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Phosphorylated SNAP25 in the CA1 regulates morphine-associated contextual memory retrieval via increasing GluN2B-NMDAR surface localization

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ABSTRACT

Although our previous studies have demonstrated both protein kinase C (PKC) and GluN2B-containing *N*-methyl-D-aspartate receptor (GluN2B-NMDAR) play crucial roles in morphine-associated learning and memory, the relationship between them remains unexplored. In this study, we validated the enhanced PKC and membrane GluN2B protein expression in the hippocampal CA1 after morphine conditioned place preference (CPP) expression in rats. Interestingly, we also found that phosphorylation of SNAP25 at Ser187 (pSer187-SNAP25), a PKC-activated target, was significantly increased following morphine CPP expression. Blocking the pSer187-SNAP25 by intra-CA1 injection of an interfering peptide impaired morphine CPP expression and accompanied by the reduced ratio of GluN2B membrane/total in the CA1. In addition, intra-CA1 blockade of pSer187-SNAP25 did not affect natural learning and memory process as evidenced by intact sucrose-induced CPP expression and normal locomotor activity in rats. Therefore, our results reveal that enhanced pSer187-SNAP25 by PKC recruits GluN2B-NMDAR to the membrane surface in the hippocampal CA1 and mediates context-induced addiction memory retrieval. Our findings in this study fill in the missing link and provide better understanding of the molecular mechanisms involved in morphine-associated contextual memory retrieval.

Keywords GluN2B, hippocampal CA1, morphine conditioned place preference, PKC, SNAP25.

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INTRODUCTION

Drug addiction hijacks normal learning and memory neural circuits, induces maladaptive changes in certain molecular targets and biases the behavior outcome towards further drug use (Nestler 2002; Kelley 2004). The high relapse rate in addicts after withdrawal is largely due to the retrieval of previously established addiction memory, provoked by re-exposure to the previous drug intake-associated contexts (Wikler 1973; O'Brien *et al.* 1992; Perry *et al.* 2014). Therefore, understanding how drug addiction tampers the normal learning and memory molecular circuits may provide novel therapeutic targets for drug craving and relapse.

The morphine conditioned place preference (CPP) model has been widely used to study the association between drug-paired context and its rewarding properties (Kim, Jang, & Park 1996; Benturquia *et al.* 2007; Solecki *et al.* 2009). Also, it has been reported that the glutamatergic signaling in the hippocampus is involved in coding context memory process (Moron *et al.* 2010; Maren, Phan, & Liberzon 2013; Portugal *et al.* 2014). Our previous studies have demonstrated that GluN2B-containing *N*-methyl-D-aspartate receptor (GluN2B-NMDAR) protein level increases specifically following the expression and reinstatement of morphine CPP in the hippocampus, and selective blockade of GluN2B-NMDAR suppresses morphine CPP expression (Ma *et al.* 2006; Ma *et al.* 2007; Shen *et al.* 2016). Moreover, GluN2B expression remains unaltered if morphine is not paired with a specific context (Portugal *et al.* 2014). Altogether, this evidence suggests that GluN2B-NMDAR plays a pivotal role in morphine reward and reward-paired context associations. Another line of our studies has shown that the

level of activated protein kinase C (PKC), which is significant for memory formation and storage (Wang, Hu, & Tsien 2006), also increases with morphine dose-dependent manner in the nucleus accumbens following morphine CPP expression (Ping *et al.* 2012). Intracerebroventricular injection of selective PKC inhibitor calphostin C suppresses the morphine-induced place preference (Narita *et al.* 2001). These results suggest that PKC is also necessary for establishing the association between morphine reward and reward-paired context.

Protein kinase C was previously reported to regulate NMDAR membrane localization through phosphorylating the synaptosomal-associated protein of 25 kDa (SNAP25) in *Xenopus* oocytes and neurons (Lan *et al.* 2001; Lu *et al.* 2001; Washbourne *et al.* 2004; Lau *et al.* 2010). SNAP25 combined with syntaxin1 and vesicle-associated membrane protein-2 (VAMP2) forms the soluble *N*-ethylmaleimide-sensitive factor attachment receptor (SNARE) core complex and drives membrane fusion (Jahn, Lang, & Sudhof 2003; Kennedy & Ehlers 2011). Furthermore, SNAP25 is specific expression on neuronal cells and also localized in the postsynaptic densities besides presynaptic terminal that is consistent with SNAP25 mediating postsynaptic receptor trafficking (Jordan *et al.* 2004; Cheng *et al.* 2006; Yamamori *et al.* 2011). Whether PKC in the hippocampal CA1 could regulate the membrane localization of GluN2B-NMDAR through modulating SNAP25 phosphorylation during morphine-associated contextual memory retrieval is still unknown.

In the present study, morphine CPP test was used as an indicator of morphine-associated contextual memory retrieval to investigate which site of SNAP25 was phosphorylated by PKC could promote GluN2B-NMDAR membrane localization in the CA1, and how the specific site affected morphine-associated memory retrieval.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats, 240–250 g on arrival, were obtained from the Laboratory Animal Center of Peking University Health Science Center and lived with food and water supplied *ad libitum*. Three rats per cage were lived in the temperature (23 ± 2°C) and humidity (50 ± 5 percent) controlled environment with a 12-hour light/dark cycle. All experimental procedures were approved by the Animal Use Committee of Peking University Health Science Center.

Drugs and peptides

Morphine hydrochloride (First Pharmaceutical Factory of Qinghai, China) was dissolved in 0.9 percent physiological saline to a final concentration of 2 mg/ml. TAT-S28/TAT-S28A, TAT-T29/TAT-T29A and TAT-S187/TAT-S187A were synthesized and purified by GL Biochem (Shanghai, China). The purity of the peptides was more than 95 percent. The TAT fusion peptides were dissolved in ddH_2O at a final concentration of 3.2 nmol/μl.

Morphine-induced conditioned place preference

Place conditioning was conducted as previously reported (Xu *et al.* 2012). The CPP apparatus contained three chambers connected through guillotine doors. The two large black conditioning chambers (A and C, 280 × 220 × 225 mm^3) with distinct visual and tactile cues were separated by a small black center choice chamber B (135 × 220 × 225 mm^3). The CPP procedure contained pre-conditioning test, conditioning and post-conditioning test. On day 0, baseline preference was assessed by placing the rats in the chamber B and allowing free access to all compartments for 15 minutes. Rats with apparent bias for either of lateral chambers were removed. On days 1–4, rats were trained for two sessions every day, 5-hour resting period between morning and afternoon sessions, with injection of morphine (4 mg/kg, i.p.) and 0.9 percent saline (2 ml/kg, i.p.) in the designated compartments. After each injection, the rats were confined to the corresponding conditioned chambers (A or C) for 45 minutes with the guillotine doors closed. All groups were counterbalanced in the time (morning versus afternoon) and rewards (morphine versus saline). On day 5, the rats for test were adapted to CPP model room for 5 minutes and then were placed in chamber B in a drug-free state with the guillotine doors removed to allow them to explore the entire apparatus freely for 15 minutes. During this time, the rats for no test remained in their home cage without any treatment. After the CPP test, brains for both the test and no test of rats were removed at the same time. Data for the CPP score were calculated as the time spent in the morphine-paired chamber divided by the total time spent in both the morphine and the saline-paired chambers during CPP test. The locomotor activity during all CPP tests was estimated by counting the beam broken times between any two adjacent compartments.

Sucrose-induced conditioned place preference

The procedure for training sucrose CPP was modified from previous studies (Ma *et al.* 2006; Zhai *et al.* 2007; Labouesse, Langhans, & Meyer 2015). The CPP apparatus is the same as mentioned previously. Rats were adapted to the feeding cages (standard plastic cages measuring 275 × 215 × 130 mm^3, with one drinking bottle

and sawdust bedding) and the different taste of 20 percent sucrose solution and pure water for 1 hour, 1 day in 3 days. Prior to each session, 20 percent sucrose solution or pure water was poured freshly, and rats were mildly food-deprived for 2 hours in all rats. The preconditioning test took place, followed by the conditioning sessions as described previously. In rewarding trials in the morning, rats were placed in the feeding cages with 20 percent sucrose solution for 25 minutes and then confined to one conditioning chamber for 20 minutes. On non-rewarding trials in the afternoon, rats were placed in the feeding cages with pure water for 25 minutes and then confined to the other chamber for 20 minutes. All groups were also counterbalanced in the time (morning versus afternoon) and rewards (sucrose versus water). On day 5, the test session was performed in the absence of sucrose and water. Calculate the preference score and the locomotor activity as described previously.

Cannula implantation and micro-infusion

The rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and secured in a Kopf stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA). Stainless steel guide cannulas (0.64 mm in outer diameter) were bilaterally implanted 0.8 mm above the hippocampal CA1 region. The CA1 coordinates were anterior/posterior, −3.6 mm; medial/lateral, ±2.2 mm; and dorsal/ventral, −2.8 mm. The cannulas were fixed to screws in the skull with dental cement. Internal cannulas were replaced with dummy cannulas to keep it patent and prevent infection. The rats recovered at least 5 days before the experiments at the home cage.

During the intra-CA1 infusion, the dummy cannulas were removed, and infusion cannulas (0.41 mm in outer diameter) were inserted. A 1 μl TAT peptide was injected bilaterally into the CA1 region, and the TAT peptide was administered in 4 minutes (0.25 μl/minutes) (Wang *et al.* 2014). The injection needle was left *in situ* for an additional 1 minute to allow drug diffusion. Rats with inaccurate microinjection sites were excluded.

Tissue dissection and membrane fraction preparation

Preparation of membrane fraction was performed according to the modified method described previously (Voulalas, Schetz, & Undieh 2011; Qi *et al.* 2015). The rats were decapitated immediately after the CPP test, and the brains were quickly removed and frozen in N-hexane (−70°C) for approximately 25 seconds. Bilateral tissue of the CA1 were obtained and homogenized in ice-cold homogenization buffer [10 mM HEPES (pH 7.4), 2 mM EDTA, 320 mM sucrose, 1× protease inhibitor cocktail (P8340, Sigma Aldrich, St. LOUIS, MO, USA) and 1 percent (v/v) phosphatase inhibitor (P1260, Applygen Technologies Inc, Beijing, China)]. The homogenate was centrifuged at 1000 *g* for 10 minutes, the supernatant was reserved and the pellet was resuspended with homogenization buffer and then centrifuged again at 1000 *g* for 5 minutes. The pooled supernatants were further centrifuged at 1000 *g* for 5 minutes to remove most nuclei and residual debris. A 30 μl of the supernatant was reserved for total protein analysis, and the other was centrifuged at 200 000 *g* for 45 minutes at 4°C. The pellet was the crude membrane fraction and then resuspended in membrane buffer [25 mM HEPES (pH 7.4), 2 mM EDTA, protease and phosphatase inhibitors], then centrifuged again at 200 000 *g* for 30 minutes. A 20–30 μl ice-cold radioimmunoprecipitation assay buffer (R0278, Sigma Aldrich) was added to the pellet, which was resuspended and saved as the membrane fraction. Both the total and membrane fractions were determined using the bicinchoninic acid assay (23225, 23227, Thermo Scientific, Rockford, IL Campus, USA), and then analyzed by western blot.

Co-immunoprecipitation

Rat hippocampal CA1 was homogenized in ice-cold nondenaturing lysis buffer (C1050, Applygen Technologies Inc). After being rotated at 4°C for 1 hour, the homogenates were centrifuged at 12 000 *g* for 10 minutes to yield the total protein extract in the supernatant. Protein samples (400 μg of protein) were incubated at 4°C for 3 hours with 4 μg of anti-PKC (sc-17769, Santa Cruz, CA, USA) or anti-GluN2B (06–600, Millipore, Billerica, MA, USA) antibody before being incubated with protein A/G Agarose (20421, Thermo Scientific) overnight at 4°C. Normal IgG served as a negative control. The next day, the beads were washed six times with TBS containing 0.1 percent Triton X-100 and then eluted with 5× sodium dodecyl sulfate loading buffer by boiling for 5 minutes and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Western blot

Protein extracts (total protein: 30 μg; membrane protein: 20 μg) were electrophoresed in 12 percent sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The blots were blocked in TBST buffer (Tris-buffered saline with 0.1 percent Tween 20) containing 5 percent nonfat milk and then incubated with primary antibody diluted in TBST overnight at 4°C (anti-PKC, 1:500; anti-GluN2B, 1:1000; anti-GluN2A, 1:1000, 4205, Cell Signaling, Danvers, MA, USA; anti-SNAP-25, 1:10000, 111111, Synaptic Systems, Goettingen, Germany; anti-β-actin, 1:4000, A2228, Sigma) or TBST with 1 percent bovine serum albumin (anti- pS187-SNAP-25, 1:1000,

ab169871, Abcam, Cambridge, UK). The blots were then washed in TBST and incubated with horseradish peroxidase-conjugated goat anti-mouse or rabbit IgG for 1 hour at room temperature (1:2000, ZB-2305 or ZB-2301 Zhongshan Biotechnology, Beijing, China) followed by development with a chemiluminescence detection kit (WBKLS0500, Millipore). The blots were washed with stripping buffer (P1650, Applygen Technologies Inc) to incubate with other antibodies. The immunoreactive bands were analyzed quantitatively by densitometry with the Quantity One® 1-D analysis software. The optical density of each band was standardized as a percentage of the total before statistical treatment.

Statistical analysis

Data were analyzed by software GraphPad Prism 6.0. Differences between groups were compared with either paired *t*-test or one-way ANOVA followed by Tukey's *post hoc* test or by two-way ANOVA followed by Bonferroni *post hoc* test. The statistical significance was set at $P < 0.05$. All values represented as mean ± SEM.

RESULTS

Morphine conditioned place preference expression enhanced the membrane GluN2B and total PKC level in the CA1

Our previous studies report that GluN2B-NMDAR protein level is increased following morphine CPP expression (Ma *et al.* 2006; Ma *et al.* 2007; Ping *et al.* 2012). Here, we firstly detected the membrane protein level of GluN2B and GluN2A subunit in the CA1 after CPP test (Fig. 1a). Twenty-eight rats were used in this experiment.

We found that the membrane level of GluN2B increased obviously (Fig. 1c left; two-way ANOVA: Drug: $F_{1,\ 24} = 6.526$, $P = 0.0174$; Treatment: $F_{1,\ 24} = 7.275$, $P = 0.0126$; Drug × Treatment: $F_{1,\ 24} = 6.153$, $P = 0.0205$) and that was positively correlated with morphine CPP score (Fig. 1c right; $r = 0.9601$, $P = 0.0006$), whereas the membrane GluN2A level remained unchanged (Fig. 1d; two-way ANOVA: Drug: $F_{1,\ 24} = 0.3331$, $P = 0.5692$; Treatment: $F_{1,\ 24} = 1.051$, $P = 0.3154$; Drug × Treatment: $F_{1,\ 24} = 0.04366$, $P = 0.8363$). That was consistent with previous results. Moreover, the total PKC was also increased significantly (Fig. 1b left; two-way ANOVA: Drug: $F_{1,\ 24} = 9.800$, $P = 0.0045$; Treatment: $F_{1,\ 24} = 8.504$, $P = 0.0076$; Drug × Treatment: $F_{1,\ 24} = 8.018$, $P = 0.0092$), in positive correlation with morphine CPP score (Fig. 1b right; $r = 0.8602$, $P = 0.0130$) in the CA1 after the morphine CPP expression.

The results showed that the protein level of total PKC and membrane GluN2B, but not the GluN2A, in the CA1 increased markedly after morphine CPP test, with significant positive correlation with morphine CPP preference score.

Phosphorylation of SNAP25 at Ser187 by PKC was critical to morphine conditioned place preference expression in the CA1

Protein kinase C-induced phosphorylation of SNAP25 is reported to regulate NMDAR localization in cultured neurons by monitoring PKC potentiation of NMDA-elicited currents (Lau *et al.* 2010). Because PKC and GluN2B increased significantly and simultaneously in hippocampus CA1 in our study, we speculated whether the phosphorylated SNAP25 filled in the pivotal gap between PKC and GluN2B-NMDAR following the morphine-associated memory retrieval. According to previous studies, SNAP25 possesses three potential PKC sites: Ser28, Thr29 and Ser187 (Lau *et al.* 2010). We designed three separate peptides targeting each site. Each peptide comprised 15 aa of SNAP25, including the phosphorylated site of PKC and FITC fluorescent label, and binded to a cell-penetrating peptide (HIV TAT protein sequence RKKRRQRRR) (Fig. 2b). The phosphorylated site of the control peptide was replaced with an alanine. The TAT-mediated peptide is proved to effectively disrupt endogenous phosphorylation in previous studies (Futaki 2005; Zhang *et al.* 2010; Ping *et al.* 2012; Liu *et al.* 2015). The concentration of peptide increased in a time-dependent manner, peaking time at 4 hours in the N2a cells after FITC-TAT-S187 treatment (Fig. S1), which is consistent with previous reports in HEK 293 cells (Liu *et al.* 2015). Next, we microinjected the TAT fusion peptides (3.2 nmol/1 μl/side) into the bilateral CA1 4 hours before CPP test (Wang *et al.* 2014) (Fig. 2a) and evaluated the CPP expression.

Results (Fig. 2c) showed that microinjection of TAT-S187 peptide [two-way repeated measures (RM) ANOVA: Drug: $F_{1,\ 14} = 4.856$, $P = 0.0448$; Treatment: $F_{1,\ 14} = 11.21$, $P = 0.0048$; Drug × Treatment: $F_{1,\ 14} = 8.269$, $P = 0.0122$] significantly inhibited the morphine CPP expression compared with the TAT-S187A group, whereas vehicle (two-way RM ANOVA: Drug: $F_{1,\ 14} = 4.619$, $P = 0.0496$; Treatment: $F_{1,\ 14} = 6.607$, $P = 0.0222$; Drug × Treatment: $F_{1,\ 14} = 7.440$, $P = 0.0163$) and TAT-S187A peptide (two-way RM ANOVA: Drug: $F_{1,\ 14} = 12.37$, $P = 0.0034$; Treatment: $F_{1,\ 14} = 18.57$, $P = 0.0007$; Drug × Treatment: $F_{1,\ 14} = 17.06$, $P = 0.0010$) did not affect the morphine CPP expression. At the same time, the TAT-S187 peptide did not alter the general locomotor activity in rats (Fig. S2). The expression of FITC-TAT-S187 and the injection sites in the CA1 were verified in the Fig. 2e & f. In this experiment, 55 rats were used. One rat died during the cannula implantation. Three rats

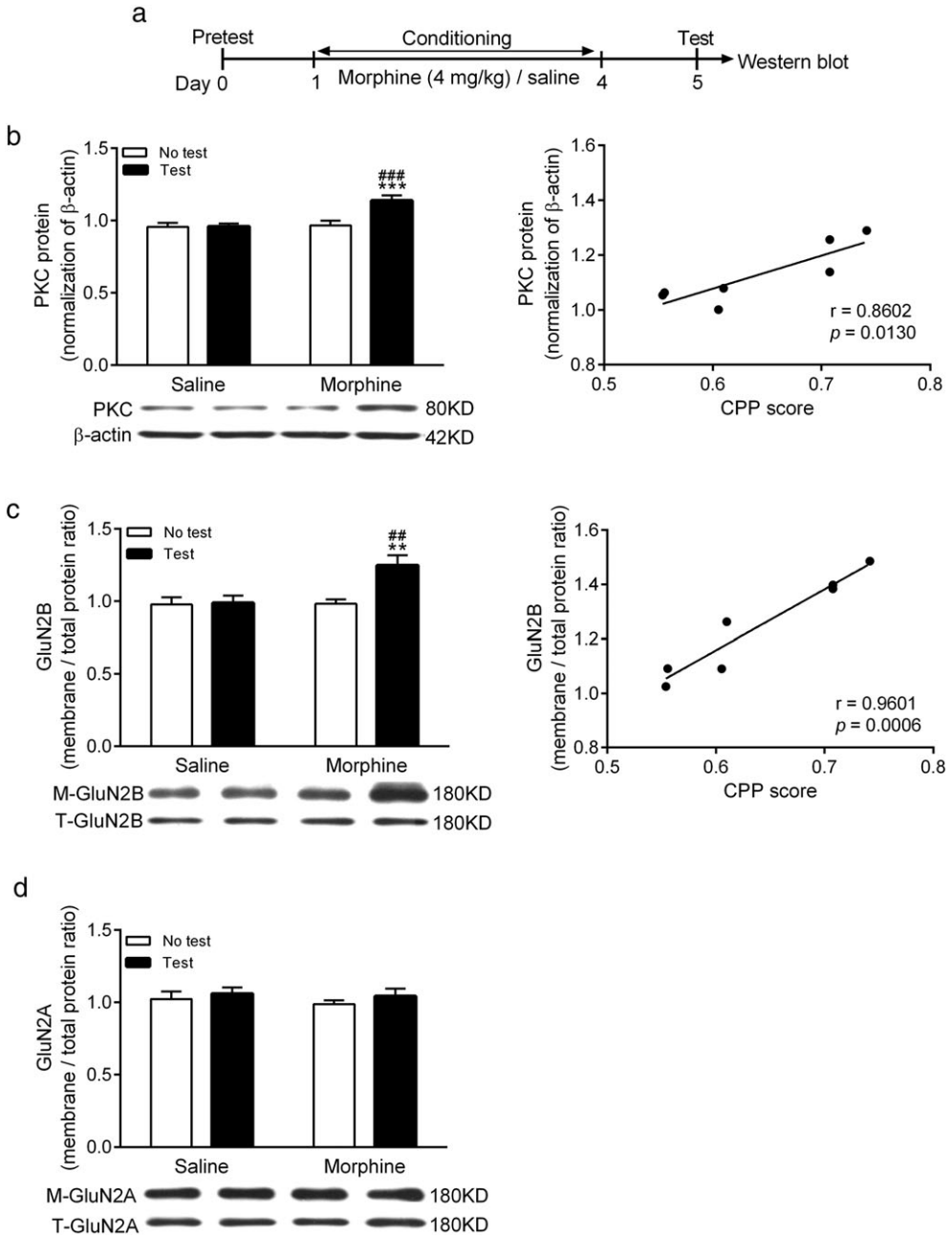

Figure 1 Morphine conditioned place preference (CPP) test enhanced the total protein kinase C (PKC) and the membrane GluN2B, not GluN2A protein expression in the CA1. (a) The timeline illustrated the experimental procedures. (b) Left, western blot analysis of PKC protein expression was elevated obviously in the CA1 after morphine CPP test. Right, correlation of morphine CPP score with PKC protein expression in the morphine CPP test group. (c) Left, western blot analysis of the membrane/total GluN2B was significantly increased in the CA1 after morphine CPP test. Right, correlation of morphine CPP score with the membrane/total GluN2B in the morphine CPP test group. (d) Western blot analysis of GluN2A membrane/total protein expression after morphine CPP test was the same among groups. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the morphine no test group, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the saline test group by two-way ANOVA and the Bonferroni *post hoc* test. $n = 7$

were excluded because of a side preference being present. Three rats were excluded from the data analysis because of incorrect cannula.

To further confirm the roles of SNAP25 Ser28 and Thr29 sites in morphine CPP test, a total of 80 rats were divided into eight groups. Among them, five rats with a side preference and three rats with missed cannula were excluded. In contrast, the microinjection of TAT-S28, TAT-S28A, TAT-T29 or TAT-T29A peptide had no effect on morphine CPP expression (Fig. 2d) and saline CPP expression and also did not alter the general locomotor activity in rats (Fig. S3).

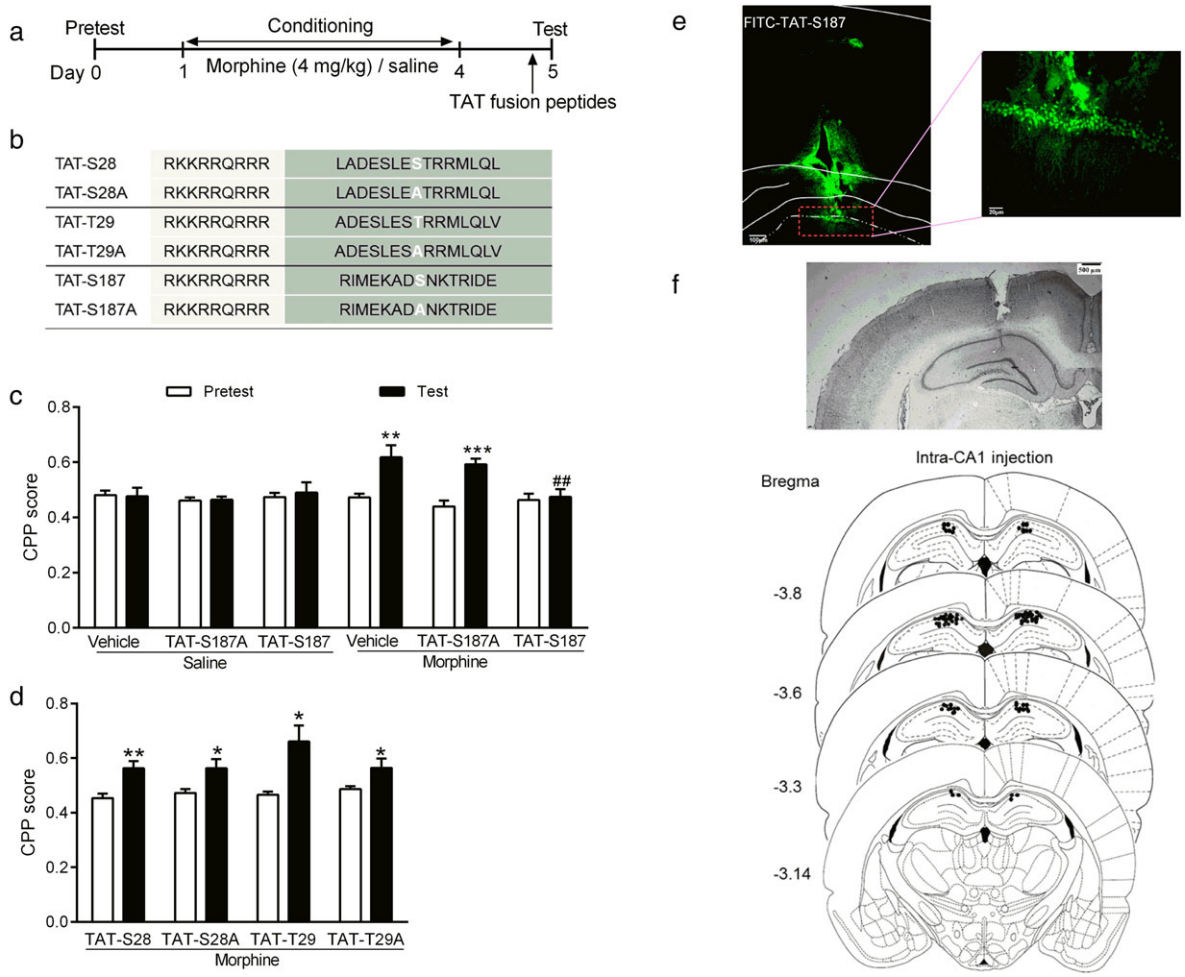

Figure 2 Intra-CA1 injection of TAT-S187 before test blocked morphine conditioned place preference (CPP) expression specifically. (a) The timeline illustrated the experimental procedures. (b) Schematic diagram of TAT-S28/TAT-T29/TAT-S187 peptides and TAT-S28A/TAT-T29A/TAT-S187A control peptides. (c) Inhibition of morphine CPP expression by intra-CA1 injection of TAT-S187 peptide. $^{**}P < 0.01$ compared with morphine pretest + vehicle group, $^{***}P < 0.001$ compared with morphine pretest + TAT-S187A group, $^{\#\#}P < 0.01$ compared with the morphine test + TAT-S187A group by two-way RM ANOVA and Bonferroni *post hoc* test. $n = 8$. (d) The expression of morphine CPP was not altered by intra-CA1 injection of TAT-S28, TAT-S28A, TAT-T29 or TAT-T29A peptide. $^{**}P < 0.01$, $^{*}P < 0.05$ versus the morphine CPP pretest by paired *t*-test. $n = 9$. (e) FITC-TAT-S187 expressed around the injection site. Left (10×), scale bar, 100 μm; right (40×), scale bar, 20 μm. The images were collected with an Olympus FV 1000 confocal microscope (Olympus Corporation, Tokyo, Japan) under the excitation FITC (488 nm). (f) Representative cannulae placement (1.25×, scale bar, 500 μm) and locations of intra-CA1 injection sites from the samples (graph C) summarized

These results indicated Ser187, but not Ser28 or Thr29, is the phosphorylation site by PKC involving in morphine CPP expression.

Phosphorylated S187-SNAP25 was involved in morphine conditioned place preference expression via increasing GluN2B-NMDAR membrane localization in the CA1

To further determine the effect of pS187-SNAP25 on the morphine CPP expression, we investigated the pS187-SNAP25 level in the CA1 after morphine CPP test using western blot. Twenty-four rats were used in this experiment. Results showed an upregulation of the ratio of pS187-SNAP25/SNAP25 in the morphine CPP test group (Fig. 3a; two-way ANOVA: Drug: $F_{1,\ 20} = 18.33$, $P = 0.0004$; Treatment: $F_{1,\ 20} = 12.31$, $P = 0.0022$; Drug × Treatment: $F_{1,\ 20} = 6.473$, $P = 0.0193$), which correlated positively with morphine CPP score (Fig. 3b; $r = 0.8759$, $P = 0.0222$).

Next, we microinjected the TAT-S187 or TAT-187A into the bilateral CA1 4 hours before CPP test to detect the protein level of membrane GluN2B, GluN2A and total pS187-SNAP25 (Fig. 3c). Brains were dissected from 36 rats obtained in the Fig. 2c experiment. The TAT-S187 peptide successfully disrupted pS187-SNAP25 expression. The ratio of pS187-SNAP25/SNAP25 decreased obviously (Fig. 3d; one-way ANOVA: $F_{2,\ 15} = 6.610$, $P = 0.0087$) compared with vehicle and TAT-S187A

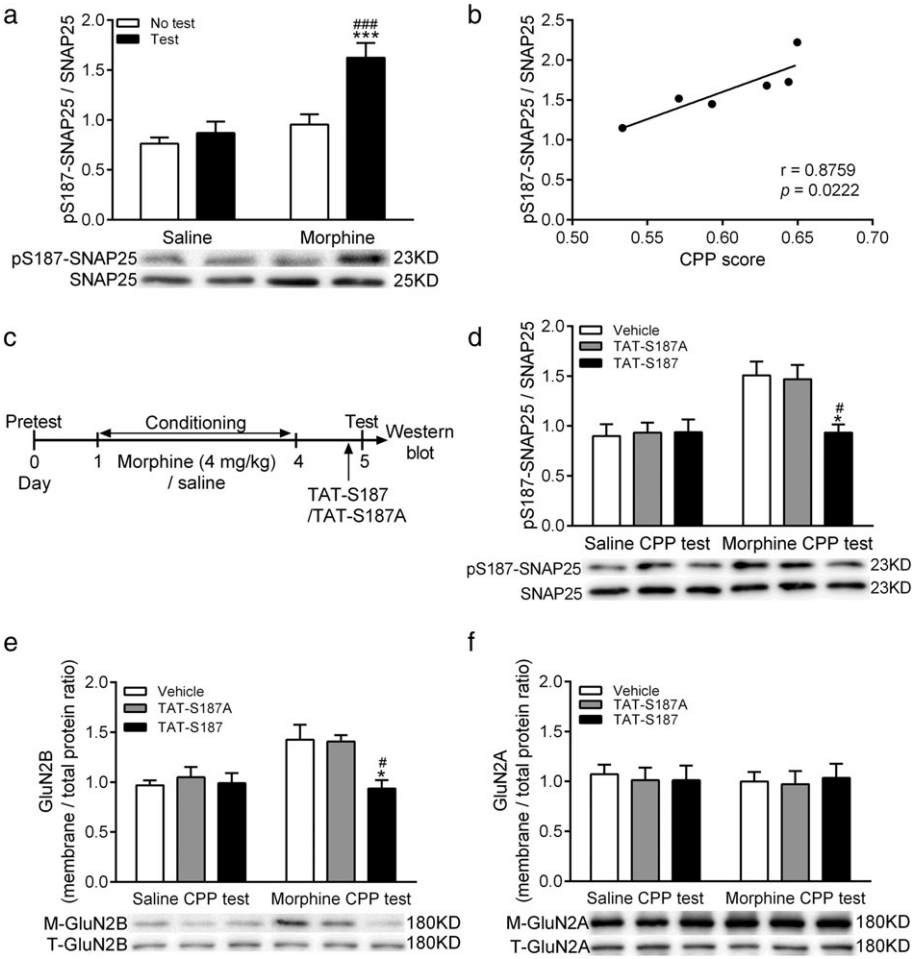

Figure 3 pS187-SNAP25 was involved in morphine conditioned place preference (CPP) expression via increasing GluN2B-NMDAR membrane localization in the CA1. (a) Re-exposure to the morphine-associated context increased the pS187-SNAP25/SNAP25 protein level in the CA1. $^{***}P < 0.001$ versus the morphine CPP no test group, $^{\#\#\#}P < 0.001$ versus the saline CPP test group by two-way ANOVA and the Bonferroni *post hoc* test. (b) Correlation of the morphine CPP score with pS187-SNAP25/SNAP25 level in the morphine test group. (c) The timeline illustrated the experimental procedures for (d), (e) and (f). Intra-CA1 microinjection of TAT-S187 abolished the increased (d) total protein pS187-SNAP25/SNAP25 and (e) membrane/total GluNR2B expression but did not change the (f) GluN2A membrane/total protein level after morphine CPP test. $^{*}P < 0.05$ versus the morphine CPP test + TAT-S187A group, $^{\#}P < 0.05$ versus the morphine CPP test + vehicle by the one-way ANOVA and the Tukey's *post hoc* test. $n = 6$ per group

group in morphine CPP test. Of note, blockade of the pS187-SNAP25 impaired membrane protein level of GluN2B. The ratio of GluN2B membrane/total reduced under the intervention with TAT-S187 (Fig. 3e; one-way ANOVA: $F_{2,\ 15} = 6.769$, $P = 0.0080$) but not its control peptide TAT-S187A in morphine CPP test. The membrane/total ratio of GluN2A was unaffected by either TAT-S187 or TAT-187A (Fig. 3f; one-way ANOVA: $F_{2,\ 15} = 0.06416$, $P = 0.9381$).

Phosphorylated S187-SNAP25 was not necessary to sucrose conditioned place preference expression in the CA1

Next, we took sucrose-induced CPP model as an example to investigate the effect of TAT-S187 peptide on the retrieval of natural rewarding memory, in comparison with morphine-associated rewarding memory. Fifty-two rats were used to train the CPP model. Among them, three rats with a side preference and one rat with missed cannula were excluded. All rats displayed obvious preference for sucrose-paired side, compared with pure water-paired side (Fig. 4b; two-way RM ANOVA, TAT-187A: Drug: $F_{1,\ 16} = 5.005$, $P = 0.0399$; Treatment: $F_{1,\ 16} = 5.529$, $P = 0.0319$; Drug × Treatment: $F_{1,\ 16} = 5.145$, $P = 0.0375$; TAT-187, Drug: $F_{1,\ 18} = 6.939$, $P = 0.0168$; Treatment: $F_{1,\ 18} = 4.923$, $P = 0.0396$; Drug × Treatment: $F_{1,\ 18} = 13.62$, $P = 0.0017$). Compared with pure water, all the rats in the sucrose CPP preferred to sucrose solution (Fig. 4c; two-way RM ANOVA, sucrose versus water: $F_{1,\ 72} = 139.3$, $P < 0.0001$) indicated that their

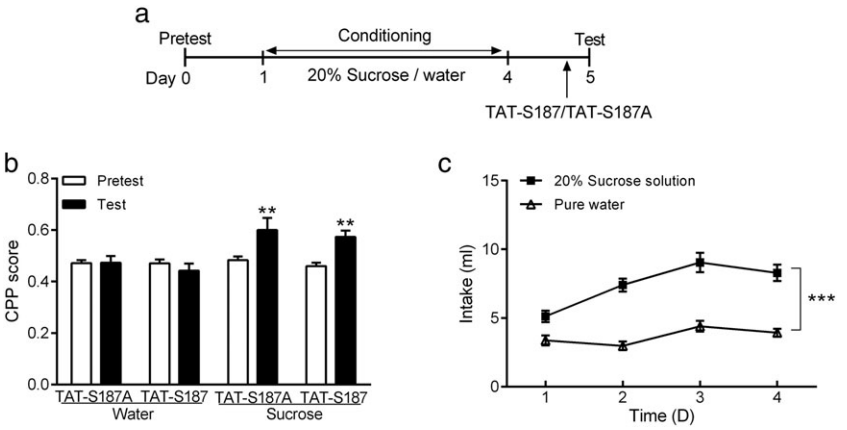

Figure 4 Phosphorylation of SNAP25 at Ser187 was not necessary to sucrose CPP expression. (a) The timeline illustrated the sucrose conditioned place preference (CPP) experimental procedures. (b) Intra-CA1 microinjection of the TAT-S187 peptide did not affect sucrose CPP expression. $^{**}P < 0.01$ versus the sucrose pretest group using by two-way repeated measures ANOVA and Bonferroni *post hoc* test. $n = 9$–10. (c) In the conditioning days, the rats preferred to 20 percent sucrose solution not pure water obviously. $^{***}P < 0.001$ versus the pure water group using by two-way repeated measures ANOVA and Bonferroni *post hoc* test. $n = 19$

natural reward system is normal. The results showed that neither intra-CA1 injection of TAT-187 nor its control peptide TAT-187A affected sucrose CPP expression, which is necessary for living.

PKC interacted with phosphorylated S187-SNAP25 accompanied by increasing GluN2B membrane localization in the CA1 after conditioned place preference expression

In order to explore the interactions between PKC, pS187-SNAP25 and GluN2B, we performed co-immunoprecipitation (Co-IP) assays with rat hippocampal CA1 extracts after CPP test. Hippocampal CA1 tissue in each experiment of Fig. 5 was respectively dissected from 12 rats.

Endogenous pS187-SNAP25 protein was immunoprecipitated by PKC antibody, but not normal mouse IgG, in rat hippocampal CA1 tissue (Fig. 5a & c). However, no precipitation of pS187-SNAP25 by GluN2B antibody was detected in hippocampal CA1 preparations in all groups (Fig. 5b & d). Of note, the intensity of pS187-SNAP25 immunoprecipitated by the same amount of PKC antibody was significantly higher in morphine CPP test compared with saline CPP test group (Fig. 5a), consistent with the enhanced expression of PKC and pS187-SNAP25 in morphine CPP test (Figs 1b & 3a). In addition, results in Fig. 5(c) showed that morphine CPP

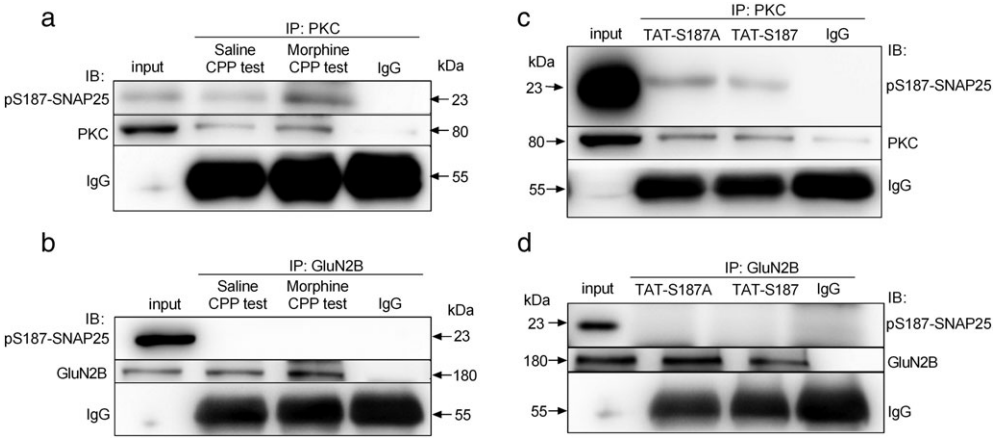

Figure 5 Protein kinase C (PKC) interacted with pS187-SNAP25 accompanied by increasing GluN2B membrane localization in the CA1 after conditioned place preference (CPP) expression. (a) pS187-SNAP25-specific antibody recognized the complex immunoprecipitated by PKC-specific antibody, but normal IgG did not, in the saline CPP test group and morphine CPP test group. Input represented the direct immunoblotting of the hippocampus CA1 extracts. (b) pS187-SNAP25-specific antibody did not recognize the complex immunoprecipitated by GluN2B-specific antibody. Similarly, (c) PKC interacted with pS187-SNAP25 and (d) GluN2B did not interact with pS187-SNAP25 in the morphine CPP test with TAT-S187A or TAT-S187 peptide. Results are representative of three independent experiments

test with TAT-S187 peptide remarkably reduced the interaction of PKC with pS187-SNAP25, compared with the TAT-S187A peptide group, confirming the intervention effect of TAT-S187 peptide. So far, our results showed that PKC interacted with pS187-SNAP25, though the interaction between pS187-SNAP25 and GluN2B was not visible in Co-IP assays with rat hippocampal CA1 tissue.

DISCUSSION

Our present study (Fig. 6) showed that PKC selectively increased the pS187-SNAP25, promoted the membrane localization of GluN2B-NMDAR in the hippocampal CA1 and mediated the retrieval of contextual-induced addiction memory but not natural reward memory. It is for the first time that we reported the cell-permeable TAT-S187 targeting pS187-SNAP25 in the CA1, blocked membrane localization of GluN2B and impeded the morphine-associated contextual memory retrieval. Unexpectedly, the interactive evidence between pS187-SNAP25 and GluN2B was not obtained by Co-IP assays, although that of PKC and pS187-SNAP25 was verified.

We observed differential changes in NMDAR subunit composition: GluN2A and GluN2B upon addiction memory retrieval in our adult rats. Previous studies also reported differential expression levels of NMDAR subunits: GluN2B subunit is highly expressed at the early stage of development, and the expression of GluN2A subunit begins only after birth and exceeds GluN2B level after adulthood (Sheng *et al.* 1994). The switch from predominance of GluN2B-containing to GluN2A-containing receptors is thought to be regulated by sensory experience and activity (Quinlan *et al.* 1999; Bellone & Nicoll 2007). Recently, Dong & Nestler (2014) put forward a neural rejuvenation hypothesis in cocaine addiction that repeated exposure to cocaine restores and awakens certain dormant developmental plasticity mechanisms within the brain's reward circuitry to form unusually strong and persistent changes in drug-related memories. The re-enrichment of GluN2B subunit in the present study was activity-dependent and also consistent with this hypothesis. It has been demonstrated that GluN2B-NMDARs preferentially traffic through recycling endosomes than GluN2A-NMDARs (Lavezzari *et al.* 2004). In addition, we have found that the GluN2B-NMDAR antagonist ifenprodil blocks morphine CPP expression without affecting the food-induced CPP expression (Ma *et al.* 2006), indicating that targeted intervention of GluN2B-NMDAR provides the possibility of treating drug addiction without affecting the natural reward memory. In summary, the high level of GluN2B, but not the GluN2A, plays an important role in morphine-associated contextual memory retrieval (Fig. 1c & d).

Protein kinase C is critical for morphine-associated contextual memory retrieval, as intracerebroventricular injection of PKC inhibitor calphostin C inhibits morphine-induced CPP in a dose-dependent manner (Narita *et al.* 2001). In our work, PKC protein was also

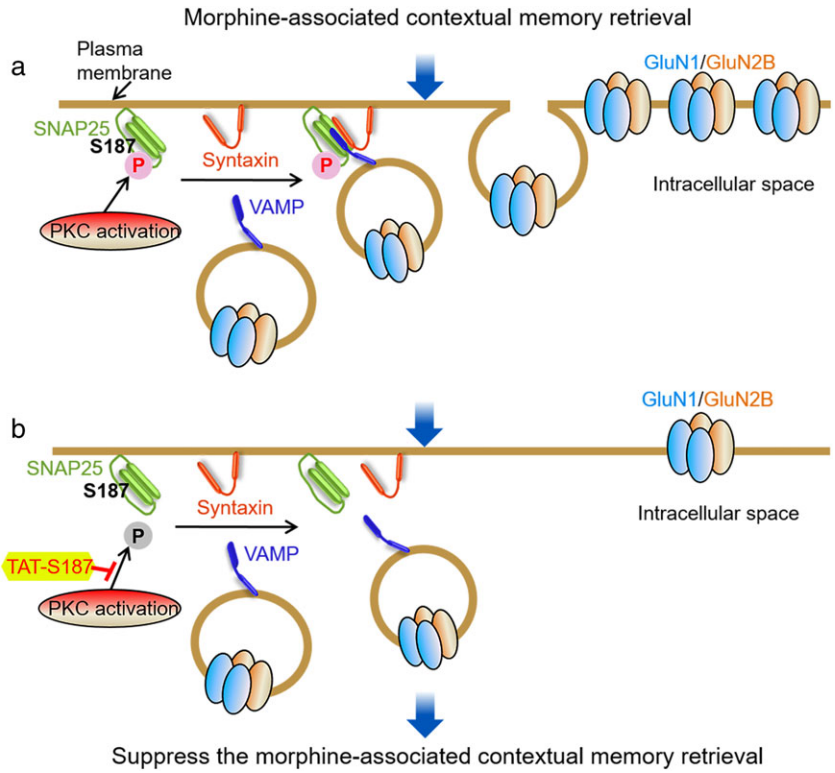

Figure 6 A schematic illustration of the research model. (a) Morphine-associated contextual memory retrieval induced protein kinase C (PKC) activation in the CA1. Next, PKC phosphorylated serine 187 in SNAP25, leading to increase the formation of SNARE, enhanced the GluN2B-NMDAR membrane localization. (b) Disrupting pS187-SNAP25 with the TAT-S187 peptide reduced surface GluN2B-NMDAR levels and suppressed the morphine-associated contextual memory retrieval

upregulated in the CA1 after morphine CPP expression (Fig. 1b). However, the specific subtypes of PKC were not examined thoroughly. So far, at least 12 kinds of PKC subtypes have been isolated and purified from different tissues and organs (Mellor & Parker 1998). Two previous studies indicate the PKC γ is involved in morphine CPP expression (Narita *et al.* 2001; Ping *et al.* 2012), but other subtypes of PKC remained largely unexplored under contextual addiction memory retrieval. Thus, it would be interesting to look into differential changes of PKC subtypes and verify which subtype predominate the p187-SNAP25 upon morphine CPP expression.

Previous *in vitro* study stated that PKC-mediated insertion of NMDARs requires pS187-SNAP25 in cultured neurons (Lau *et al.* 2010). Our study not only confirmed that pS187-SNAP25 was the key component linking PKC and GluN2B membrane location but also provided *in vivo* evidence for their interactions in the hippocampus CA1. We discovered that pSer187-SNAP25 was critical to morphine CPP expression, and re-exposure to morphine-associated context increased the protein level of pS187-SNAP25 in the CA1 (Figs 2 & 3a). In addition, cold-water restraint stress induces phosphorylation of SNAP25 in the hippocampus, and the level of phosphorylation increases with increasing amounts of stress (Yamamori *et al.* 2014). These studies indicate that whether in morphine-associated contextual memory or negative emotion, phosphorylated SNAP25 has an important significance. Furthermore, intra-CA1 injection of TAT-S187 peptide blocked morphine CPP expression (Fig. 2c) and reduced the GluN2B subunit membrane localization (Fig. 3e). Researchers may argue that chronic morphine treatment is shown to decrease pS187-SNAP25 and SNARE complex formation in the hippocampus (Xu *et al.* 2004), while our results showed that pS187-SNAP25 was upregulated upon morphine CPP expression. We believe the discrepancy lies in that chronic morphine-dependent model emphasizes on drug properties to induce opiate dependence and tolerance, while morphine CPP model focuses on the association between the drug reward properties and drug-paired context. Therefore, even though chronic morphine use may decrease the level of pSNAP25, the PKC-dependent pS187-SNAP25 still increases significantly upon morphine context-induced memory retrieval.

The roles of SNAP25 played in neurotransmitter release through exocytosis (Sudhof 1995) and in postsynaptic receptors trafficking (Lan *et al.* 2001) are well studied. However, the role of pS187-SNAP25 on neurotransmitter is still controversial. Researchers report different outcomes as different cell types or different stimulus conditions were employed (Gonelle-Gispert *et al.* 2002; Hepp, Cabaniols, & Roche 2002; Finley, Scheller, & Madison 2003; Shu *et al.* 2008). Importantly, we confirmed that intra-CA1 injection of TAT-S187 had no effect on the general locomotor (Fig. S2) or sucrose-induced CPP expression in rats (Fig. 4), further supporting that specific role of pS187-SNAP25 in intervening drug-associated but not natural-associated contextual memory retrieval.

Given the important role of pS187-SNAP25 by PKC in the morphine CPP expression and its effect on GluN2B membrane localization, we further studied the interaction between PKC and pS187-SNAP25, pS187-SNAP25 and GluN2B subunit. Unexpectedly, our results showed that pS187-SNAP25 had no interaction with GluN2B by Co-IP in the CA1 after CPP test (Fig. 5b & d). The main reason that the interaction between them was not detected may be due to technical limitations in the Co-IP assay we used. First, Co-IP is based on the close integration of protein complexes with each other, meaning that the loose binding of the protein component is likely to be undetectable. We speculate that pS187-SNAP25 and GluN2B are not closely combined. Second, it is necessary to use the different members of the complex to detect independently, and the results should be able to verify each other in order to ensure the reliability and rigor of Co-IP experiment. However, because pS187-SNAP25-specific antibody for Co-IP is not commercially available, we could not use pS187-SNAP25 antibody to pull down PKC or GluN2B. Nonetheless, TAT-S187 peptide could inhibit morphine CPP expression and reduce GluN2B membrane localization synchronously, indicating that there must be some connection between them. Moreover, the functional interactions between SNAP25 and NMDARs have been confirmed using electrophysiological techniques (Lan *et al.* 2001; Lau *et al.* 2010). Thus, the functional regulation between pS187-SNAP25 and GluN2B in this study remains to be studied in the future.

In conclusion, the results in the present study revealed that activated PKC in the CA1 could selectively increase pS187-SNAP25 to promote GluN2B-NMDAR membrane localization, which was necessary to morphine-associated contextual memory retrieval. The findings provide new insights into the molecular mechanisms underlying drug addiction memory retrieval and relapse after withdrawal.

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Conflict of Interest

The authors declare no conflict of interest.

Authors Contribution

CC and XW conceived and designed the research; XW performed the experiments; YL, MJ and XS assisted with experiments and data analysis; YL provided technical support; XW drafted the manuscript; CC edited and revised the manuscript. NW provided language help.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1. Representative fluorescence images showed that 5 μM FITC-TAT-S187 expressed in the N2a cell for 2 h 40X (left), 4 h 40X (middle) and 4 h 60X (right). The 5 μM FITC-TAT-S187 could express effectively in the N2a for 4 h. Scale bar, 20 μm.

Figure S2. Intra-CA1 injection of TAT-S187 or TAT-S187A peptide before CPP test had no effect on locomotor activity in rats. $n = 8$. The data were analyzed by two-way ANOVA and Bonferroni *post-hoc* test.

Figure S3. Intra-CA1 injection of TAT-S28, TAT-S28A, TAT-T29 or TAT-T29A peptide before CPP test had no effect on saline CPP expression and locomotor activity in rats. (a) Microinjection of TAT-S28, TAT-T29 or control peptide into the CA1 had no effect on saline CPP test. $n = 8–9$. (b), (c) No significant difference of total locomotor activity between the pretest and test each group was detected after CPP test. $n = 8–9$.The data were analyzed by paired *t* test.