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ORIGINAL PAPER

AMPA Receptor in Ventromedial Prefrontal Cortex Plays Different Roles in the Recent and Remote Retrieval of Morphine-Associated Memory

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Abstract

Previous studies demonstrate that drug addiction can share the neural circuits in the brain with normal learning and memory. Re-exposure to drug-associated contexts, one way to retrieve the drug-associated memory, can trigger strong psychic craving and even relapse in addicts after prolonged abstinence. The ventromedial prefrontal cortex (vmPFC) has been shown to be involved in time-dependent reinstatement of drug self-administration. This work is designed to investigate the role of AMPA receptor (AMPAR) in the vmPFC in the recent and remote retrieval of morphine-associated memory. Rats were re-exposed to the morphine-paired context 1 day (recent) and 3 weeks (remote) after morphine conditioned place preference (CPP) training. Results showed that membrane expression of GluA1 and GluA2 in the vmPFC was decreased following the recent retrieval, while the membrane expression of GluA1 and GluA2 in the vmPFC was increased following the remote retrieval of morphine-associated memory. Furthermore, the microinfusion of Tat-GluA2-3Y, a GluA2 endocytosis inhibitor, into the vmPFC impaired the recent retrieval of morphine-associated memory. The microinfusion of AMPAR antagonist NBQX into the vmPFC prevented the remote retrieval of morphine-associated memory. Taking together, the present study proved that AMPAR in the vmPFC played different roles in the recent and remote retrieval of morphine-associated memory.

Keywords Conditioned place preference (CPP) · Recent retrieval · Remote retrieval · AMPAR · Ventromedial prefrontal cortex (vmPFC) · Memory

Introduction

Drug addiction is a chronic relapsing brain disorder. Repeated drug intake can cause an association between the neutral context cues (e.g., locations, paraphernalia) with the efficacy of the drug, and then form drug-associated memory [1, 2]. Previous studies have shown that the changes of the molecule and synaptic plasticity involved in drug-associated memory were similar to that observed in physiological memory [3, 4]. It's known that memory is not immediately formed, it occurs within minutes or hours after learning, then progressively reorganized across the brain systems, thought to persist weeks or months [5]. Therefore, re-exposure to the drug-associated context cues after drug conditioning can induce psychic craving for the drug and drug-taking/seeking behavior in addicts or animals, even after long periods of abstinence [6, 7]. Obviously, disrupting or weakening the associations is expected to prevent drug-associated memory retrieval and suppress the relapse susceptibility.

The ventromedial prefrontal cortex (vmPFC) has been reported to be dynamically involved in drug-associated memory retrieval. Koya et al. have found that re-exposure to context cues increased signal-regulated kinase (ERK) phosphorylation in the vmPFC 30 days but not 1 day after cocaine self-administration [8]. And the inactivation of the vmPFC with muscimol + baclofen can suppress the cue-induced reinstatement after 30 days of withdrawal. Moreover, significantly increased expression of protein kinase C epsilon type (PKCε) and phosphatidylinositol 3-kinase (PI3K) in the vmPFC [9, 10] is detected 30 days but not 3 days after cocaine self-administration, and intervening in the vmPFC with drugs to inhibit these molecular changes block the reinstatement.

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Additionally, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), trafficking into and out membrane is documented to mediate the majority of rapid excitatory neurotransmission in the central nervous system [11, 12]. Recently in vivo study with two-photon microscopy shows that changes in membrane level of GluA1 are positively correlated with changes in spine size following the whisker stimulation [13]. Synaptic plasticity has been proved to contribute to synaptic efficacy alterations, like long-term potentiation (LTP) and long-term depression (LTD) that have been thought as the cellular basis of learning and memory [14–16]. Interestingly, AMPAR plasticity in medial prefrontal cortex is considered to be crucial for cue-induced relapse to heroin-seeking [17, 18]. GluA2 in the vmPFC is decreased in context-induced reinstatement of heroin seeking after 3 weeks of withdrawal. And the intra-vmPFC injection of Tat-GluA2-3Y peptide to inhibit the GluA2 endocytosis suppress context-induced heroin reinstatement [17].

Recent work in our lab also shows that context-induced reinstatement of heroin seeking causes a decreased GluA2 expression in the infralimbic cortex (IL, a part of the vmPFC), and pharmacological disconnection of the ventral CA1-IL pathway attenuates context-induced heroin reinstatement [18]. In general, although these data indicate that the expression of AMPAR changes after re-exposure to drug-associated context, direct evidence for the role of AMPAR in the vmPFC in the recent and remote retrieval of morphine-associated memory is unclear. In this study, conditioned place preference (CPP) model, a widely used drug-conditioned learning and memory model was conducted. Recent retrieval was performed 1 day, whereas remote retrieval was performed 3 weeks after morphine conditioning to allow time for system consolidation. Then, the changes of GluA1 and GluA2 in the vmPFC were detected by immunoblotting, and the changes in the vmPFC were intervened with the corresponding pharmacological approach to reveal the role of GluA1 and GluA2 in the vmPFC in the recent and remote retrieval of morphine-associated memory.

Materials and Methods

Subjects

Male Sprague Dawley rats, weighing 230–250 g at the beginning, were purchased from the Laboratory Animal Center of the Peking University Health Science Center. Three rats per cage were housed in a temperature and humidity controlled animal facility under a 12/12 h light/dark cycle (lights off at 7 p.m.). Food and water provided ad libitum. All experiments procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

CPP Apparatus

The CPP apparatus was a black, rectangular polyvinyl chloride (PVC) box ($795 \times 230 \times 250$ mm^3) containing three chambers (A, B and C) separated by guillotine doors. Two large black conditioning chambers (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). Chamber A had four light-emitting diodes (LEDs) forming a square on the wall and a stainless steel mesh floor, chamber C had four LEDs forming a triangle on the wall and a stainless-steel rod floor, while chamber B had a plain floor. Fourteen photon beams were placed across the chambers and were 47.5 mm apart. Using a computer interface, the time spent in each chamber and the numbers of entrances into each of the three compartments were recorded by infrared beam crossings.

Morphine CPP Procedure

The morphine CPP procedure was performed and modified as previously described [19]. Rats were handled for at least 3 days before the experiments. On day 0, rats were placed in the center choice chamber B with the guillotine doors removed to allow access to the entire apparatus for 15 min and time spent in each chamber were recorded (pre-conditioning test, pretest). These data were used to separate animals into groups with approximately equal biases for each chamber. Rats with a bias for either of the lateral chambers (> 540 s) were excluded from the experiments.

Beginning on day 1, rats were scheduled for training sessions twice daily (08:30 and 16:30) for 4 days (day 1–4). Rats received morphine (4 mg/kg, i.p., First Pharmaceutical Factory of Shenyang, China) before being confined to one side of two lateral chambers for 45 min, and normal saline (2 ml/kg, i.p.) to the other side of two lateral chambers. The morphine-paired sides were counter-balanced among all groups.

Subsequently, recent and remote retrieval of morphine-associated memory was assessed 1 day and 3 weeks after the last conditioning session, respectively. Same as those described for the preconditioning test, rats were placed in chamber B with the guillotine doors removed to allow access to the entire apparatus for 15 min and time spent in each chamber were recorded (post-conditioning test, test). The CPP score was defined as the time (in seconds) spent in the morphine-paired chamber minus the time spent in the saline-paired chamber.

Preparation of Total and Membrane Protein

For western blotting experiments, the rats were decapitated immediately, 30 and 60 min after recent and remote retrieval, the brains were quickly removed and frozen. Bilateral tissue punches (12 gauges) of the vmPFC were obtained from 60 µm thick sections taken on a sliding freezing microtome. The extraction of the membrane fraction and the total protein were conducted using the procedure described previously [20, 21]. Tissue punches were homogenized in 170 µl of ice-cold homogenization buffer (10 mM HEPES, pH 7.4; 2 mM EDTA; 320 mM sucrose; 1% protease inhibitor cocktail) using a Potter–Elvehjem tissue grinder with a Teflon pestle. The homogenate was centrifuged at 1000×*g* for 10 min; the supernatant was preserved, and the pellet was re-suspended with the homogenization buffer and then centrifuged again at 1000×*g* for 5 min. The pooled supernatant was further centrifuged at 1000×*g* for 5 min to remove residual debris. 30 µl of the supernatant was preserved for total protein analysis, and the remaining volume centrifuged at 200,000 g for 45 min. The pellet consisting crude membrane fraction was re-suspended in the membrane buffer (25 mM HEPES, pH 7.4; 2 mM EDTA; 1% protease inhibitor cocktail) and centrifuged again at 200,000×*g* for 30 min. The resulting pellet, which was re-suspended with 35 µl of ice-cold RIPA buffer (Applygen Technologies Inc, C1053), was preserved as the membrane fraction. Both the total and membrane fractions were quantified using the BCA assay (Thermo; 23,225).

Western Blotting

Total and membrane protein of each sample was loaded and electrophoresed on 10% SDS-PAGE gels. Then the proteins were transferred to the polyvinylidene difluoride (PVDF) membrane. Non-specific binding sites were blocked in Tris-buffered saline (TBST, 50 mM Tris-HCl, pH 7.4; 150 mM NaCl and 0.05% Tween 20) with 5% non-fat milk for 1 h at room temperature. Then the PVDF membranes were incubated with the following primary antibodies overnight at 4 ℃: GluA1 (1:1000, Millipore, AB1504), GluA2 (1:1000, Millipore, MAB397), Na–K ATPase (1:3000, AB7671, Abcam) and β-actin (1:4000, Sigma, A2228). On the next day, the PVDF membranes were washed thrice in TBST, followed by 1 h of incubation at room temperature with horseradish peroxidase-conjugated AffiniPure goat anti-mouse or anti-rabbit IgG (1:2000, Zhongshan Biotechnology, ZB-5305, 2301). Then the PVDF membranes were washed thrice and treated with Super Enhanced chemiluminescence detection kit (Applygen Technologies Inc, P1010) and visualized by exposure to Kodak film. The intensity of bands on the autoradiogram was quantified with Quantity One. To correct for the inconsistency in loading, the membrane protein was normalized to the Na–K ATPase protein and the total protein was normalized to the β-actin protein.

Surgery

Rats weighing 260–280 g were anesthetized with pentobarbital sodium solution (40 mg/kg, i.p.) and mounted on a Kopf stereotaxic apparatus (Kopf Instruments, Tujunga, CA). The scalp was incised and retracted and small holes were drilled on the skull for bilateral emplacement of stainless-steel guide cannulae (0.8 mm in outer diameter) to the vmPFC. The coordinates were anterior/posterior, + 3.0 mm; medial/lateral, ± 0.7 mm; and dorsal/ventral, − 3.5 mm, 1.0 mm above the intended site of injection [22]. Guide cannulae were fixed to the skull with dental acrylic cement. Stainless-steel obturators (0.4 mm in outer diameter) were inserted to the guide cannulae to avoid occlusion and prevent infection. Rats were injected with penicillin (2.0×10^5 U/rat, s.c.) after surgery and allowed for 7 days recovery.

Intracranial Microinfusions

Obturators were removed and microinjectors (outer diameter, 0.41 mm; inner diameter, 0.25 mm; RWD Life Science Co., Ltd) were inserted bilaterally, extending 1.0 mm beyond the tip of guide cannulae. The microinjectors were connected to 1.0 µl Hamilton syringes via PE-20 tubing. The tubing was back-filled with saline, with a small air bubble separating the saline from the drug or vehicle. The microinfusion was performed through an infusion pump at a speed of 0.1 µl/min while the rat was gently held. The microinjectors were left in place for an additional 2 min to allow for drug diffusion. The obturators were then reinserted, and the rat was placed back to its home cage.

A total volume of 0.3 µl per side was microinfused into the vmPFC. Synthetic peptide derived from the rat GluA2 C terminus (GluA2-3Y; 15 pM; 869YKEGYNVYG877; AnaSpec, AS-64429) fused to the cell membrane transduction domain of the HIV-1 TAT protein, or an HIV-TAT-fused GluA2 negative control peptide (GluA2-3A; 15 pM; AKEGANVAG; AnaSpec, AS-64984), was microinfused into the vmPFC 1 h prior to the recent retrieval [18, 23]. 6-nitro-7-sulfamoylbenzo[f]quinoxaline-2, 3-dione (NBQX, 1 µM dissolved in PBS; Sigma-Aldrich, N171), the selective AMPA receptor antagonist, was microinfused into the vmPFC 10 min prior to the remote retrieval [24].

Histology

Histological verification of cannulae location was performed after behavioral tests. Rats were anesthetized with chloral hydrate (35 mg/kg, i.p.) and perfused intracardially with

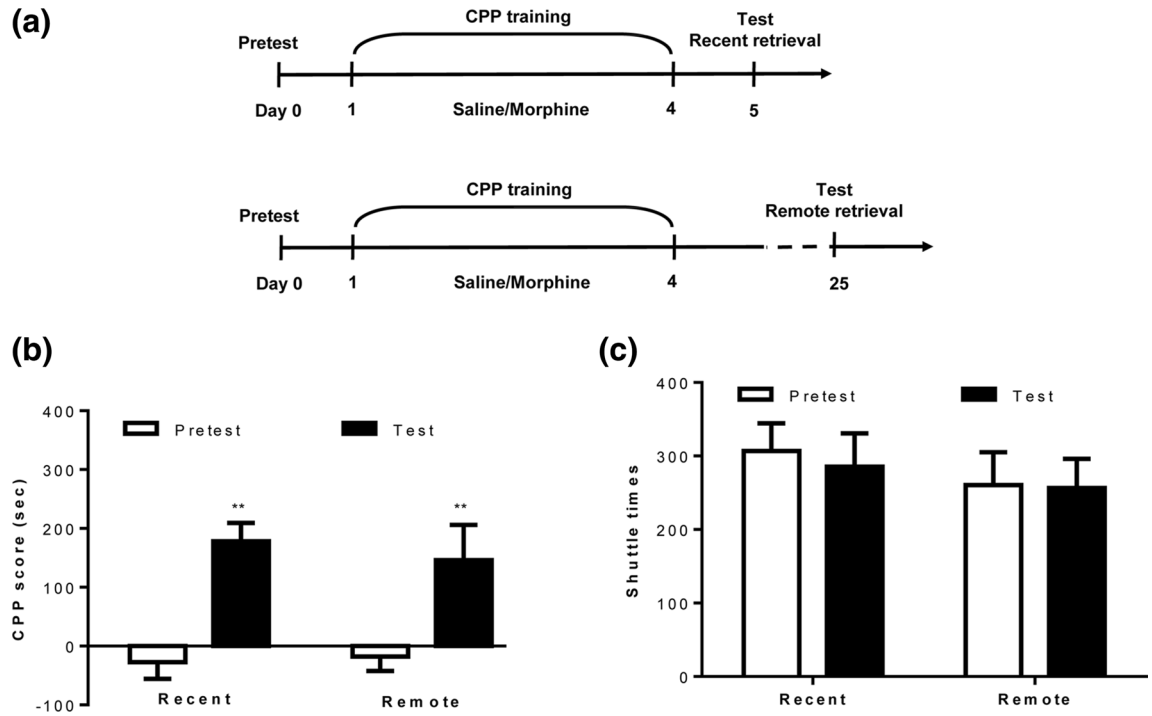

Fig. 1 Recent and remote retrieval of morphine-associated memory. **a** The schematic timeline of the recent (upper, n = 13) and remote (lower, n = 15) retrieval of morphine-associated memory. **b** Both recent and remote retrieval of morphine-associated memory induced a significant conditioned-place preference. **c** There were no significant changes in locomotion activity across groups. Data were analyzed by two-way RM ANOVA and Bonferroni *post-hoc* test. $^{**}P < 0.01$, compared with the pretest

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 (40 μm thick) were cut on a cryostat (− 22 °C) and wet-mounted on glass microscope slides. Slides were stained with cresyl violet. The sections were examined with light microscopy using × 10 magnification. The most ventral point of the microinjector was mapped onto schematics from a rat brain atlas (Paxinos and Watson [22]). Only animals exhibiting correct placement within ventral prelimbic or within the infralimbic cortex or at their interface were included in the statistical analyses.

Statistics

All statistical tests were conducted with Prism 6 (GraphPad Software). Data were presented as mean ± SEM. Results from Figs. 1, 4 and 5 were analyzed with two-way repeated-measures (RM) ANOVA followed by Bonferroni *post-hoc* tests, results from Figs. 2 and 3 were analyzed with one-way ANOVA followed by Newman-Keuls multiple comparisons tests. The accepted level of statistical significance was $P < 0.05$.

Result

Recent and Remote Retrieval of Morphine-Associated Memory

Rats were trained to associate the context with the efficacy of morphine. Here we detected the retrieval of morphine-conditioned memory at two time-points: 1 day (day 5, recent retrieval) after conditioning and 3 weeks (day 25, remote retrieval) after conditioning (Fig. 1a). Two-way RM ANOVA revealed a significant effect of Time (test vs. pretest, $F_{1, 26} = 23.24$, $P < 0.0001$), but there was no significant effect of Retrieval (recent vs. remote, $F_{1, 26} = 0.07204$, $P = 0.7905$), and Time x Retrieval interaction ($F_{1, 26} = 0.2931$, $P = 0.5928$). Bonferroni post-hoc test demonstrated that a significant increase of CPP score was found both in recent retrieval group (test vs. pretest, $t = 3.663$, $P = 0.0022$) and remote retrieval group (test vs. pretest, $t = 3.14$, $P = 0.0084$). The above results showed that significant conditioned-place preference was induced following the recent and remote retrieval of morphine-associated memory (Fig. 1b).

Moreover, we also analyzed the shuttle times of rats following the recent and remote retrieval of

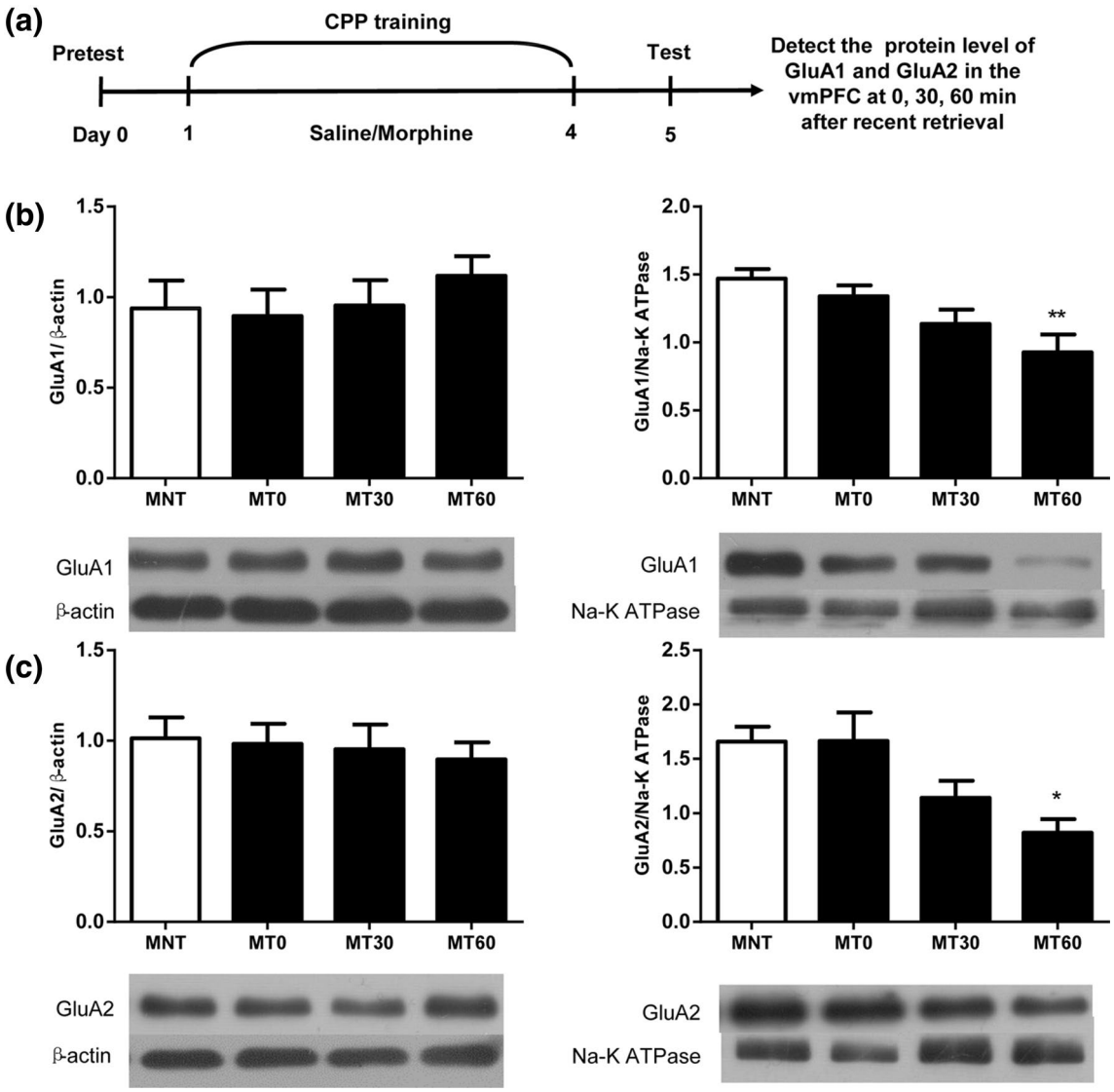

Fig. 2 Changes in total and membrane protein level of GluA1 and GluA2 in the vmPFC following the recent retrieval of morphine-associated memory. **a** Schematic diagram for experimental design. **b** The membrane protein level of GluA1 (right) in the vmPFC was significantly decreased 60 min after the recent retrieval of morphine-associated memory, while the total protein level of GluA1 (left) had no changes. **c.** The membrane protein level of GluA2 (right) in the vmPFC was significantly decreased 60 min after the recent retrieval of morphine-associated memory, while the total protein level of GluA2 (left) had no changes. Representative results of western blotting were shown on the bottom of the panel. Data were analyzed by one-way ANOVA and Newman-Keuls multiple comparisons test. $^{**}P<0.01$, $^{*}P<0.05$, compared with MNT. MNT: morphine conditioning + no test (n = 5); MT0: morphine conditioning + 0 min post-test (n = 5); MT30: morphine conditioning + 30 min post-test (n = 5); MT60: morphine conditioning + 60 min post-test (n = 5)

morphine-associated memory. Two-way RM ANOVA revealed that there was no significant effect of Time ($F_{1, 26} = 0.2161$, $P = 0.6459$), Retrieval ($F_{1, 26} = 0.5061$, $P = 0.4832$), and Time x Retrieval interaction ($F_{1, 26} = 0.09876$, $P = 0.7558$), suggesting that the locomotor activity of rats had no significant changes following the recent and remote retrieval of morphine-associated memory (Fig. 1c).

Changes in Total and Membrane Protein Level of GluA1 and GluA2 in the vmPFC Following the Recent Retrieval of Morphine-Associated Memory

The total and membrane protein level of GluA1 and GluA2 in the vmPFC were analyzed before (MNT), immediately (MT0), 30 min (MT30) and 60 min (MT60) after the recent retrieval of morphine-associated memory (Fig. 2a). One-way ANOVA revealed that the total protein level of GluA1 ($F_{3, 16} = 0.4971$,

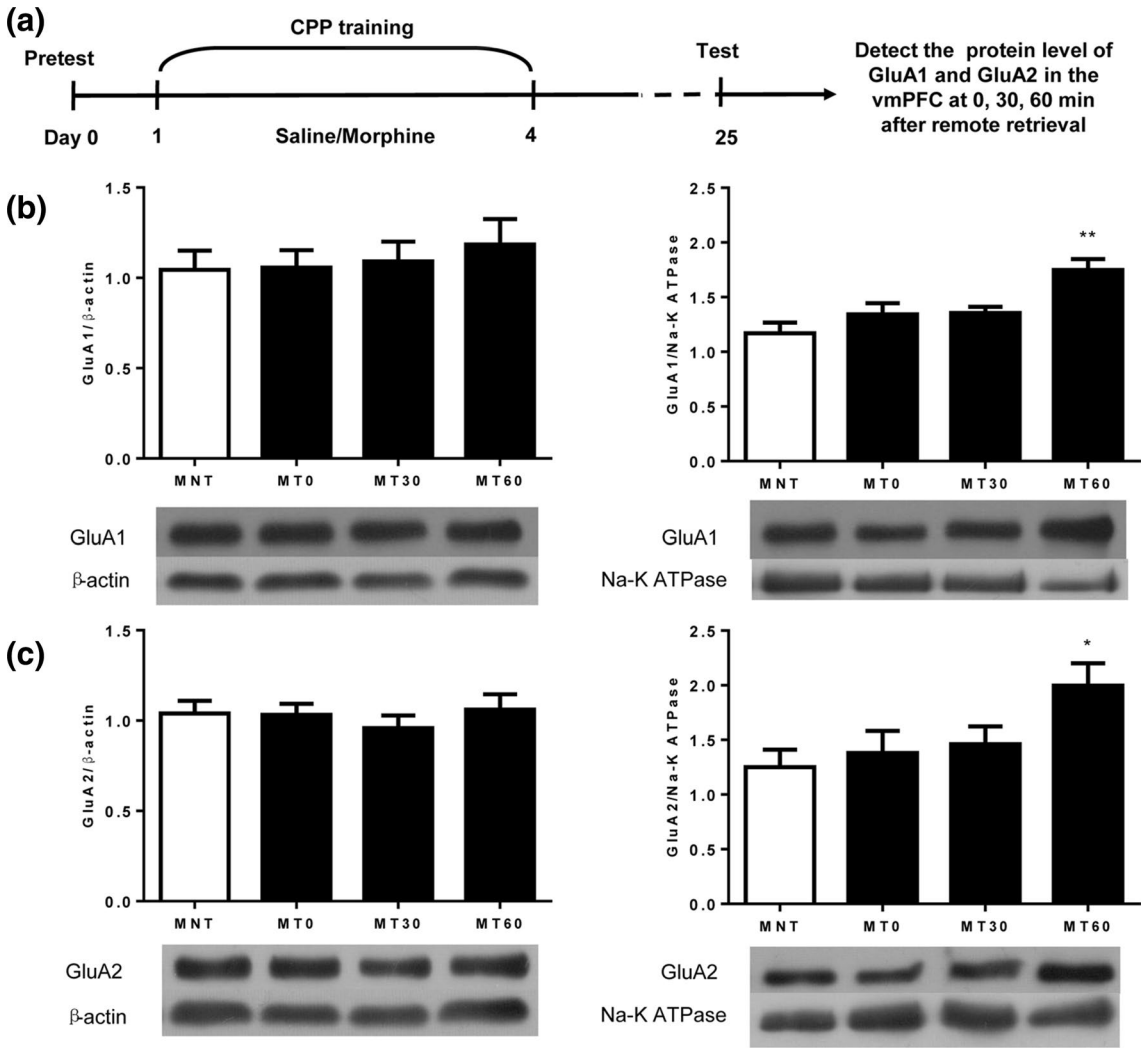

Fig. 3 Changes in total and membrane protein level of GluA1 and GluA2 in the vmPFC following the remote retrieval of morphine-associated memory. **a** Schematic diagram for experimental design. **b** The membrane protein level of GluA1 (right) in the vmPFC was significantly increased 60 min after the remote retrieval of morphine-associated memory, while the total protein level of GluA1 (left) had no changes. **c** The membrane protein level of GluA2 (right) in the vmPFC was significantly increased 60 min after the remote retrieval of morphine-associated memory, while the total protein level of GluA2 (left) had no changes. Representative results of western blotting were shown on the bottom of the panel. Data were analyzed by one-way ANOVA and Newman-Keuls multiple comparisons test. $^{**}P<0.01$, $^{*}P<0.05$, compared with MNT. MNT: morphine conditioning + no test (n = 6); MT0: morphine conditioning + 0 min post-test (n = 6); MT30: morphine conditioning + 30 min post-test (n = 6); MT60: morphine conditioning + 60 min post-test (n = 6)

$P = 0.6894$; Fig. 2b, left) and GluA2 ($F_{3,\ 16} = 0.1807$, $P = 0.9079$; Fig. 2c, left) had no significant differences across groups. One-way ANOVA revealed a significant difference in the membrane protein level of GluA1 ($F_{3,\ 16} = 5.537$, P = 0.0084; Fig. 2b, right). Newman-Keuls multiple comparisons test confirmed that the membrane protein level of GluA1 in the MT60 group (q = 5.37, P < 0.01) was decreased compared with the MNT group. And the membrane protein level of GluA2 was also decreased compared to the MNT group (One-way ANOVA: $F_{3,\ 16} = 5.292$, P = 0.01; Newman-Keuls multiple comparisons test: MT60 vs. MNT, q = 4.662, P < 0.05; Fig. 2c, right). In summary, the membrane protein level of GluA1 and GluA2 in the vmPFC was significantly decreased following the recent retrieval of morphine-associated memory while the total protein level of GluA1 and GluA2 had no significant changes.

Changes in Total and Membrane Protein Level of GluA1 and GluA2 in the vmPFC Following the Remote Retrieval of Morphine-Associated Memory

The total and membrane protein level of GluA1 and GluA2 in the vmPFC were analyzed before (MNT), immediately (MT0), 30 min (MT30) and 60 min (MT60) after the

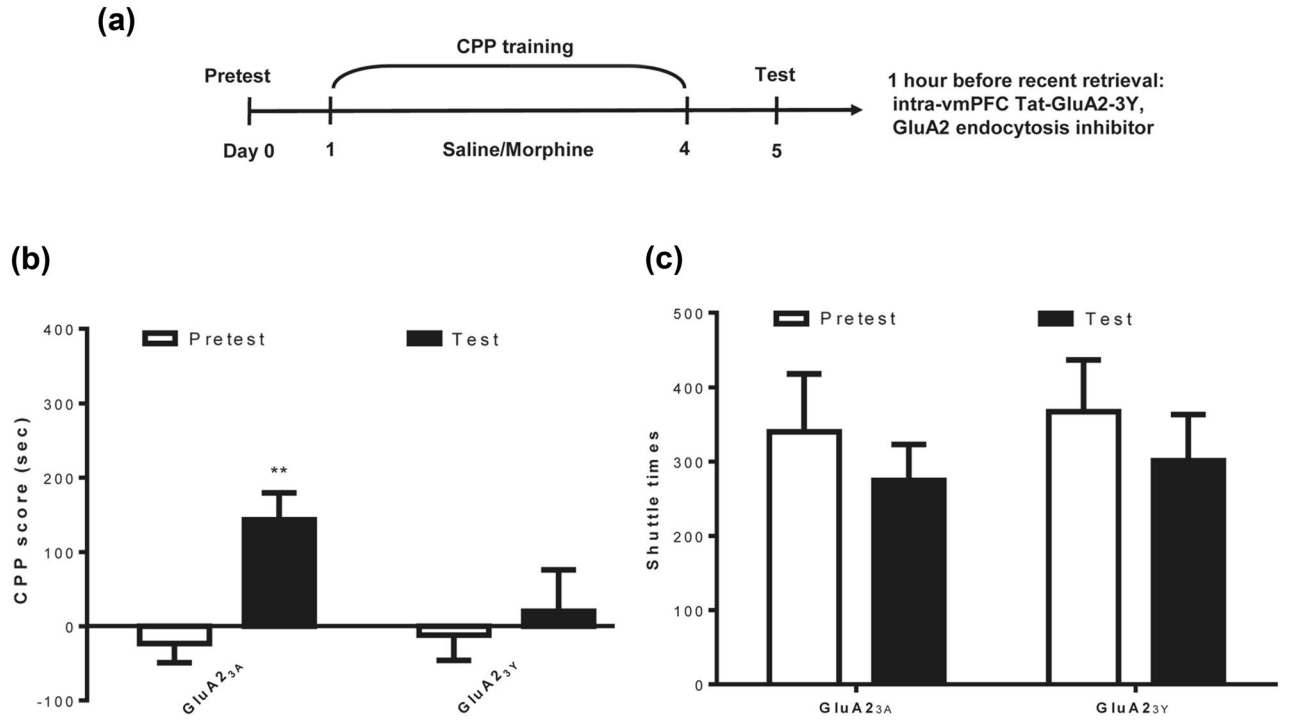

Fig. 4 Effect of micro-infusion Tat-GluA2-3Y into the vmPFC on the recent retrieval of morphine-associated memory. **a** Schematic diagram for experimental design. Tat-GluA2-3Y (15 pmol/side, the GluA2 endocytosis inhibitor, n = 10) or Tat-GluA2-3A (15 pmol/side, the negative control peptide, n = 9) was microinfused into the vmPFC 1 h before the recent retrieval of morphine-associated memory. **b** Conditioned-place preference was blocked by bilateral microinfusion of Tat-GluA2-3Y but not Tat-GluA2-3A into the vmPFC before the recent retrieval. **c** There were no significant changes in locomotion activity across groups. Data were analyzed by two-way RM ANOVA and Bonferroni *post-hoc* test. $^{**}P < 0.01$, compared with pretest

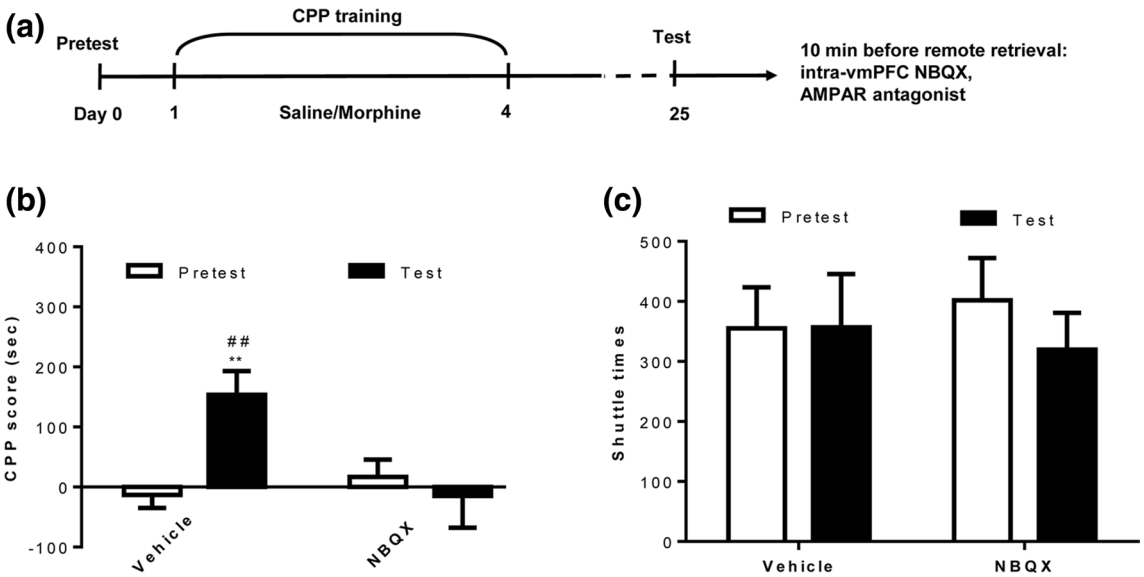

Fig. 5 Effect of micro-infusion NBQX into the vmPFC on the remote retrieval of morphine-associated memory. **a** Schematic diagram for experimental design. NBQX (1 μM/side, the AMPAR antagonist, n = 10) or vehicle (0.3 μl/side, PBS, n = 10) was microinfused into the vmPFC 10 min before the remote retrieval of morphine-associated memory. **b** Conditioned-place preference was blocked by bilateral microinfusion of NBQX but not vehicle into the vmPFC before the remote retrieval. **c.** There were no significant changes in locomotion activity across groups. Data were analyzed by two-way RM ANOVA and Bonferroni *post-hoc* test. $^{**}P < 0.01$, compared with pretest, $^{\#\#}P < 0.01$, compared with NBQX

remote retrieval of morphine-associated memory (Fig. 3a). One-way ANOVA revealed that the total protein level of GluA1 ($F_{3, 20} = 0.3042$, $P = 0.822$; Fig. 3b, left) and GluA2 ($F_{3, 20} = 0.3886$, $P = 0.7624$; Fig. 3c, left) had no significant differences across groups. One-way ANOVA revealed a significant difference in the membrane protein level of GluA1 ($F_{3, 20} = 7.307$, P = 0.0017; Fig. 3b, right). Newman-Keuls multiple comparisons test showed that the membrane protein level of GluA1 in the MT60 group (q = 6.394, P < 0.01) was increased compared to the MNT group. Further, the membrane protein level of GluA2 was also increased (One-way ANOVA: $F_{3, 20} = 3.185$, P = 0.0461; Newman-Keuls multiple comparisons test: MT60 vs. MNT, q = 4.061, P < 0.05; Fig. 3c, right). In summary, the membrane protein level of GluA1 and GluA2 in the vmPFC was significantly increased following the remote retrieval of morphine-associated memory while the total protein level of GluA1 and GluA2 had no significant changes.

Effect of Micro-Infusion Tat-GluA2-3Y into the vmPFC on the Recent Retrieval of Morphine-Associated Memory

In this experiment, we assessed the effect of decreased membrane expression of GluA2 in the vmPFC on the recent retrieval of morphine-associated memory. Tat-GluA2-3Y and its control peptide Tat-GluA2-3A were microinjected into the vmPFC, respectively. GluA2-3Y mimics the C-terminal tail of GluA2 and has been proved to prevent the loss of membrane expression of GluA2 [25, 26]. As shown in Fig. 4a, Tat-GluA2-3Y or Tat-GluA2-3A was microinjected into the vmPFC 1 h before the recent retrieval of morphine-associated memory. Two-way RM ANOVA revealed a significant effect of Time (test vs. pretest, $F_{1, 17} = 8.141$, P = 0.011), but there was no effect of Drug (Tat- GluA2-3Y vs. Tat- GluA2-3A, $F_{1, 17} = 1.519$,$P = 0.2346$), and Time x Drug interaction ($F_{1, 17} = 3.721$, $P = 0.0706$). Bonferroni *post-hoc* test demonstrated that a significant increase of CPP score was found in the Tat- GluA2-3A group (test vs. pretest, t = 3.296, P = 0.0085). But there was no significant difference of CPP score in the Tat- GluA2-3Y group (test vs. pretest, t = 0.6714, $P > 0.9999$). The above results showed that the decreased membrane GluA2 was essential for the recent retrieval of morphine-associated memory (Fig. 4b).

Two-way RM ANOVA analysis of shuttle times revealed that there was no significant effect of Time ($F_{1, 17} = 3.193$, $P = 0.0918$), Drug ($F_{1, 17} = 0.1013$, $P = 0.7541$), and Time x Drug interaction ($F_{1, 17} = 0.01213$, $P = 0.9973$). Unchanged locomotor activity of rats excluded the possibility that the failed conditioned-place preference in the Tat-GluA2-3Y group was due to the inhibition of locomotor activity (Fig. 4c).

Effect of Micro-infusion NBQX into the vmPFC on the Remote Retrieval of Morphine-Associated Memory

In this experiment, we assessed the effect of increased membrane expression of GluA1 and GluA2 in the vmPFC on the remote retrieval of morphine-associated memory. As shown in Fig. 5a, NBQX and vehicle were microinjected into the vmPFC 10 min before the remote retrieval of morphine-associated memory. Two-way RM ANOVA revealed a significant effect of Time (test vs. pretest, $F_{1, 18} = 5.132$, P = 0.0361), Time x Drug interaction ($F_{1, 18} = 11.16$, P = 0.0036), but there was no effect of Drug (NBQX vs. vehicle, $F_{1, 18} = 2.502$, $P = 0.1311$). Bonferroni *post-hoc* test detected a significant increase of CPP score in the vehicle group (test vs. pretest, t = 3.964, P = 0.0018). However, no significant difference of CPP score was found in the NBQX group (test vs. pretest, t = 0.76, $P = 0.9142$). Moreover, a significant decreased CPP score was found in the test group (NBQX vs. vehicle, t = 3.187, P = 0.0059). These data demonstrated that increased membrane expression of GluA1 and GluA2 was involved in the remote retrieval of morphine-associated memory (Fig. 5b).

Two-way RM ANOVA analysis of shuttle times revealed no significant effect of Time ($F_{1, 18} = 0.8207$, $P = 0.3769$), Drug ($F_{1, 18} = 0.002499$, $P = 0.9607$), and Time x Drug interaction ($F_{1, 18} = 0.883$, $P = 0.3598$). Locomotor activity of rats was not influenced by intra-vmPFC microinfusion of NBQX or vehicle following the remote retrieval of morphine-associated memory (Fig. 5c).

Histological verification of cannulae locations was performed after the behavioral pharmacological test. A representative histological site of microinjector placement in the vmPFC was shown in Fig. 6a. The final sample sizes used in the statistical analyses were indicated in Fig. 6b.

Discussion

The present work revealed that membrane expression of GluA1 and GluA2 in the vmPFC decreased following the recent retrieval, while the membrane expression of GluA1 and GluA2 increased following the remote retrieval of morphine-associated memory. Further, time selective intervention in the changes of GluA1 and GluA2 by means of pharmacology inhibited the recent and remote retrieval respectively. And the intervention had no effect on the locomotor activity of rats. These findings indicated that the role of AMPAR in the vmPFC was different and

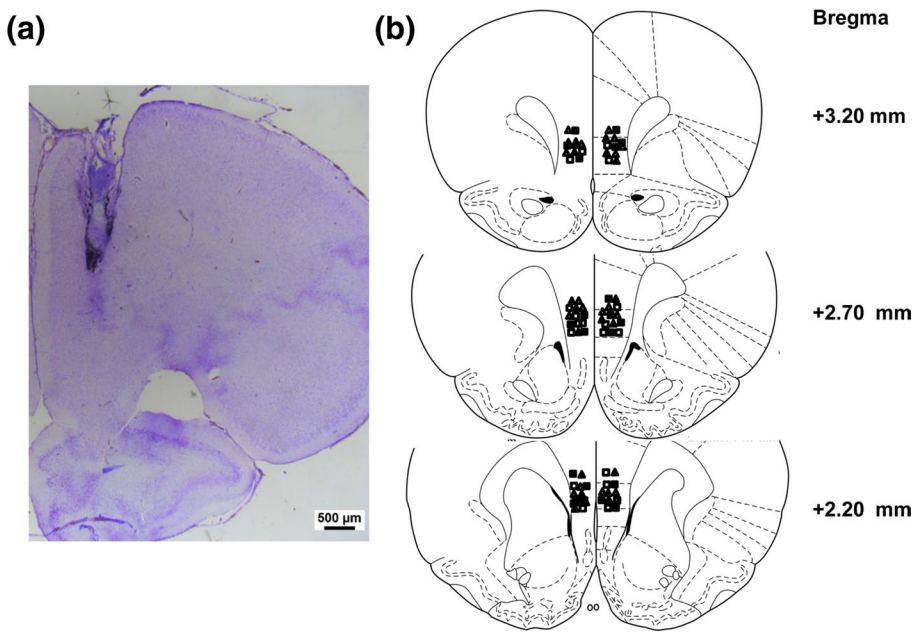

Fig. 6 Histological verification of microinjector placements within the vmPFC. **a** A representative histological site of microinjector placement in the vmPFC. Scale bar, 500 μm **b** Histological examination was conducted after the completion of behavior tests. Diagram depicted the microinjector placements of rats included in behavioral pharmacological experiments for the intra-vmPFC infusion of Tat-GluA2-3Y (filled square, n = 10), Tat-GluA2-3A (open square, n = 9), VEH (open triangle, n = 10) and NBQX (filled triangle, n = 10)

important in the recent and remote retrieval of morphine-associated memory.

The vmPFC is Involved in Both Recent and Remote Retrieval of Drug-Associated Memory

The vmPFC can index and bind information stored in multiple connected cortical areas to retrieve coherent remote memories [27]. This point is corroborated by other studies that inactivation of the vmPFC with muscimol + baclofen reduces cue-induced cocaine-seeking after 30 days of drug withdrawal in rats [8]. Selective inactivation of the neuronal ensembles, which are activated by the heroin-associated context, inhibits context-induced drug relapse [28]. Moreover, selective inhibition of the PKCε or PI3K in the vmPFC, which are both documented to be involved in AMPAR trafficking, also impairs the reinstatement of cocaine seeking behavior in rats after 30 days of withdrawal [9, 10]. Consistently, the present work showed that microinfusion of AMPAR antagonist NBQX into the vmPFC prevented the remote retrieval of morphine-associated memory. Overall, these data further reveal that vmPFC is involved in the remote retrieval of drug-associated memory.

Noteworthy, only a few works study the role of vmPFC in the recent retrieval of drug-associated memory. Van den Oever et al. report that optogenetic inhibition of pyramidal cells in the vmPFC can suppress the recent retrieval of cocaine-associated memory [29]. On the contrary, local stimulation of the vmPFC with bicuculline + saclofen (GABAa and GABAb receptor antagonists) increases cocaine seeking after 1 day of withdrawal [8]. In our work, inhibiting the endocytosis of GluA2 with Tat- GluA2-3Y in the vmPFC blocked the recent retrieval of morphine-associated memory. These data suggest that vmPFC is involved in the recent retrieval of drug-associated memory.

Retrograde and anterograde amnesia studies demonstrate that the hippocampus is essential for the storage and retrieval of a new memory, but this role is temporally graded [30–32]. Intracellular recordings in anesthetized rats reveal a monosynaptic connection between the hippocampal neurons and pyramidal cells in the vmPFC, and that tetanic stimulation of the hippocampus produced LTP of the monosynaptic EPSP [33]. Additionally, functional magnetic resonance imaging (fMRI) study detects increased functional connectivity between the vmPFC and the hippocampus during the recall of declarative memory 48 h after learning in healthy volunteers [34]. The studies indicate that the vmPFC can communicate with the hippocampus, and the communication is enhanced during the recent memory retrieval, implying that the vmPFC is mobilized by the recent memory retrieval.

Change of AMPAR-Related Plasticity in the vmPFC is Different in the Recent and Remote Retrieval of Drug-Associated Memory

Lai et al. using transcranial two-photon microscopy find that auditory-cued fear conditioning eliminates postsynaptic dendritic spines in the mouse frontal association cortex. On the contrary, fear extinction with the same auditory cue increases the spine formation on the same dendritic branches [35], indicating that fear conditioning and extinction can lead to opposed changes at the level of synapses. As mentioned in the introduction, changes in membrane level of GluA1 are positively correlated with changes in

spine size following the whisker stimulation [13]. Membrane GluA2 can be involved in increase spine size and density in mature cultured hippocampal neurons via extracellular N-terminal domain (NTD) [36]. Therefore, membrane GluA1 and GluA2 may participate in coding different behaviors via modifying the spine plasticity. In support with that, we found that the recent and remote retrieval of morphine-associated memory elicited opposed changes in expression level of membrane GluA1 and GluA2 in the vmPFC.

Then how can GluA1 and GluA2 be trafficked into and out of the membrane? Phosphorylated ERK can link to AMPAR synaptic insertion during hippocampal LTP, increased ERK phosphorylation is in parallel with increased membrane AMPAR expression in rats after cocaine withdrew for 14 days [37]. PKCε, an upstream regulator of the ERK, overexpression of that can induce a time- and dose-dependent increase of phosphorylated ERK [38]. PI3K is shown to complex with AMPAR at the synaptic membrane, and the activation of AMPAR-coupled PI3K is required for membrane expression of AMPAR and LTP [39, 40]. Taking together, PKCε, phosphorylated ERK, and PI3K are involved in the membrane trafficking of AMPAR. Interestingly, the expressions of phosphorylated ERK, PKCε, PI3K, and AMPAR in the vmPFC are increased following the remote retrieval of drug-associated memory [8–10]. The expressions of PKCε and AMPAR in the vmPFC are decreased following the recent retrieval of drug-associated memory [9]. All in all, the different changes of AMPAR related molecules in recent and remote retrieval of drug-associated memory are in agreement with fear-associated memory [41–43], implying that neural plasticity is different in the recent and remote memory retrieval.

However, the increased membrane expression of GluA2 in induced by the remote memory retrieval in our work is inconsistent from some previous studies that expression of GluA2 in the vmPFC is decreased after re-exposure to context 21 days after heroin self-administration [17, 18]. The discrepancy between these results may be due to the differences in the route of drug administration (experimenter-delivered here vs. self-administration), the number of sessions and the duration of a session (4 sessions × 45 min here vs. 15 sessions × 3 h). Besides, rats are withdrawn in their home cage in our study while the extinction training is conducted in their studies.

In conclusion, our study found that the recent and remote retrieval of morphine-associated memory elicited opposed changes of membrane GluA1 and GluA2 in the vmPFC, which is necessary for the recent and remote memory retrieval. The retrieval of morphine-associated memory is reorganized over time in the vmPFC, and the finding is helpful for unlocking the mechanisms involved in the recent and remote memory retrieval.

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Compliance with Ethical Standards

Conflict of interest: Authors declare that they have no competing interests.

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