimulation (Fig. 2b). These results suggest that the facilitated LTP in the vSUB-NAc pathway was not due to the enhancement of presynaptic function.

To determine whether the facilitated LTP that is induced by morphine CPP expression shared similar molecular mechanisms with NMDAR-dependent LTP that was previously described in the NAc (Kombian & Malenka 1994; Schramm *et al*. 2002), the NMDAR antagonist AP-5 was microinjected into the NAc shell 30 min before LTP induction. We found that the field potential magnitude did not significantly change after the 10-Hz stimulation (Fig. 2d). These results indicate that the induction of facilitated LTP depends on NMDAR activation. It was reported that increasing the GluN2B subunit favored the induction of hippocampal LTP (Tang *et al*. 1999; Wang *et al*. 2009). To further explore whether the enhanced LTP is because of the activation of GluN2B-NMDAR in the NAc shell, the GluN2B-NMDAR antagonist RO25-6981 was infused into the NAc shell 30 min before the 10-Hz stimulation. As shown in Fig. 2e, the LTP in the morphine CPP rats

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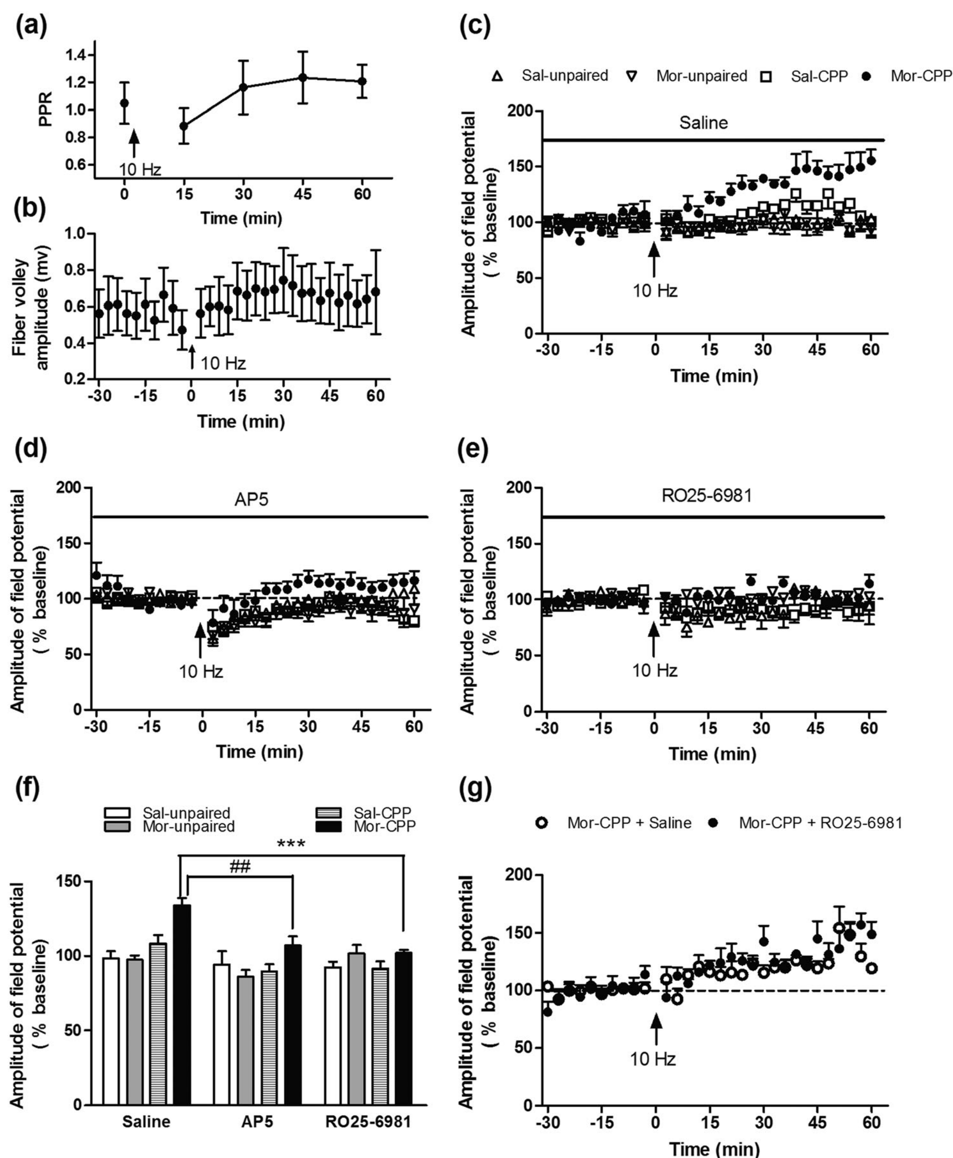

Figure 2 Intra-NAc shell injection of AP-5 and RO25-6981 inhibited facilitated LTP in morphine CPP rats. (a) Paired-pulse ratio did not change after the 10-Hz stimulation in the morphine CPP rats. PPR was measured with pairs of presynaptic stimulation pulses at intervals of 50 ms. (b) Fiber volley amplitude did not change after the 10-Hz stimulation in the morphine CPP rats. (c) Saline had no effect on the facilitated LTP in Mor-CPP rats. AP-5 (d) and RO25-6981(e) abolished the facilitated LTP in Mor-CPP rats. The field potential amplitude 60 min after the 10 Hz is plotted as a percent of the baseline (−30 min to 0 min). (f) Summary of the magnitude of LTP in all of the groups (n = 5–6). The field potential amplitude of 60 min after 10 Hz represented as a percent of the baseline. ***$P < 0.001$, ##$P < 0.01$, compared with the Mor-CPP group, which had received a microinjection of saline, by two-way ANOVA and the Bonferroni *post hoc* test. (g) RO25-6981 microinjection in the NAc core had no effect on the induction of the facilitated LTP in the vSUB-NAc pathway, n = 5. Mor-CPP indicates morphine CPP, and Mor-unpaired indicates that the rats received morphine injection in the homecage. The same logic applies for Sal-CPP and Sal-unpaired. Data points shown are as the mean ± SEM

was prevented by RO25-6981. Statistical analysis (two-way ANOVA) revealed a significant effect of the interaction between the treatment (Sal-unpaired, Mor-unpaired, Sal-CPP, Mor-CPP) and the microinjected drugs (saline, AP-5, RO25-6981) [$F_{(6, 49)} = 2.73$, $p = 0.0228$]. Significant differences were also found within the treatment [$F_{(3, 49)} = 10.46$, $p < 0.0001$] and between the microinjected drugs [$F_{(2, 49)} = 10.24$, $p = 0.0002$]. Compared with the saline-treated group, the increase in the field potential magnitude after the 10-Hz stimulation in the morphine CPP rats that received AP-5 or RO25-6981 treatment was much lower (Fig. 2f: n = 5–6; Bonferroni *post hoc* test, saline *vs* AP-5, $p < 0.01$; saline *vs* RO, $p < 0.001$). In addition, the NAc core was chosen to be a negative control region to the shell. When RO25-6981 was administered into the core region of the NAc (while the recording electrode was still in the NAc shell), the facilitated LTP was not impaired (Fig. 2g). These results demonstrate that the activation of the

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NMDARs, especially the GluN2B-NMDARs, is required to generate the facilitated LTP in the vSUB-NAc pathway of the morphine CPP rats.

GluN2B subunits were selectively increased in the synaptosomal component of the NAc shell after the morphine CPP expression

Subsequently, we detected the protein level of the GluN2A and 2B subunit in synaptosomal fraction extracted from the NAc shell and core, respectively. The results showed that the GluN2B protein level was significantly increased in the NAc shell after the morphine CPP expression [n = 6; Fig. 3a; Drug: $F_{(1, 20)} = 5.94$, $p = 0.0243$; Treatment: $F_{(1, 20)} = 5.39$, $p = 0.0309$; Drug × Treatment: $F_{(1, 20)} = 5.88$, $p = 0.0249$], whereas the GluN2A level remained unchanged (Fig. 3c). Both the GluN2A and 2B subunit levels in the NAc core of the morphine CPP rats were the same as those in other control groups (Fig. 3b and d).

Inactivation of unilateral vSUB combined with the inhibition of GluN2B-NMDAR in the contralateral NAc shell abolished the morphine CPP expression

To confirm the role of GluN2B-NMDARs in the vSUB-NAc pathway in the expression of the morphine CPP, we performed a neurochemical disconnection experiment, in which the vSUB was injected unilaterally with the GABA receptor agonists baclofen and muscimol, whereas the GluN2B-NMDAR antagonist RO25-6981 was administered into the contralateral NAc shell. The disconnection procedure was performed 15 min before the CPP test. As depicted in Fig. 4, the sham disconnection, unilateral vSUB/NAc and ipsilateral injection group successfully established morphine CPP (n = 8–10; *t*-test, pre-test *vs* post-test: sham, $p = 0.0435$; unilateral NAc shell, $p = 0.0081$; unilateral vSUB, $p = 0.0008$; ipsilateral injection, $p = 0.0019$). At the same time, the disconnection group failed to show preference for the morphine-paired chamber. This result suggest that activation of

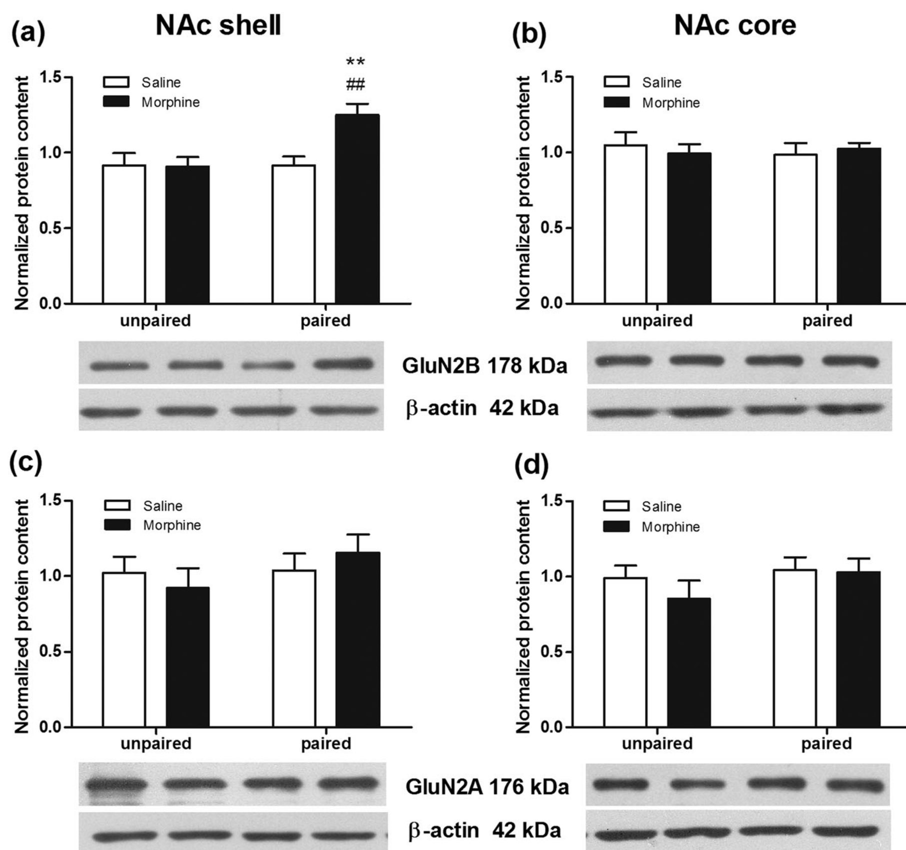

Figure 3 GluN2B protein level was selectively increased in the synaptosomal component of the NAc shell in the morphine CPP rats. (a) The GluN2B protein level in the NAc shell was significantly increased in the morphine-paired (morphine CPP) rats but not in the saline-paired (saline CPP) and saline/morphine-unpaired groups. (b) GluN2B protein expression in the NAc core was the same among the groups. (c) and (d) The GluN2A protein level in the NAc shell and core was the same among the groups. The data points shown are the mean ± SEM, ** $p < 0.01$, compared with the saline CPP group, # $p < 0.05$, compared to the saline CPP group by two-way ANOVA and the Bonferroni *post hoc* test, n = 5–6. Morphine-paired indicates morphine CPP, and Mor-unpaired indicates rats that received morphine injection in the homecage. The same logic applies for Sal-CPP and Sal-unpaired

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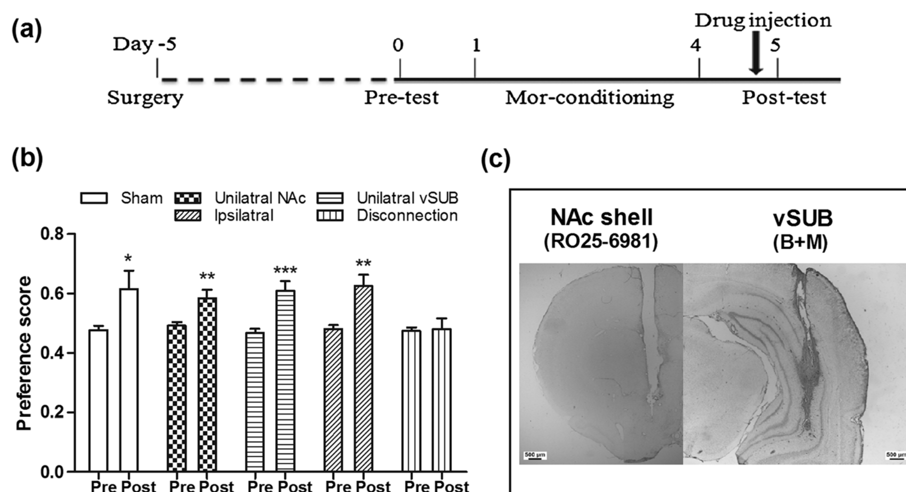

Figure 4 Inhibiting the GluN2B-NMDARs in the vSUB-NAc pathway through a neurochemical disconnection prevented the morphine CPP expression. (a) A schematic representation of the experimental procedures. (b) Unilateral baclofen + muscimol (B + M) injection into the vSUB combined with contralateral RO25-6981 injection into the NAc shell (disconnection group) abolished the expression of morphine CPP. The CPP performance of the disconnection sham controls, unilateral NAc shell/vSUB, and ipsilateral groups were not altered. (c) Histological sites of cannula placement in the NAc shell and vSUB. Data points shown are the mean ± SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the pretest by t-test, n = 8–10. Pre indicates pre-test, and Post indicates post-test on Day 5

GluN2B-NMDARs in the vSUB-NAc pathway is necessary for morphine CPP expression.

DISCUSSION

In the present study, we demonstrated, for the first time, that re-exposure to morphine-associated-context facilitated the LTP of the vSUB-NAc pathway via GluN2B-NMDAR activation in the NAc shell. In accordance with the electrophysiological adaption, the protein level of the GluN2B subunit in the synaptosomal component of the NAc shell was significantly increased after morphine CPP expression. Neurochemical disconnection of the vSUB-NAc pathway with the GluN2B-NMDAR antagonist and GABA agonist prevented the morphine CPP expression.

Similar to our earlier report (Ma *et al.* 2006), we found that the protein level of GluN2B but not GluN2A subunit in the synaptosomal component of the NAc was obviously increased after the morphine CPP expression. Furthermore, this change was subregion-specific, in other words, found only in the NAc shell but not the core. It is well known that the NAc shell receives strong glutamatergic input from the ventral hippocampus. The CPP model is considered to imitate the associative learning and memory between the drug effect and drug-associated context. Given that the hippocampus is critical for the primary rewarding effects of opiates (Corrigall & Linseman 1988; Semenova *et al.* 1999), as well as associative memory storage and retrieval (Stella & Treves 2011), our studies focus on the changes in the synaptic function in the vSUB-NAc pathway of morphine CPP rats.

GluN2B-NMDARs were found to be important for LTP induction (Tang *et al.* 1999; Clayton *et al.* 2002; Wang *et al.* 2009), although there were notable conflicting results as well (Liu *et al.* 2004; Massey *et al.* 2004; Mallon *et al.* 2005). Our findings that the increased GluN2B protein levels in morphine CPP rats led to enhanced LTP were in accordance with previous studies. Shen *et al.* observed a LTP-like neuroplasticity that required the up-regulation and activation of the GluN2B-NMDAR during the drug-induced heroin seeking (Shen *et al.* 2011).Wills *et al.* also showed that chronic ethanol exposure enhanced LTP formation in the bed nucleus of the stria terminalis (BNST) through the extrasynaptic GluN2B-NMDARs (Wills *et al.* 2012). Both of the enhanced LTP and increased GluN2B subunits in the NAc shell were not observed in the rats that received the unpaired morphine treatment, which indicates that these changes were specific to the retrieval of morphine-associated context memory. Growing evidence showed that glutamatergic synapse modification in addiction models is important for mediating the addiction process (Massey *et al.* 2004; Kauer & Malenka 2007; Luscher & Malenka 2011; Wills *et al.* 2012; Nestler 2013; van Huijstee & Mansvelder 2014). Here, we found that inhibition of the GluN2B-NMDARs in the vSUB-NAc pathway through a neurochemical disconnection could prevent morphine CPP expression. The observation that GluN2B-NMDAR antagonist could block the LTP facilitation that occurs in the vSUB-NAc pathway of morphine CPP rats led us to speculate that the facilitated LTP in the vSUB-NAc pathway could contribute to the retrieval of morphine-associated-context memory.

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The composition of NMDAR in the synaptic area switched from the predominance of GluN2B subunits over GluN2A subunits during the course of postnatal development (Barria & Malinow 2002; van Zundert *et al.* 2004; Dumas 2005). Recently, Dong and Nestler proposed a neural rejuvenation hypothesis of cocaine addiction (Dong & Nestler 2014). According to this hypothesis, drugs of abuse could awaken and then utilize the highly efficient plasticity mechanisms that are normally associated with brain development within the reward circuitry to produce abnormally robust and stable forms of memories that are related to addiction. The results in the present study agreed well with this hypothesis. Re-enrichment of the GluN2B subunits at the NAc shell resulted in the LTP facilitation in the vSUB-NAc pathway after the re-exposure to the morphine-associated context. Inactivation of the GluN2B-NMDARs reversed the synaptic modulation and blocked the retrieval of morphine-conditioned memory. Therefore, we speculated that when the synaptic GluN2B-NMDARs in the NAc shell were increased, the glutamatergic synaptic plasticity from the vSUB to NAc shell became more sensitive to the drug-paired context and the synaptic connection between the hippocampus and NAc, which is mainly involved in encoding the drug-associated information about the context cues and drug-reward, might be strengthened persistently. This process could participate in the acquisition of drug-associated memory or the reconsolidation of the newly formed drug-related memory. The facilitated LTP was observed after the retrieval of morphine CPP, it is possible that the potentiation of the synaptic transmission promotes the restabilization of the reactivated drug-associated memory and strengthens the memories. Drugs that could reverse the change in the synaptic plasticity could disrupt the reconsolidation process and then erase the drug-associated memory. Combined with our previous findings that the GluN2B-NMDAR antagonist ifenprodil suppressed morphine CPP without affecting the food CPP (Ma *et al.* 2006), these results suggest that GluN2B-NMDAR-targeted interventions can be a potential treatment for drug addiction without affecting the memory that is associated with the natural reward, which is necessary for living.

In addition, the activation of the dopamine D1 receptor is believed to selectively enhance the surface expression and function of GluN2B-NMDARs in prefrontal and striatum neurons by tyrosine phosphorylation of GluN2B at Tyr1472 (Dunah *et al.* 2004; Hallett *et al.* 2006; Gao & Wolf 2008; Lei *et al.* 2009). Cocaine administration could induce ERK activation through the D1R/Src family kinases/GluN2B pathway (Pascoli *et al.* 2011). Moreover, intra-NAc administration of the D1 agonist SKF38393 produced a significant enhancement in the LTP at the HPC-NAc input (Goto & Grace 2005). Our previous studies found that the tissue content of DA and its metabolites was significantly increased in the NAc after morphine CPP expression (Ma *et al.* 2009). Therefore, we proposed that the elevated DA that followed the morphine CPP test might promote the surface expression and function of GluN2B-NMDARs. With respect to investigating the detailed mechanisms that underlie the elevation of the synaptic GluN2B-NMDARs and the synaptic plasticity, further research is still required.

Obviously, this work has some limitations. First, in addition to the vSUB, NAc also receives glutamatergic inputs from the infralimbic cortex (IL), the ventral tegmental area (VTA), and the basolateral amygdale (BLA) (Gipson *et al.* 2014). Enhancement of prefrontal cortex (PFC)-NAc shell glutamatergic synaptic transmission after withdrawal from exposure to cocaine was suggested by previous studies (Kourrich *et al.* 2007; Suska *et al.* 2013). Morphine-induced increase in GluN2B subunits was found in the NAc shell in our study, so the glutamatergic synaptic plasticity may not only occur in the vSUB-NAc pathway. Whether synaptic changes of other projections (IL-NAc or BLA-NAc pathway) are necessary as well for morphine CPP remains to be determined. Second, cellular mechanism underlying GluN2B-dependent plasticity in the present study should be investigated using ex vivo whole-cell patch clamp recordings in the following works. Furthermore, it was reported that induction of NAc long-term depression (LTD) requires GluN2B-NMDAR activation (Jeanes *et al.* 2011). So there is anther possibility we can not exclude, the increased GluN2B subunit in morphine CPP rats may reflect the occurrence of LTD-like process, which could facilitate the subsequent induction of LTP. Detecting the AMPA/NMDA ratio with whole-cell patch clamp after the morphine CPP expression will help us to verify this assumption.

In summary, this study demonstrated that re-exposure to the morphine-associated context facilitated the LTP of the vSUB-NAc pathway via GluN2B-NMDAR activation in the NAc shell. The inhibition of the GluN2B-NMDARs of the vSUB-NAc pathway prevented morphine CPP expression. Our data indicate that GluN2B-NMDAR-dependent synaptic modulation in the vSUB-NAc pathway contributes to the retrieval of morphine-associated-context memory. These findings provide important information about the mechanisms that underlie the morphine-associated-context memory. The GluN2B-NMDARs might represent a useful therapeutic target for erasing morphine-related memory or intervening relapse to drug abuse.

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Conflict of Interest

The authors declare no competing financial interests.

Authors Contribution

Cai-Lian Cui and Yi-Jing Li conceived and designed the research; Yi-Jing Li, Xing-Jie Ping and Qi Chong performed the experiments; Fang Shen and Lin-Lin Sun assisted with data analysis; Xiao-Wei Sun, Fei-Fei Ge and Guo-Gang Xing provided technical support; Yi-Jing Li drafted the manuscript; Cai-Lian Cui and Yi-Jing Li edited and revised the manuscript.

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