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Essential role of the NO signaling pathway in the hippocampal CA1 in morphine-associated memory depends on glutaminergic receptors

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ABSTRACT

The nitric oxide (NO)/soluble guanylyl cyclase (sGC)/cGMP-dependent protein kinase (PKG) signaling pathway has been reported to play a key role in memory processing. However, little is known about its role in drug-associated reward memory. Here, we report the following. 1) The NO pathway in the CA1 is critical for the retrieval of morphine-associated reward memory. Specifically, the nNOS, sGC and PKG protein levels in the CA1 were increased after the expression of morphine conditioned place preference (CPP). Intra-CA1 injection of an NOS, sGC or PKG inhibitor prevented morphine CPP expression. 2) The involvement of the NO pathway in morphine CPP requires NR2B-containing NMDA receptors (NR2B-NMDARs). NR2B-NMDAR expression was elevated in the CA1 following morphine CPP expression, and intra-CA1 injection of the NR2B-NMDAR antagonist Ro25-6981 not only blocked morphine CPP expression but also inhibited the up-regulation of nNOS, sGC and PKG. Moreover, the Ro25-6981-induced blockade of morphine CPP was abolished by intra-CA1 injection of a NOS substrate or an sGC activator. 3) The NR2B-NMDAR stimulated the NO pathway by up-regulating the phosphorylation of Akt^{Ser473}. Morphine CPP expression enhanced the $pAkt^{Ser473}$ level, which has been corroborated to regulate nNOS activity, and this effect was reversed by intra-CA1 injection of Ro25-6981. 4) GluR1 acted downstream of the NO pathway. The membrane level of GluR1 in the CA1 was increased after morphine CPP expression, and this effect was prevented by pre-injection of a PKG inhibitor into the CA1. Additionally, co-immunoprecipitation revealed an interaction between PKG and GluR1; this result further indicated a role of PKG in regulating GluR1 trafficking. Collectively, the results of our study demonstrated that the activation of the NR2B-NMDAR/NO/sGC/PKG signaling pathway is necessary for the retrieval of morphine-associated reward memory.

1. Introduction

Over the years, one of the most significant conceptual advances has been the understanding that drug addiction can be deemed as a pathological usurpation of normal learning and memory function of the brain, in that it shares numerous similarities with physiological learning and memory processes (Kelley, 2004). Reward-associated learning and memory have been demonstrated to play an essential role in the development and persistence of drug addiction (Hyman et al., 2006). The conditioned association between the contexts paired with drug taking and the effects of an addictive drug is of great importance to the maintenance of drug-induced reward memory and the high risk of relapse, which is usually triggered by drug-associated cues even after a long period of abstinence (De Vries and Shippenberg, 2002; Robbins and Everitt, 1999; See et al., 2003). Disrupting the formation of drug-related learning and memory may help prevent drug addiction and relapse.

The hippocampus is a renowned brain structure that plays pivotal roles in the formation of several types of long-term memory, including reward-related memory (Ito et al., 2008). For example, blockade of dopamine receptors in the dorsal hippocampus impaired the acquisition, expression and the late consolidation of cocaine-induced CPP (Kramar et al., 2014a, 2014b). Similarly, blocking hippocampal glucocorticoid receptors

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prevented morphine-induced CPP-related behavior (Dong et al., 2006). An imaging study also showed that opioid exposure increased the activation of the hippocampus in opioid-naïve healthy volunteers (Becerra et al., 2006). Presumably, addictive drugs may employ learning and memory mechanisms in the hippocampus to form addiction memory (Davis and Gould, 2008).

NO is a highly diffusible gas molecule synthesized by a family of enzymes termed NO synthase (NOS), which use L-arginine (L-arg) as substrate. NO can serve as a type of neuromodulator in the brain in response to neuronal activation (Cai et al., 1999). Neuronal NOS (nNOS) is ubiquitously and abundantly expressed throughout the CNS, especially in the cerebellum and the hippocampus. It has been reported that NO exerts various modulatory effects on several types of learning and memory processes (Choopani et al., 2008; Harooni et al., 2009; Majlessi et al., 2008; Ota et al., 2010b). Emerging evidence has also demonstrated that NO is involved in drug-induced reward memory. For example, administration of the nNOS inhibitor 7-NI blocked the acquisition of CPP induced by nicotine (Martin and Itzhak, 2000), alcohol (Itzhak and Martin, 2000), or cocaine (De Vries and Shippenberg, 2002) in mice. Similarly, 7-NI suppressed the acquisition, expression (Li et al., 2002b), and reinstatement (Li et al., 2002a) of D-methamphetamine-induced CPP in rats. Additionally, mice lacking the nNOS gene were found to be resistant to cocaine-induced CPP (Itzhak et al., 1998). In terms of the role of NO in opioid-induced CPP, systemic use of 7-NI or the nonselective NOS inhibitor L-N^G-nitroarginine blocked the acquisition of morphine CPP in rats (Kivastik et al., 1996) and male mice (Manzanedo et al., 2004).

The majority of NO-mediated physiological processes are thought to result from its activation of guanylate cyclase (GC) and, in turn, the activation of protein kinase G (PKG) by cGMP (Boulton et al., 1995; East and Garthwaite, 1991; Edwards et al., 2002). This pathway has also been assumed to be a prominent mechanism by which NO functions in memory processes (Bernabeu et al., 1996, 1997; Izquierdo et al., 2000). To the best of our knowledge, only two reports, which were published by our laboratory, studied the role of the NO/sGC/PKG signaling pathway in morphine-associated reward memory. Our previous experiments showed that the inhibition of the NO/sGC/PKG signaling pathway in the nucleus accumbens blocked the acquisition of morphine CPP (Shen et al., 2014) but that the suppression of this pathway in the CA1 region of the hippocampus disturbed the consolidation of morphine CPP (Shen et al., 2012). Nonetheless, neither of these studies investigated whether the NO pathway in the CA1 is involved in the expression of morphine CPP, which represents the retrieval of morphine-associated reward memory. More importantly, the underlying mechanism(s) through which the NO pathway affects morphine-associated reward memory remains undetermined.

Signal transduction initiated by ionotropic glutamate receptors, such as the NMDA and AMPA receptors, plays a major role in the mechanisms underlying synaptic plasticity and drug addiction (Vanhoutte and Bading, 2003). NMDARs regulate the level of NO by tethering to nNOS via the scaffolding protein postsynaptic density-95 (PSD-95). In this complex, NMDAR-mediated Ca^{2+} influxes regulate nNOS activity and NO production (Bredt and Snyder, 1989; Christopherson et al., 1999; Kornau et al., 1997; Rameau et al., 2004). In addition, the control of NO production by the NMDAR involves the regulatory phosphorylation of nNOS (Rameau et al., 2003, 2004). The NMDAR boosts the phosphorylation level of Ser1412 at the C-terminal of nNOS by recruiting and activating Akt on the plasma membrane, which then phosphorylates nNOS (Hisatsune et al., 1999; Waxman and Lynch, 2005). Furthermore, changes in synaptic strength underlying the cellular mechanism of learning and memory, such as LTP and LTD, are thought to involve the rapid movement of AMPA receptors (AMPARs) (Malinow and Malenka, 2002). NO exerts its effects by activating PKG or S-nitrosylating proteins (Bredt, 2003). S-nitrosylation of N-ethylmaleimide-sensitive factor (NSF) increases the membrane expression of glutamate receptor 2 (GluR2), a subunit of the AMPAR, whereas the activation of the NO/PKG signaling pathway increases GluR1 expression in synaptic puncta (Huang et al., 2005). However, how these molecules are involved in morphine-associated memory and the mechanism by which they regulate the NO/PKG signaling pathway are largely unknown.

Here, we studied the role of the NO/sGC/PKG signaling pathway in the expression of morphine CPP and the mechanisms underlying this role. Our data indicated that the retrieval of morphine-associated memory requires a series of cellular events in the CA1. Briefly, up-regulation of the NR2B-NMDAR stimulates the phosphorylation of Akt^{Ser473}, leading to the activation of NO/sGC/PKG signaling, which then enhances the trafficking of GluR1-AMPAR in the CA1.

2. Materials and methods

2.1. Subjects

Male Sprague Dawley rats of 220–250 g at the time of surgery were used in the study. The animals were obtained from the Laboratory Animal Center of the Peking University Health Science Center and were housed 4 per cage under a 12/12 h light/dark cycle (lights on at 7 p.m.); food and water were provided *ad libitum*. The room temperature was maintained at 23 ± 2 °C, and the relative humidity was kept between 45% and 55%. Five days prior to the experiments, the rats were handled with care. The behavioral tests were conducted during the dark period. All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local committee of animal use and protection. Animal suffering and the number of animals used were minimized.

2.2. Drugs

Morphine hydrochloride was purchased from the First Pharmaceutical Factory of Qinghai, China, and was dissolved in sterile saline to its final concentrations. Guanosine 30,50-cyclic monophosphorothioate, b-phenyl-1, N2-etheno-8-bromo-Rp-isomer, sodium salt (Rp-8-Br-PET-cGMPS); 7-NI; 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ); 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1); and L-arg were obtained from Sigma–Aldrich (St. Louis, MO). Ro25-6981 was obtained from Tocris Bioscience. Rp-8-Br-PET-cGMPS was dissolved in distilled water at a stock concentration of 2 μg/μl. 7-NI and ODQ were dissolved in 100% dimethyl sulfoxide (DMSO) to stock concentrations of 4 μg/μl and 0.748 μg/μl, respectively, which were then diluted 1:1 in artificial cerebrospinal fluid (ACSF) prior to infusion. YC-1, L-arg and Ro25-6981 were dissolved in ACSF to stock concentrations of 0.304 μg/μl, 0.05 μg/μl and 2 μg/μl, respectively. The composition of ACSF was as follows: 115 mM sodium chloride, 3.3 mM potassium chloride, 1 mM magnesium sulfate, 2 mM calcium chloride, 25.5 mM sodium bicarbonate, 1.2 mM sodium dihydrogen phosphate, and 10 mM glucose (Ota et al., 2010a).

2.3. Apparatus for CPP

Identical CPP apparatuses were used in the morphine- and sucrose-induced CPP sessions. Conditioning was conducted in black rectangular PVC boxes (795 × 230 × 250 mm^3), each of which was composed of three chambers 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 a stainless steel mesh floor (225×225 mm^2) and four light-emitting diodes (LEDs), forming a square on the wall; Chamber C had a stainless steel rod floor (15-mm apart) and four LEDs, forming a triangle on the wall; and Chamber B had a flat stainless steel floor. Fourteen photo beams, spaced 47.5 mm apart, were placed across the chambers. The infrared-beam crossings were recorded by a computer to calculate the time that a given rat spent in each chamber.

2.4. Cannulation and microinjections

Rats were anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneal) and secured in a Kopf stereotaxic apparatus (Kopf Instruments, Tujunga, CA). The incisor bar was lowered 3.3 mm below horizontal zero to achieve a flat skull position. Stainless steel guide cannulas (outer diameter, 0.67 mm) were bilaterally implanted 0.5 mm above the CA1 region of the hippocampus. The CA1 region coordinates (Paxinos and Watson, 2005) were as follows: anteroposterior, −3.8 mm; mediolateral, ±2.2 mm; and dorsoventral, −2.4 mm. The cannulas were fixed to the skull with dental cement and screws. Internal cannulas were replaced with dummy cannulas, which were 0.5 mm longer than the guide cannulas, to keep the cannulas patent and to prevent infection. The rats were allowed at least 5–7 days to recover before further experiments were performed.

In studies involving intra-CA1 infusions, the dummy cannulas were removed, and infusion cannulas (outer diameter, 0.3 mm) were inserted. The cannulas were connected to 1.0-μl Hamilton syringes using PE 20 tubing. The tubing was backfilled with saline such that a small air bubble separated the saline solution from the drug solution. Drugs were administered using an infusion pump at a rate of 0.25 μl/min. After infusion, the infusion cannulas were left in place for an additional1 min to allow the drugs to diffuse thoroughly from the cannulas. The dummy cannulas were then reinserted, and the rats were returned to their original home cage. Only the data from rats that received histologically verified injections were included for further analyses (Fig. S1).

2.5. Behavioral training and tests for morphine CPP

2.5.1. Preconditioning test phase

On Day 0, the rats were allowed to explore the entire apparatus freely for 15 min. To assess the unconditioned chamber preference, the time (in seconds) a rat spent in each chamber and the number of shuttles it made was recorded. Untreated rats are not supposed to show a preference towards any chamber. Thus, rats with an apparent bias for either of the lateral chambers (greater than 5%) were excluded from the experiments. Other rats were assigned to groups according to their behavior to balance the unconditioned preference. The chambers selected for pairing with morphine were counterbalanced within each group.

2.5.2. Conditioning phase

The animals were subjected to two training sessions every day (at 8:30 a.m. and 3:30 p.m.) for 4 days (Days 1–4). The rats in the morphine-conditioning group were confined to one lateral chamber after treatment with morphine (4 mg/kg, intraperitoneal) and to the other lateral chamber after saline injection. The animals in the control group received their injections in both lateral chambers before the training sessions. To counterbalance the treatment within the morphine-conditioned groups, half of the group received morphine training in Compartment A and saline training in Compartment C, and half received training in the opposite chambers. Furthermore, half of the rats were conditioned with morphine in the morning and saline in the afternoon, and the other half received these treatments in the reverse order.

2.5.3. Expression test phase

On Day 5, each animal was placed in the center choice chamber with the guillotine door open to allow free access to the entire apparatus for 15 min, and the time it spent in each chamber was recorded. The CPP score of a given rat was defined as the time it spent in the morphine-paired chamber divided by the total time it spent in both the morphine- and saline-paired chambers during CPP testing.

To rule out the possibility that any treatment would affect morphine CPP expression in the animals by influencing locomotion, we also recorded the locomotor activity of every rat on every CPP test. The locomotor activity of a rat during the CPP test was represented by the total number of crossings it made between any two adjacent compartments.

2.6. Western blotting

For western blotting experiments, the rats were decapitated immediately after the CPP tests, and the brains were quickly removed and frozen in N-hexane (−70 °C) for approximately 40 s. Bilateral tissue punches (16 gauge) of the CA1 were obtained from 80-μm thick sections generated using a sliding freezing microtome. The extraction of the membrane fraction and the total protein were conducted using procedures described previously (Lv et al., 2011; Tyrrell et al., 2001; Voulalas et al., 2011). The concentrations of samples were determined using the bicinchoninic acid (BCA) assay (Pierce) and were analyzed directly via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred to polyvinylidene difluoride membranes. The membranes were blocked in Tris-buffered saline (TBS) (50 mM Tris–HCl, pH 7.4, 150 mM NaCl and 0.05% Tween 20) with 5% dry milk and incubated in the following primary antibodies overnight at 4 °C: anti-nNOS (1:1000, #4231, Cell Signaling), anti-sGC (1:1500, #160897, Cayman Chemical), anti-PKGII/cGKII (1:500, sc-25430, Santa Cruz Biotechnology), anti-pNOS^{Ser1412} (1:1000, ab5583, Abcam), anti-Akt (1:1000, #4691, Cell Signaling), anti-pAkt^{Ser473} (1:2000, #4060, Cell Signaling), anti-NR1 (1:2000, #05-432, Millipore), anti-NR2A (1:2000, #05-901R, Millipore), anti-NR2B (1:2000, #AB1557P, Millipore), anti-GluR1 (1:2000, #8850S, Cell Signaling) and anti-β-actin (1:2000, #A2228, Sigma). After three washes for 5 min in TBS/0.1% Tween 20, the membranes were then incubated in an anti-rabbit or anti-mouse secondary antibody conjugated to horseradish peroxidase (1:2000; Zhongshan Biotechnology) and were developed using West Dura chemiluminescent substrate (Pierce Laboratories). Densitometry was performed to calculate the band intensity. To control for inconsistencies between the loaded samples, the optical densities were normalized to β-actin protein expression. Data for the treated animals were normalized to the average values for the naive controls. Protein concentrations were determined using the bicinchoninic acid (BCA) assay (Pierce).

2.7. Co-immunoprecipitation

The extraction of protein from the CA1 region was the same as the protocol described for western blotting. The total protein was lysed in 500 μl of freshly prepared lysis buffer (pH 7.4, 25 mM Tris, 50 mM β-glycerophosphate, 1.5 mM EGTA, 0.5 mM EDTA, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 μg/ml leupeptin and pepstatin, 10 μg/ml aprotinin, 100 μg/ml PMSF, and

1% Triton X-100). Equal amounts of protein (500 μg) in a total volume of 750 μl of lysis buffer were incubated with gentle rocking overnight at 4 °C in 15 μg of rabbit polyclonal IgG or anti-PKGII/cGKII (1:50, Santa Cruz Biotechnologies). Thereafter, protein A-Sepharose beads suspended in lysis buffer (1:1 blend, 60 μl in total) were added to each sample and incubated for an additional 12 h with gentle rocking at 4 °C. The beads were then pelleted by brief centrifugation, washed three times with ice-cold lysis buffer and boiled for 5 min in 50 μl of 2× sample buffer. Beads alone incubated in IgG were used as a negative control. Then, the beads and the protein fraction of the CA1 (the positive control) were separated via SDS-PAGE and transferred to PVDF membranes. The membranes were blocked in TBS containing 5% dry milk and incubated in the anti-GluR1 (1:2000, #8850S, Cell Signaling) or anti-PKGII antibody (1:500, Santa Cruz Biotechnologies) overnight at 4 °C. The following protocols were the same as those performed for western blotting.

2.8. Statistical analyses

Data were expressed as the means ± the standard error of the mean (SEM). To assess the effects of different drugs (inhibitors, antagonists, substrates, etc.) on morphine-induced CPP (e.g. Fig. 1), data were analyzed by three-way ANOVA with treatment (two-level: morphine or saline), drug (two-level: drug or vehicle) as between-subject factors and time (two level: pretest or test) as within-subject variables. If the treatment × drug × time interaction was significant, paired *t*-test was used to examine the effect of inhibitors at different time. To assess the effects of morphine conditioning and the CPP expression test on the protein expression level (e.g. Fig. 2), data were analyzed by three-way ANOVA with treatment (two-level: morphine or saline), conditioning (two-level: conditioning or without conditioning) and test (two-level: test or without test) as between-subject factors. If the treatment × conditioning × test interaction was significant, *t*-test was used to examine the effect of morphine-induced CPP on protein expression. Three-way ANOVA were performed with IBM SPSS (version 20, IBM Corporation, Armonk, NY, USA). The results from the effects of drugs on the change of protein expression level after morphine/saline CPP tests (e.g. Fig. 3E) were analyzed via two-way ANOVA followed by the Bonferroni *post hoc* test. One-way ANOVA followed by the Newman–Keuls *post hoc* test was used to analyze the results from the Morris water maze. The *t*-test was used to analyze the results from the expression test of sucrose CPP. Except for three-way ANOVA, statistical analyses were processed using the software GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Graphs were also produced using GraphPad Prism 5.0. The accepted level of significance was $p < 0.05$.

Additional methods are described in detail in the supplementary materials.

3. Results

3.1. Intra-CA1 infusion of 7-NI, ODQ or Rp-8-Br-PET-cGMPS blocked the expression of morphine CPP

Rats were divided into morphine- and saline-treated CPP groups. After 4 days of CPP conditioning, rats received bilateral intra-CA1 infusion of 7-NI (nNOS inhibitor, 1 μg/0.5 μl/side), ODQ (sGC inhibitor, 0.187 μg/0.5 μl/side), Rp-8-Br-PET-cGMPS (PKG inhibitor, 1 μg/0.5 μl/side) or the respective vehicles 20 min before the test on day 5 (Fig. 1A).

When 7-NI or 50% DMSO in ACSF (vehicle) was micro-infused into the CA1, three-way ANOVA revealed that the effects of treatment ($F_{1, 31} = 10.677$, $p < 0.01$), inhibitor ($F_{1, 31} = 5.403$, $p < 0.05$) and time ($F_{1, 31} = 5.403$, $p < 0.01$) reached the statistical significance. Moreover, the treatment × inhibitor × time interaction was significant ($F_{1, 31} = 5.732$, $p < 0.05$). Paired *t*-test showed that there

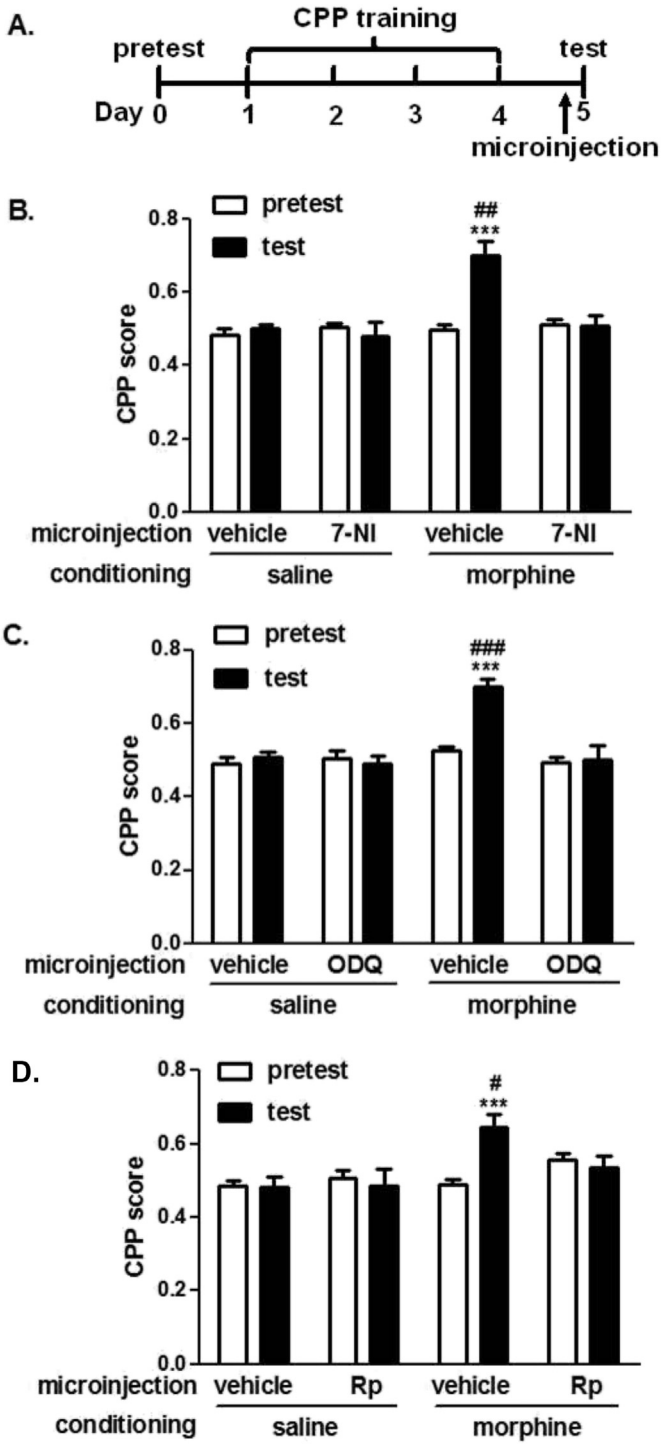

Fig. 1. Microinjecting 7-NI, ODQ or Rp-8-Br-PET-cGMPS into the CA1 before the expression test blocked morphine CPP expression. (A) Diagram outlining the behavioral procedures. (B–D) Microinjection of 7-NI (B), ODQ (C), or Rp-8-Br-PET-cGMPS (D), but not vehicle, into the CA1 blocked the expression of morphine CPP. The data are expressed as the means ± SEM, n = 7–10. The blank and solid columns represent the data from the pre- and post-conditioning tests, respectively. $^{***}p < 0.001$, pretest vs. test (paired *t*-test); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared to the morphine-conditioned rats receiving intra-CA1 microinjection of drugs (*t*-test). Rp, Rp-8-Br-PET-cGMPS.

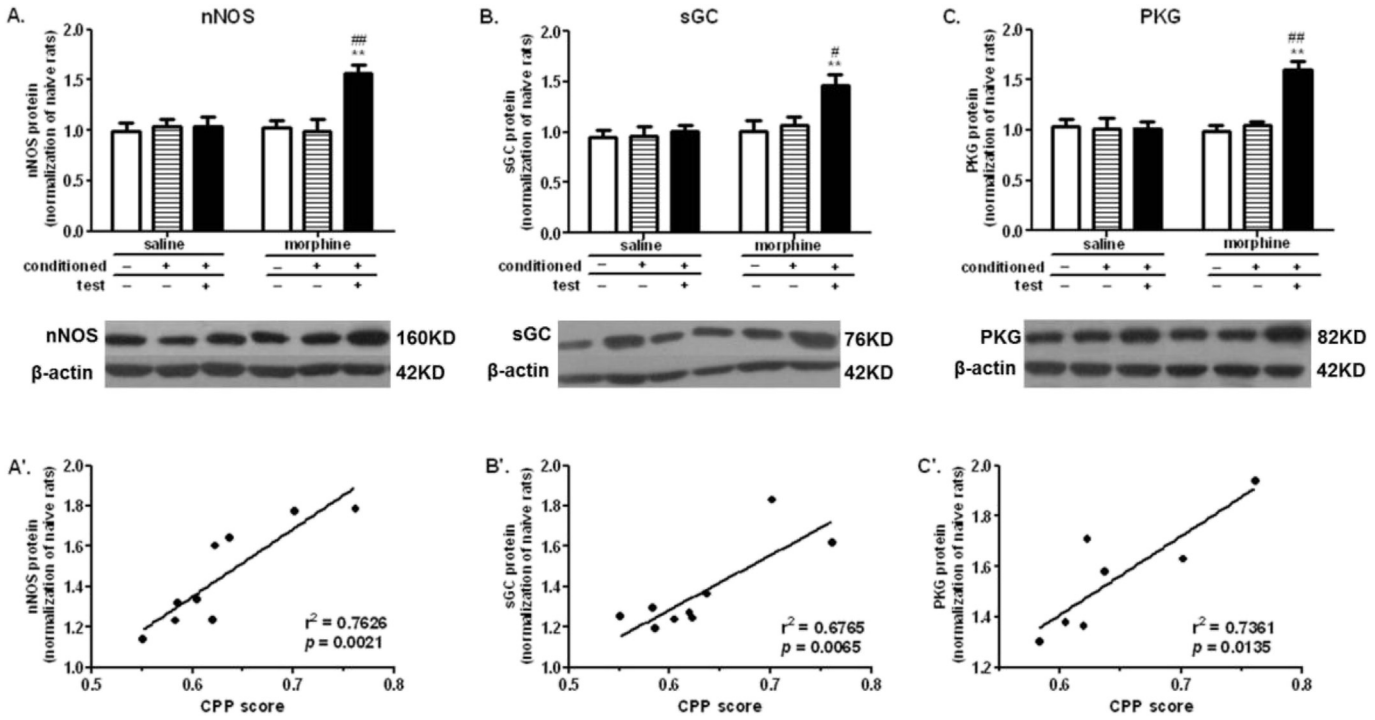

Fig. 2. Re-exposure to the morphine-conditioning context induced nNOS, sGC and PKG protein expression in the CA1. Re-exposure to the morphine-conditioning context significantly increased nNOS (A), sGC (B) and PKG (C) protein expression in the CA1 of morphine-conditioned rats. The data are expressed as the means ± SEM, n = 4–6. $^{**}p < 0.01$, compared to the saline-treated group with re-exposure; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared to the morphine-conditioned group without re-exposure (*t*-test). Representative bands from western blots are shown under each column. (A′–C′) Correlation of the nNOS (A′), sGC (B′) and PKG (C′) levels in the CA1 with the morphine CPP expression test score. The data were expressed as the means of the nNOS, sGC and PKG levels and the CPP expression test score, and the mean values were analyzed via linear regression.

was a significant increase in the CPP score on the expression test of morphine-conditioned rats infused into the CA1 with vehicle ($t = 6.084$, $p < 0.001$), compared with the score on the pretest. Further comparisons suggested that there was a significant decrease in the CPP score of the expression test of morphine-conditioned rats infused into the CA1 with 7-NI compared to vehicle ($t = 3.873$, $p < 0.01$, *t*-test). These results indicated that intra-CA1 administration of 7-NI blocked morphine-induced place preference compared to vehicle (Fig. 1B).

When ODQ or 50% DMSO in ACSF (vehicle) was micro-infused into the CA1, three-way ANOVA revealed that the effects of treatment ($F_{1, 36} = 12.680$, $p < 0.001$), inhibitor ($F_{1, 36} = 13.641$, $p < 0.001$) and time ($F_{1, 31} = 9.381$, $p < 0.01$) reached the statistical significance. Moreover, the treatment × inhibitor × time interaction was significant ($F_{1, 36} = 5.415$, $p < 0.05$). Paired *t*-test showed that there was a significant increase in the CPP score in the morphine-conditioned rats intra-CA1 infused with vehicle ($t = 5.711$, $p < 0.001$) on the test compared with the pretest. Further comparisons suggested that there was a significant decrease in the CPP score of the expression test of morphine-conditioned rats infused into the CA1 with ODQ compared to vehicle ($t = 4.542$, $p < 0.001$, *t*-test). These results indicated that intra-CA1 administration of ODQ blocked morphine-induced place preference compared to vehicle (Fig. 1C).

When Rp-8-Br-PET-cGMPS or distilled water (vehicle) was micro-infused into the CA1, three-way ANOVA revealed that the effects of treatment ($F_{1, 26} = 8.968$, $p < 0.01$), inhibitor ($F_{1, 26} = 5.441$, $p < 0.05$) and time ($F_{1, 26} = 4.349$, $p < 0.05$) reached the statistical significance. Moreover, the treatment × inhibitor × time interaction was significant ($F_{1, 26} = 7.309$, $p < 0.05$). Paired *t*-test showed that there was a significant increase in the CPP score in the morphine-conditioned rats infused into the CA1 with vehicle ($t = 3.965$, $p < 0.001$) on the test compared with the pretest. Further comparisons suggested that there was a significant decrease in the CPP score of the expression test of morphine-conditioned rats infused into the CA1 with Rp-8-Br-PET-cGMPS compared to vehicle ($t = 2.270$, $p < 0.05$, *t*-test). These results indicated that intra-CA1 administration of Rp-8-Br-PET-cGMPS blocked morphine-induced place preference compared to vehicle (Fig. 1D).

In addition, we also found that intra-CA1 administration of 7-NI, ODQ or Rp-8-Br-PET-cGMPS affected neither the general behavior (Fig. S2) nor the spatial memory (Fig. S3) of the animals. Furthermore, we also investigated the effects of 7-NI, ODQ or Rp-8-Br-PET-cGMPS on the retrieval of memory induced by non-drug/natural rewards (such as sucrose). The results showed that intra-CA1 injection with these inhibitors had no effect on sucrose-induced CPP (Fig. S4).

3.2. The nNOS, sGC and PKG protein levels in the CA1 were increased following the morphine CPP expression test

The rats were divided into six subgroups to evaluate the nNOS, sGC and PKG protein levels in the CA1 after the morphine CPP expression test.

For nNOS, three-way ANOVA showed that morphine conditioning and the CPP expression test significantly increased the nNOS protein expression in the CA1 [treatment, $F_{1, 22} = 8.319$, $p < 0.01$; test, $F_{1, 22} = 9.698$, $p < 0.01$; treatment × test interaction, $F_{1, 22} = 9.733$, $p < 0.01$], whereas morphine conditioning without the CPP expression test did not change the nNOS protein level [conditioning, $F_{1, 22} = 0.007$, ns; treatment × conditioning interaction, $F_{1, 22} = 0.227$, ns]. Moreover, *t*-test revealed that nNOS protein was increased in the rats that received both morphine conditioning and the CPP expression test compared to the saline-

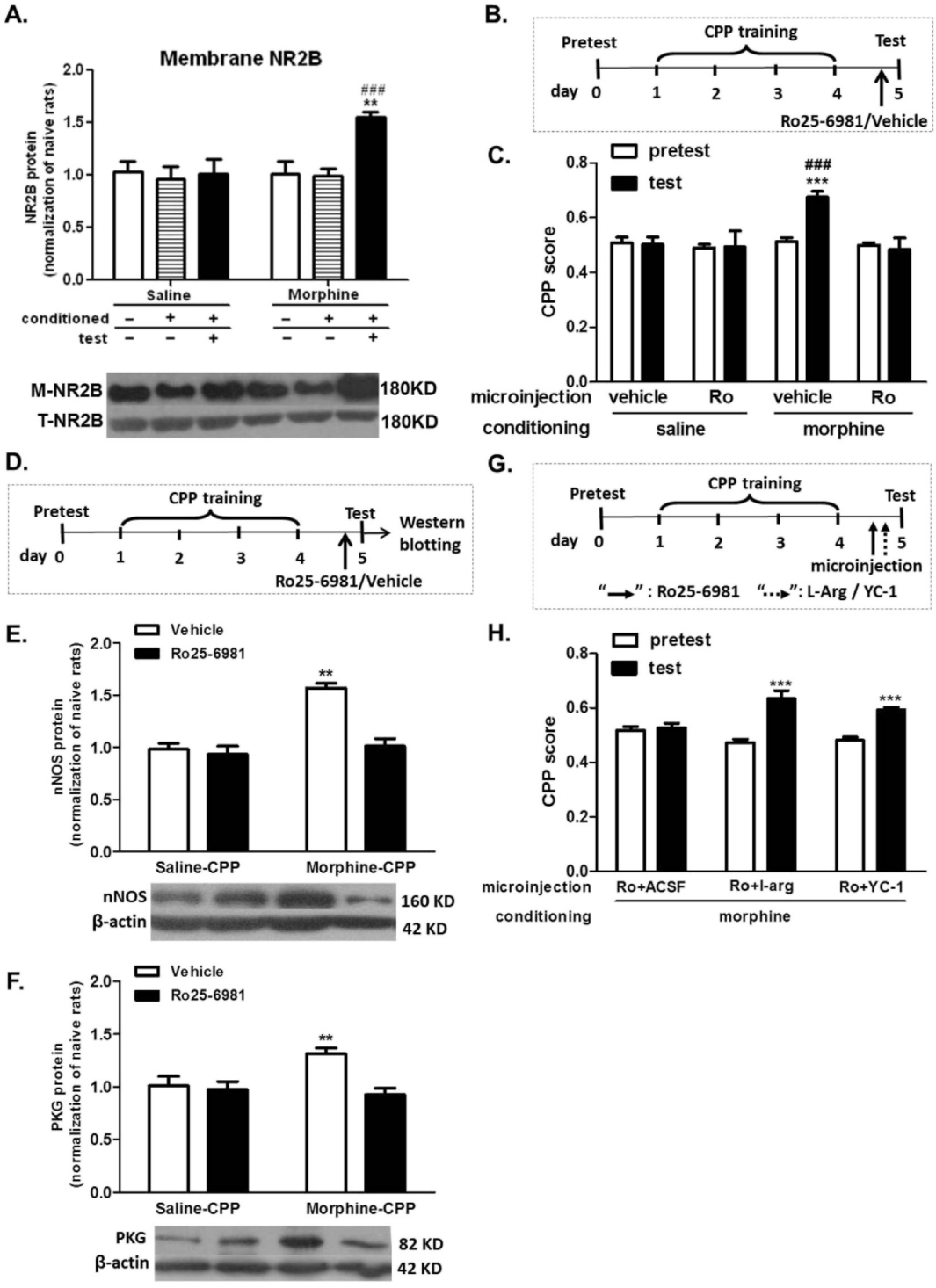

Fig. 3. NR2B-NMDAR was involved in the retrieval of morphine CPP via activation of the NO/sGC/PKG signaling pathway in the CA1. (A) Re-exposure to the morphine-conditioning context significantly increased the membrane NR2B levels. (B) Diagram outlining the behavioral procedures. (C) Intra-CA1 microinjection of Ro25-6981 blocked the expression of morphine CPP. (D) Diagram outlining the behavioral procedures. (E, F) Intra-CA1 microinjection of Ro25-6981 abolished the elevation of the protein expression of nNOS (E) and PKG (F) after the morphine CPP expression tests. (G) Diagram outlining the behavioral procedures. (H) Pre-microinjection of L-arg or YC-1 into the CA1 reversed the blockade of morphine CPP expression induced by micro-infusion of Ro25-6981 prior to the expression tests. Representative bands from western blots are shown under each column. The data are expressed as the means ± SEM; n = 3–6 in A, E and F; n = 8–11 in C and H. In A, $^{**}p < 0.01$, compared to the saline-treated group with re-exposure; $^{###}p < 0.001$, compared to the morphine-conditioned group without re-exposure (*t*-test). In C, $^{***}p < 0.001$, pretest vs. test; $^{###}p < 0.001$, compared to the morphine-conditioned rats receiving intra-CA1 microinjection of Ro25-6981 (*t*-test). In E and F, $^{**}p < 0.01$, compared to the morphine-conditioned rats receiving intra-CA1 microinjection of Ro25-6981. In H, $^{***}p < 0.001$, pretest vs. test. Ro, Ro25-6981.

treated rats that underwent the expression test ($t = 3.826$, $p < 0.01$) or the morphine-treated rats that did not undergo the expression test ($t = 4.883$, $p < 0.01$) (Fig. 2A). In addition, the nNOS protein level strongly positively correlated with the CPP expression test score ($r^2 = 0.7626$, $p = 0.0021$) (Fig. 2A′).

For sGC, three-way ANOVA showed that morphine conditioning

and the CPP expression test significantly increased the sGC protein expression in the CA1 [treatment, $F_{1, 21} = 8.135$, $p = 0.01$; test, $F_{1, 21} = 5.881$, $p < 0.05$], although no treatment × test interaction was found ($F_{1, 21} = 3.251$, $p = 0.086$). However, morphine conditioning without the CPP expression test did not change the sGC protein level [conditioning, $F_{1, 21} = 0.099$, ns; treatment × conditioning interaction, $F_{1, 21} = 0.076$, ns]. Moreover, *t*-test demonstrated that sGC protein was increased in rats that received both morphine conditioning and the CPP expression test compared to the saline-treated rats that underwent the expression test ($t = 3.630$, $p < 0.01$) or the morphine-treated rats that did not undergo the expression test ($t = 2.877$, $p < 0.05$) (Fig. 2B). In addition, the sGC protein level strongly positively correlated with the CPP expression test score ($r^2 = 0.6765$, $p = 0.0065$) (Fig. 2B′).

For PKG, three-way ANOVA showed that morphine conditioning and the CPP expression test significantly increased the PKG protein expression in the CA1 [treatment, $F_{1, 23} = 10.361$, $p < 0.01$; test, $F_{1, 23} = 11.639$, $p < 0.01$; treatment × test interaction, $F_{1, 23} = 11.054$, $p < 0.01$], whereas morphine conditioning without the CPP expression test did not change the PKG protein level [conditioning, $F_{1, 23} = 0.043$, ns; treatment × conditioning interaction, $F_{1, 23} = 0.009$, ns]. Moreover, *t*-test demonstrated that PKG protein was increased in rats that received both morphine conditioning and the CPP expression tests compared to the saline-treated rats that underwent the expression test ($t = 4.744$, $p < 0.01$) or the morphine-treated rats that did not undergo the expression test ($t = 4.883$, $p < 0.01$) (Fig. 2C). In addition, the PKG protein level strongly positively correlated with the CPP expression test score ($r^2 = 0.7361$, $p = 0.0135$) (Fig. 2C′). These results in Fig. 2 showed that nNOS, sGC and PKG expression were increased in the CA1 after context-induced drug seeking behavior and that the expression levels of these molecules positively correlated with the morphine CPP expression test score.

Additionally, we also examined the expression levels of nNOS, sGC and PKG in the CA3, another region of the hippocampus, after the expression test. The results showed that the morphine-induced CPP had no effect on the expression levels of these proteins in the CA3 (Fig. S5).

3.3. NR2B-NMDAR in the CA1 played an important role in the expression of morphine CPP by activating the NO pathway

To investigate whether the NMDAR was involved in the expression of morphine CPP, we tested the protein levels of NR2B in the CA1 after the CPP test. Three-way ANOVA showed that morphine conditioning and the CPP expression test significantly increased the membrane NR2B protein expression [treatment, $F_{1, 18} = 6.229$, $p < 0.05$; test, $F_{1, 18} = 8.291$, $p = 0.01$; treatment × test interaction, $F_{1, 18} = 5.767$, $p < 0.05$], whereas morphine conditioning without the CPP expression test did not change the membrane NR2B protein level [conditioning, $F_{1, 18} = 0.146$, ns; treatment × conditioning interaction, $F_{1, 18} = 0.027$, ns]. Moreover, *t*-test demonstrated that the membrane NR2B protein was increased in rats that received both morphine conditioning and the CPP expression test compared to the saline-treated rats that underwent the expression test ($t = 4.233$, $p < 0.01$) or the morphine-treated rats that did not undergo the expression test ($t = 6.487$, $p < 0.001$) (Fig. 3A). Additionally, morphine- and saline-conditioned rats received bilateral microinjections of Ro25-6981 (1 μg/side/0.5 μl) or vehicle 20 min before the test on day 5 (Fig. 3B). Three-way ANOVA revealed that the effects of treatment ($F_{1, 31} = 5.536$, $p < 0.05$), antagonist ($F_{1, 31} = 8.583$, $p < 0.01$) and time ($F_{1, 31} = 5.805$, $p < 0.05$) reached the statistical significance. Moreover, the treatment × antagonist × time interaction was significant ($F_{1, 31} = 8.828$, $p < 0.01$). Paired *t*-test showed a dramatic increase in the CPP expression test score compared with the pretest score in the rats micro-infused with saline ($t = 4.211$, $p < 0.001$). Further comparisons suggested that there was a significant decrease in the CPP score of the expression test of morphine-conditioned rats infused into the CA1 with Ro25-6981 compared to vehicle ($t = 4.229$, $p < 0.001$). These results indicated that intra-CA1 administration of Ro25-6981 blocked morphine-induced place preference compared to vehicle (Fig. 3C).

Further, to investigate the effect of blocking the NR2B-NMDAR on the protein expression of NO pathway members, the rats were microinjected into the CA1 with Ro25-6981 or vehicle 20 min before the test. The brains were rapidly removed immediately after the expression test for western blotting (Fig. 3D).

For nNOS, two-way ANOVA showed significant effects of the conditioning treatment ($F_{1, 17} = 25.48$, $p < 0.001$), of treatment with vehicle vs. Ro25-6981 ($F_{1, 17} = 21.03$, $p < 0.001$) and the interaction of these two factors ($F_{1, 17} = 14.70$, $p < 0.01$). A Bonferroni *post hoc* test demonstrated that there was a significant increase in the nNOS level after the expression test in the morphine-conditioned rats infused into the CA1 with vehicle ($t = 6.088$, $p < 0.001$, vehicle vs. Ro25-6981) (Fig. 3 E). For PKG, two-way ANOVA did not reveal any significant effects of the conditioning treatment ($F_{1, 20} = 3.107$, $p > 0.05$), but there was a significant difference between treatment with vehicle and Ro25-6981 ($F_{1, 20} = 8.665$, $p < 0.01$) and a significant interaction between these two factors ($F_{1, 20} = 5.995$, $p < 0.05$). A Bonferroni *post hoc* test demonstrated that there was a significant increase in the PKG level after the expression test in the morphine-conditioned rats infused into the CA1 with vehicle ($t = 3.813$, $p < 0.01$, vehicle vs. Ro25-6981) (Fig. 3F).

Moreover, we investigated whether intra-CA1 injection of the NOS substrate L-arg (0.025 μg/side/0.5 μl) or the sGC activator YC-1 (0.152 μg/side/0.5 μl) could rescue the expression of morphine CPP when blocked with Ro25-6981. All rats were conditioned with morphine at 4 mg/kg. On day 5, 40 min before the expression test, the rats first received intra-CA1 injection of Ro25-6981; 20 min later, they were infused with L-arg, YC-1 or vehicle (Fig. 3G). Two-way ANOVA did not reveal significant differences between the treatments ($F_{2, 52} = 1.692$, $p > 0.05$), but there was a significant difference between the pretest and test scores ($F_{1, 52} = 49.79$, $p < 0.001$) and a significant interaction effect between treatment and time ($F_{2, 52} = 10.58$, $p < 0.001$). A Bonferroni *post hoc* test showed a significant increase in the CPP test score in rats injected into the CA1 with Ro25-6981 in combination with either L-arg ($t = 6.758$, $p < 0.001$) or YC-1 ($t = 5.184$, $p < 0.001$), compared with the pretest score (Fig. 3H). These results in Fig. 3 revealed the essential role of the NR2B-NMDAR in the expression of morphine CPP via activation of the NO/sGC/PKG pathway.

Furthermore, we also investigated the effects of morphine-induced CPP on the expression level of NR1 and NR2A. The results showed that no change in the levels of NR1 or NR2A was detected in the rats received both morphine conditioning and the CPP expression test (Fig. S6).

3.4. Akt in the CA1 acted downstream of NR2B-NMDAR to play an important role in the expression of morphine CPP

As shown in Fig. 4A–C, after 4 days of conditioning, rats underwent the CPP expression test on day 5. Immediately after the expression test, the rats′ brains were rapidly removed for western blotting experiments (Fig. 4A). For $pAkt^{Ser473}$, three-way ANOVA showed that morphine conditioning and the CPP expression test significantly increased the $pAkt^{Ser473}$ protein expression [treatment, $F_{1, 24} = 8.104$, $p < 0.01$; test, $F_{1, 24} = 12.744$, $p < 0.01$; treatment × test interaction, $F_{1, 24} = 17.166$, $p < 0.001$], whereas morphine conditioning without the CPP expression test did not

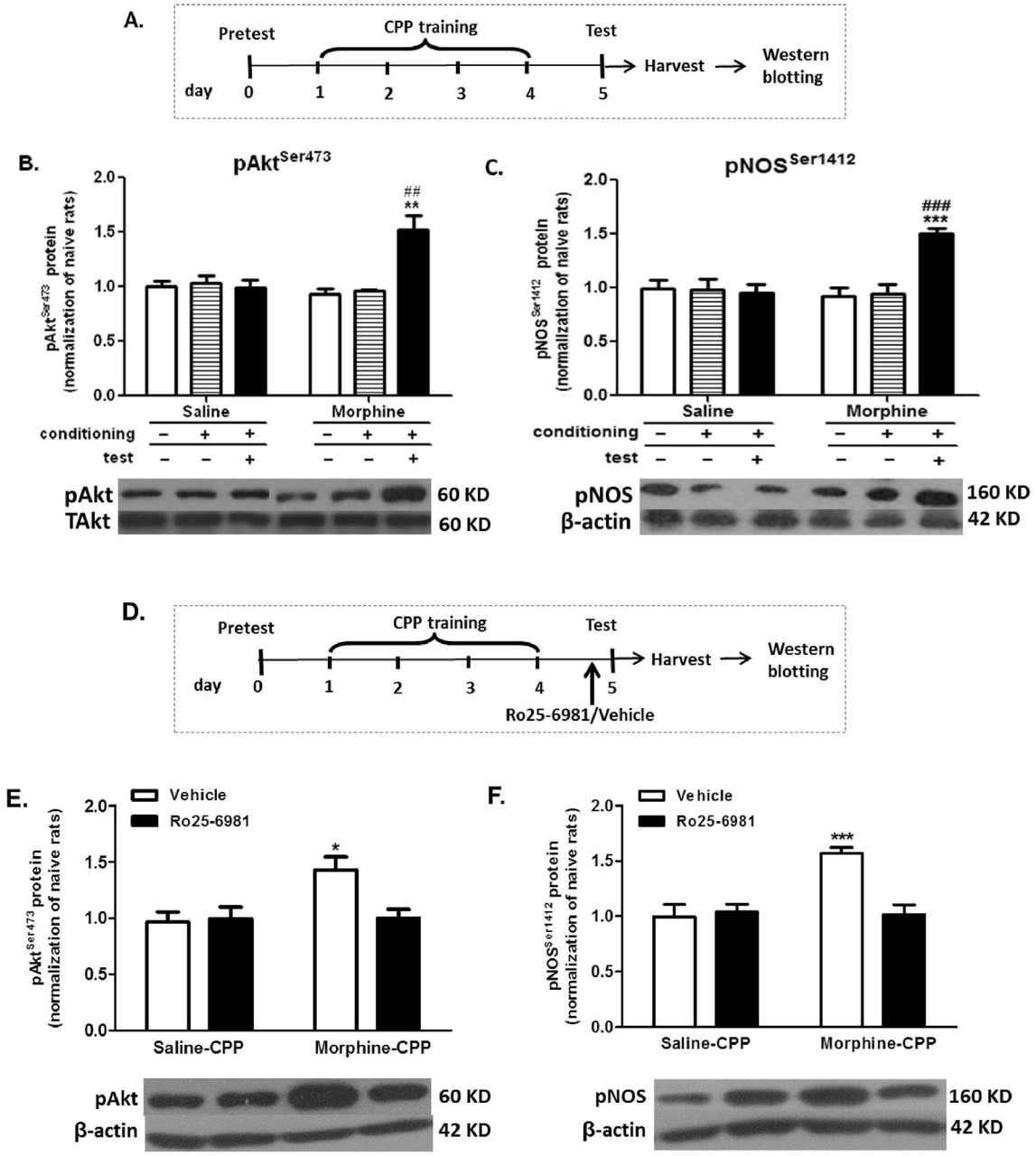

Fig. 4. NR2B-NMDAR regulated the phosphorylation of Akt and nNOS in the CA1 of morphine-conditioned rats. (A) Diagram outlining the behavioral procedures. (B, C) Re-exposure to the morphine-conditioning context significantly increased the levels of pAKt^{Ser473} (B) and pNOS^{Ser1412} (C) in the CA1 of morphine-conditioned rats. (D) Diagram outlining the behavioral procedures. (E, F) Intra-CA1 microinjection of Ro25-6981 blocked the elevation of the pAKt^{Ser473} (E) and pNOS^{Ser1412} levels (F) in the CA1 of morphine-conditioned rats. Representative bands from western blots are shown under each column. The data are expressed as the means ± SEM, n = 5 in each group. In Fig. B and C, $^{**}p < 0.01$, $^{***}p < 0.001$, compared to the saline-treated group with re-exposure; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to the morphine-conditioned group without re-exposure (*t*-test). In Fig. E and F, $^{*}p < 0.05$, $^{***}p < 0.001$, compared to the morphine-conditioned rats receiving intra-CA1 microinjection of Ro25-6981 (two-way ANOVA, Bonferroni *post hoc* test).

change pAkt^{Ser473} protein level [conditioning, $F_{1,\ 18} = 0.163$, ns; treatment × conditioning interaction, $F_{1,\ 18} = 0.001$, ns]. Moreover, *t*-test demonstrated that the pAkt^{Ser473} was increased in rats that received both morphine conditioning and the CPP expression tests compared to the saline-treated rats that underwent the expression test ($t = 3.631$, $p < 0.01$) or the morphine-treated rats that did not undergo the expression test ($t = 4.392$, $p < 0.01$) (Fig. 4B). For pNOS^{Ser1412}, three-way ANOVA showed that morphine conditioning and the CPP expression test significantly increased the pNOS^{Ser1412} protein expression [treatment, $F_{1,\ 25} = 7.661$, $p = 0.01$; test, $F_{1,\ 25} = 10.893$, $p < 0.01$; treatment × test interaction, $F_{1,\ 25} = 12.895$, $p = 0.001$], whereas morphine conditioning without the CPP expression test did not change the pNOS^{Ser1412} [conditioning, $F_{1,\ 25} = 0.003$, ns; treatment × conditioning interaction, $F_{1,\ 18} = 0.046$, ns]. Moreover, *t*-test demonstrated that the pNOS^{Ser1412} was increased in rats that received both morphine conditioning and the CPP expression test compared to the saline-treated rats that underwent the expression test ($t = 5.838$, $p < 0.001$) or the morphine-treated rats that did not undergo the expression test ($t = 5.540$, $p < 0.001$) (Fig. 4C).

As shown in Fig. 4D–F, all rats were conditioned with morphine at 4 mg/kg. On day 5, 20 min before the test, the rats received intra-

CA1 injection of Ro25-6981 or vehicle (Fig. 4D). For pAkt^{Ser473}, two-way ANOVA did not reveal significant effects of the conditioning treatment ($F_{1, 16} = 4.024$, $p > 0.05$), but there was a significant difference between vehicle and Ro25-6981 treatment ($F_{1, 16} = 5.731$, $p < 0.05$) and a significant interaction between these two factors ($F_{1, 16} = 5.349$, $p < 0.05$). A Bonferroni *post hoc* test demonstrated that there was a significant increase in the pAkt^{Ser473} level after the expression test in the morphine-conditioned rats intra-CA1 infused with vehicle ($t = 3.054$, $p < 0.05$, vehicle vs. Ro25-6981) (Fig. 4E). For pNOS^{Ser1412}, two-way ANOVA showed significant effects of the conditioning treatment ($F_{1, 16} = 10.70$, $p < 0.01$), vehicle vs. Ro25-6981 treatment ($F_{1, 16} = 9.036$, $p < 0.01$) and the interaction of these two factors ($F_{1, 16} = 12.58$, $p < 0.01$). A Bonferroni *post hoc* test demonstrated that there was a significant increase in the pNOS^{Ser1412} level after the expression test in the morphine-conditioned rats infused into the CA1 with vehicle ($t = 4.634$, $p < 0.001$, vehicle vs. Ro25-6981) (Fig. 4F).

The data presented in Fig. 4 showed that during morphine CPP expression, the NR2B-NMDAR regulated nNOS expression by activating Akt in the CA1.

3.5. GluR1 in the CA1 acted downstream of the NO/sGC/PKG signaling pathway to play an important role in the expression of morphine CPP

For the total GluR1 level, three-way ANOVA showed that morphine conditioning and the CPP expression test had no effect on the total GluR1 [treatment, $F_{1, 25} = 3.098$, ns; conditioning, $F_{1, 25} = 0.001$, ns; test, $F_{1, 25} = 0.001$, ns; treatment × conditioning interaction, $F_{1, 25} = 0.075$, ns; treatment × test interaction, $F_{1, 25} = 0.456$, ns] (Fig. 5A). However, for the membrane GluR1 level, three-way ANOVA showed that morphine conditioning and the CPP expression test significantly increased the membrane GluR1 [treatment, $F_{1, 20} = 20.307$, $p < 0.001$; test, $F_{1, 20} = 15.941$, $p = 0.001$; treatment × test interaction, $F_{1, 20} = 11.308$, $p < 0.01$], whereas morphine conditioning without the CPP test had no effect on the membrane GluR1 [conditioning, $F_{1, 20} = 0.973$, ns; treatment × conditioning interaction, $F_{1, 20} = 1.359$, ns]. Moreover, *t*-test demonstrated that the membrane GluR1 level was increased in rats that received both morphine conditioning and the CPP expression test compared to the saline-treated rats that underwent the expression test ($t = 5.046$, $p < 0.001$) or the morphine-treated rats that did not undergo the expression test ($t = 5.402$, $p < 0.001$) (Fig. 5B).

For the total GluR2 level, three-way ANOVA showed that morphine conditioning and the CPP expression test had no effect on the total GluR2 [treatment, $F_{1, 29} = 0.047$, ns; conditioning, $F_{1, 29} = 0.007$, ns; test, $F_{1, 29} = 0.304$, ns; treatment × conditioning interaction, $F_{1, 29} = 0.260$, ns; treatment × test interaction, $F_{1, 29} = 0.001$, ns] (Fig. 5C). Similarly, three-way ANOVA showed that morphine conditioning and the CPP expression test had no effect on the membrane GluR2 [treatment, $F_{1, 30} = 0.155$, ns; conditioning, $F_{1, 30} = 0.097$, ns; test, $F_{1, 30} = 0.609$, ns; treatment × conditioning interaction, $F_{1, 30} = 0.074$, ns; treatment × test interaction, $F_{1, 30} = 0.159$, ns] (Fig. 5D).

As shown in Fig. 5E and F, all rats were conditioned with morphine at 4 mg/kg. On day 5, 20 min before the expression test, the rats received intra-CA1 injection of Rp-8Br-PET-cGMPS or vehicle (Fig. 5E). For the membrane GluR1 level, two-way ANOVA revealed significant effects of the conditioning treatment ($F_{1, 17} = 9.669$, $p < 0.01$), vehicle vs. Rp-8Br-PET-cGMPS treatment ($F_{1, 17} = 10.45$, $p < 0.01$) and the interaction of these two factors ($F_{1, 17} = 5.586$, $p < 0.05$). A Bonferroni *post hoc* test demonstrated that there was a significant increase in the GluR1 level after the expression test in morphine-conditioned rats intra-CA1 infused with vehicle ($t = 4.046$, $p < 0.05$, vehicle vs. Rp-8Br-PET-cGMPS) (Fig. 5F).

We further investigated whether there is an interaction between PKG and GluR1 in the CA1. As shown in Fig. 5G, the protein extract from the CA1 region of rats was precipitated using a control IgG or an anti-PKG antibody. GluR1 was detected by an anti-GluR1 antibody in the protein samples precipitated using the anti-PKG antibody; this result indicated that PKG associates with GluR1 in the CA1.

4. Discussion

In this study, we demonstrated that the NO/sGC/PKG pathway in the hippocampal CA1 plays an essential role in the retrieval of morphine-associated memory and that its role depends on the local expression of ionotropic glutamate receptors. Specifically, we observed that intra-CA1 microinjection of 7-NI, ODQ or Rp-8-Br-PET-cGMPS before the morphine CPP expression test prevented the retrieval of morphine-induced memory. In addition, when morphine-conditioned rats were re-exposed to the morphine-paired chambers, the nNOS, sGC and PKG protein levels in the CA1 were significantly increased compared with the levels in the saline-conditioned animals or the morphine-conditioned animals without re-exposure on the test day. These findings implied that the NO pathway in the CA1 is essential for the retrieval of morphine-associated memory. These results are analogous to the finding that inhibiting NO synthesis via intra-CA1 injection of L-NAME, a NOS inhibitor, impaired the retrieval of short- and long-term memory on an inhibitory avoidance task (Harooni et al., 2009). However, our findings are inconsistent with the observation by Kleppisch et al. that mice carrying a hippocampus-specific deletion of PKG showed normal performance on a discriminatory water maze task and exhibited intact contextual fear conditioning (Kleppisch et al., 2003). This discrepancy may result from the variations in the experimental techniques used to knock down the PKG level in the hippocampus and in the behavioral paradigms employed.

Additionally, we examined the protein levels of these proteins in the CA3, another region of the hippocampus, after the morphine CPP expression test (Fig. S5). Our results revealed that the retrieval process had no effect on their expression in the CA3; thus, the activity of the NO/sGC/PKG signaling pathway in the CA1 region is selectively required for the retrieval of previously formed morphine-associated memory. This regional specificity could be ascribed to the function of the dorsal hippocampus. Morphine conditioning trains the animals to form drug-related memory by associating the euphoric effect of the drug with the drug-paired environment, and this memory formation apparently requires the processing of spatial information, a well-known function of the CA1 (Morris et al., 1986; Shen et al., 1994). According to these findings, we conclude that the NO/sGC/PKG signaling pathway in the CA1 plays an essential and specific role in the context-induced retrieval of addiction memory.

In the control group, intra-CA1 microinjection of 7-NI, ODQ or Rp-8-Br-PET-cGMPS had no effect on the expression of saline-induced CPP; these results suggested that the reagents themselves did not result in the rats' preference or aversion to one of the paired chambers. Therefore, the most likely reason that intra-CA1 injection of these reagents blocked the expression of morphine CPP is the inhibition of the NO/sGC/PKG signaling pathway.

To rule out the possibility that the inhibition of the NO/sGC/PKG signaling pathway blocked the expression of morphine CPP by impairing the animals' spatial memory ability, experiments were conducted in which 7-NI, ODQ or Rp-8-Br-PET-cGMPS was microinjected into the CA1 20 min prior to the probe trial of the Morris

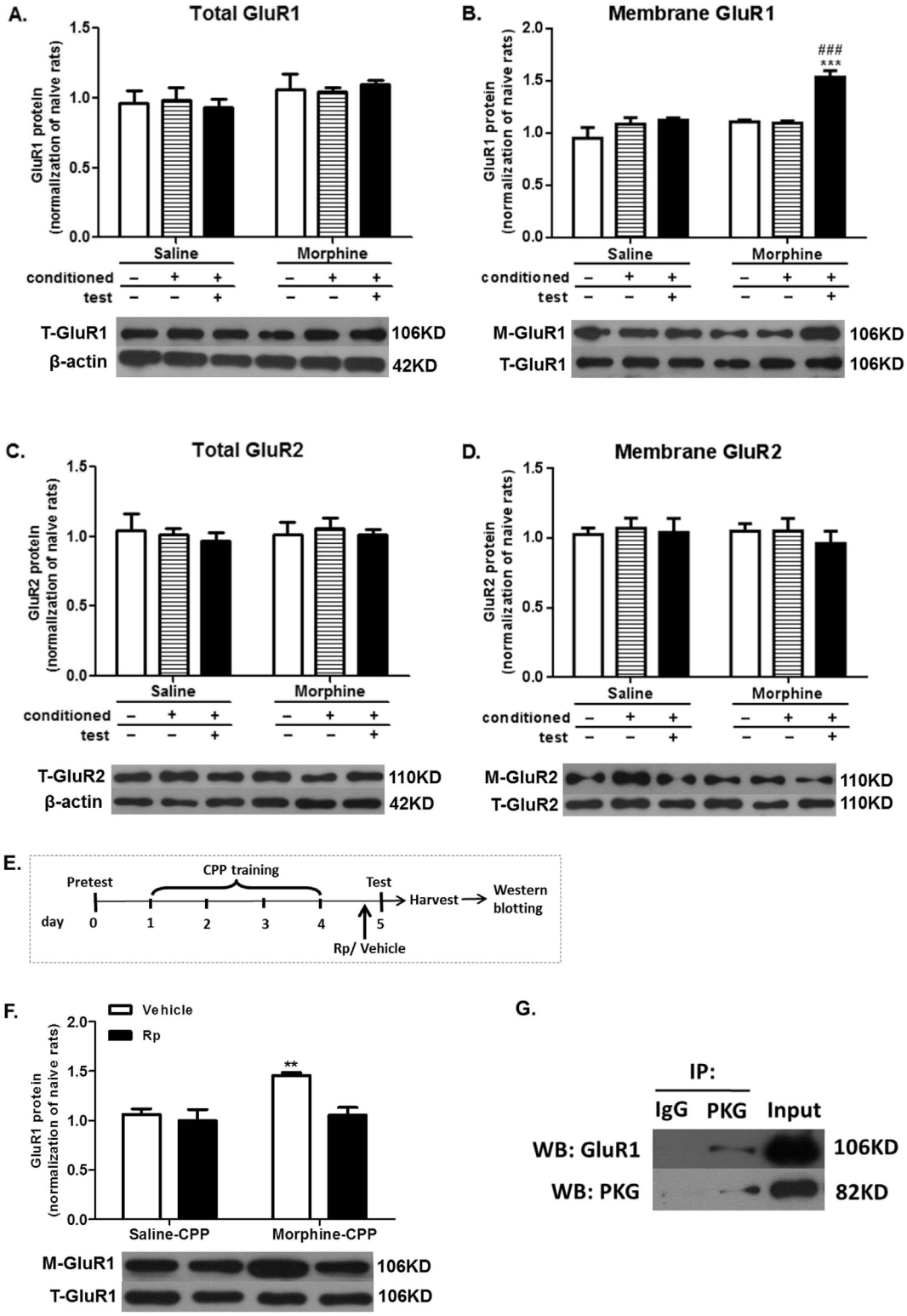

Fig. 5. The NO/sGC/PKG signaling pathway was involved in the retrieval of morphine CPP by regulating the trafficking of GluR1-containing AMPARs. (A, B) Re-exposure to the morphine-conditioning context significantly increased the membrane GluR1protein levels (B) but had no effect on the total GluR1 levels (A) in the CA1 of morphine-conditioned rats. (C–D) Re-exposure to the morphine-conditioning context had no effect on the total GluR2 (C) or membrane GluR2 protein levels (D) in the CA1 of morphine-conditioned rats. (E) Diagram outlining the behavioral procedures. (F) Intra-CA1 microinjection of Rp-8-Br-PET-cGMPS blocked the elevation of membrane GluR2 protein expression in the CA1 of morphine-conditioned rats. (G) PKG associated with GluR1 in the CA1 of the rat brain. Rat brain tissue was precipitated with a control IgG or an anti-PKG antibody, and the bound proteins were detected by an anti-GluR1 antibody. Representative bands from western blots are shown under each column. The data are expressed as the means ± SEM, n = 4–6. In Fig. B, $^{***}p < 0.001$, compared to the saline-treated group with re-exposure; $^{\#\#\#}p < 0.001$, compared to the morphine-conditioned group without re-exposure (*t*-test). In Fig. F, $^{**}p < 0.01$, compared to the morphine-conditioned rats receiving intra-CA1 microinjection of Rp-8-Br-PET-cGMPS (two-way ANOVA, Bonferroni *post hoc* test).

water maze task. The results showed that intra-CA1 administration of 7-NI, ODQ or Rp-8-Br-PET-cGMPS did not affect the duration that rats spent in the target quadrant or their swimming speed; these findings suggested that disrupting NO/sGC/PKG signaling does not impair the spatial memory of rats (Fig. S3). Another possible explanation is that the deficit in the expression of morphine CPP resulted from a marked augmentation of the rats' locomotor activity, which made it difficult to detect the difference in the time spent between the chambers because more time was spent shuttling (Bozarth, 1987). However, neither morphine- nor saline-conditioned rats receiving intra-CA1 microinjection of 7-NI, ODQ or Rp-8-Br-PET-cGMPS showed any increase in the number of crossings between adjacent compartments (Fig. S2). Thus, we conclude that the most tenable reason for our data is that 7-NI, ODQ or Rp-8-Br-PET-cGMPS selectively blocks morphine CPP due to specific suppression of morphine-associated reward memory.

Furthermore, we investigated the effects of 7-NI, ODQ and Rp-8-Br-PET-cGMPS on the retrieval of memory induced by non-drug/natural rewards (such as sucrose). Our results showed that sucrose-conditioned rats microinjected with 7-NI, ODQ or Rp-8-Br-PET-cGMPS displayed equivalent preference scores to those of the rats that received intra-CA1 infusion of vehicle (Fig. S4). This finding indicated that NO/sGC/PKG signaling in the CA1 is not involved in the retrieval of natural reward-associated memory. It can thus be speculated that disparate neural circuits may be involved in the regulation of memories induced by addictive drug and non-drug rewards. In fact, previous studies have elucidated that in subjects self-administering either cocaine or a natural reinforcer, the two types of reward activated different populations of cells in the NAc, and only less than 10% of cells responded to both rewards (Carelli et al., 2000).

Previous studies have demonstrated that NOS is tethered to the NMDAR by the scaffolding protein PSD-95 to form a complex. In this complex, NMDAR-mediated Ca^{2+} influxes regulate nNOS activity and NO production (Christopherson et al., 1999; Rameau et al., 2004). Additionally, most NMDARs in the brain are thought to be heteromeric complexes in which NR1 serves as a constructive subunit and NR2A-D serve as functional subunits to increase NMDAR-mediated currents (Narita et al., 2000). The distributions of NR1, NR2A and NR2B, but not NR2C or NR2D, are consistent with the brain regions related to reward (Ma et al., 2007; Mori and Mishina, 1995). Therefore, we investigated whether the involvement of NO/sGC/PKG signaling in the retrieval of morphine reward memory involves the activation of NMDARs. The results revealed that rats conditioned with morphine showed a significant increase in the levels of the NR2B subunit in the CA1. Moreover, intra-CA1 injection of Ro25-6981, a selective NR2B-NMDAR antagonist, blocked the expression of morphine CPP. In contrast, no change in the levels of NR1 or NR2A was detected in morphine-conditioned rats (Fig. S6). These results are in line with those from our previous study, in which the level of NR2B-NMDAR in the hippocampus was elevated in morphine CPP rats but the inhibition of NR2B-NMDAR in the dorsal hippocampus blocked morphine CPP (Ma et al., 2006). Although NR1 subunits are thought to exist in all functional NMDARs, we failed to observe a significant change in it in rats following morphine CPP. The existing data are not sufficient to clearly explain why the increase in the NR2B protein level was not accompanied by a similar increase in the NR1 subunit level. One possibility is that some NMDARs are composed of the NR1 subunit and that other subunits, such as NR3A, might be down-regulated following morphine CPP, leading to an unchanged total level of NR1 subunits. Our data also showed that intra-CA1 injection of Ro25-6981 eliminated the elevation of nNOS and PKG expression induced by morphine conditioning but that pre-microinjection of L-arg or YC-1 into the CA1 abolished the inhibitory effect of Ro25-6981 infusion on the expression of morphine CPP. The above findings suggested that the NR2B-NMDAR acts upstream of NO/sGC/PKG signaling to regulate the retrieval of morphine reward memory; this conclusion is analogous to the findings in the study by Rameau et al., in which the NMDAR antagonist MK801 blocked the increase in the cGMP levels induced by glutamate (Rameau et al., 2007). Therefore, the present study demonstrated the involvement of the NR2B-NMDAR in the expression of morphine CPP by activating the NO/sGC/PKG signaling pathway.

Further experiments were conducted to investigate how NR2B-NMDAR regulates the activity of NO/sGC/PKG signaling. Rameau et al. demonstrated that the NMDAR mediated the activity of NOS via phosphorylation of NOS at Ser1412 and Ser847 (Rameau et al., 2007). As expected, we found that the phosphorylation level of $NOS^{ser1412}$ was increased in the CA1 after the morphine CPP expression test and that intra-CA1 administration of Ro25-6981 abrogated the increase in the $pNOS^{ser1412}$ levels. These results indicated that the NMDAR-dependent phosphorylation of $nNOS^{ser1412}$ in the CA1 is an essential process during the retrieval of morphine reward memory. These results are in accordance with the previous findings that the control of NO production by the NMDAR at neural synapses involves the regulatory phosphorylation of nNOS (Rameau et al., 2003, 2004). Moreover, other researchers have found that NR2B is responsible for the phosphorylation of phosphoinositide and the activation of the serine/threonine protein kinase Akt, which phosphorylates nNOS at Ser1412 (Hisatsune et al., 1999; Waxman and Lynch, 2005). Similar results were observed in our study, in which the increase in the $pAkt^{ser473}$ levels in the CA1 caused by the retrieval of morphine reward memory was

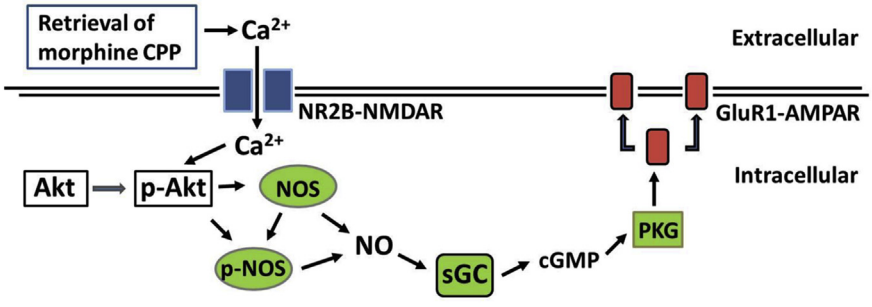

Fig. 6. Model for the regulation of GluR1 trafficking by NR2B-NO signaling in the CA1 during the retrieval of morphine-associated reward memory. The proposed sequence of events that occurs after NMDAR stimulation is shown. On the one hand, activation of the NMDAR induces the phosphorylation of Akt^{Ser473}, and phosphorylated Akt further triggers the phosphorylation of $nNOS^{Ser1412}$. On the other hand, NMDAR causes the elevation of nNOS protein expression. Finally, elevated NO/sGC/PKG signaling leads to an increase in the membrane GluR1 levels. These events may represent the cellular mechanisms by which NO/sGC/PKG signaling is involved in the regulation of the retrieval of morphine-associated memory.

reversed by microinjecting Ro25-6981 into the CA1. This finding implied that NR2B-NMDAR up-regulates the activity of nNOS by phosphorylating Akt^{Ser473}. Akt is activated via phosphorylation on two residues, Thr308 and Ser473 (Alessi et al., 1996). However, phosphorylation of Akt at Ser473 is critical because only when Akt and 3-phosphoinositide-dependent protein kinase 1 (PDK1) are stably colocalized does the phosphorylation of Ser473 lead to the PDK1-driven phosphorylation of Thr308 (Scheid and Woodgett, 2003). Our finding is consistent with the results of a previous study, in which the activation of the NO pathway by morphine was dependent on Akt stimulation (Cunha et al., 2010).

Several groups have shown that the NO pathway is involved in hippocampal LTP (Stanton et al., 2003; Zhuo et al., 1994). Moreover, it is known that the regulated trafficking of AMPARs is an important process that underlies the activity-dependent modification of synaptic strength, which is a cellular mechanism involved in learning and memory (Serulle et al., 2008). Therefore, the protein levels of GluR1 and GluR2 were also measured in this study. Our results displayed that morphine conditioning and re-exposure to the morphine-paired context significantly increased the membrane GluR1 levels in the CA1 without changing the total GluR1 or GluR2 subunit levels or the membrane GluR2 level. Further, this elevation of the membrane GluR1 subunit levels in the CA1 was abolished by intra-CA1 microinjection of Rp-8Br-PET-cGMPS before the morphine CPP test. This result indicated that the up-regulation of the membrane GluR1 levels induced by PKG is required for the retrieval of morphine-associated memory. Furthermore, a co-immunoprecipitation assay revealed an interaction between PKG and GluR1 in the CA1, as we had speculated. These findings are consistent with the results of previous studies, which showed the trafficking of GluR1 is governed by NO, cGMP, and PKG and that their interaction with GluR1, which is controlled by the NMDAR and NO, plays an important role in hippocampal synaptic plasticity (Rameau et al., 2007; Serulle et al., 2008, 2007). Moreover, the inhibited formation of the PKG-GluR1 complex by a PKG dominant-negative inhibitory peptide in hippocampal slices caused a reduction in tetanus-evoked LTP in the CA1 (Serulle et al., 2008, 2007). Based on this evidence, it is reasonable to speculate that the involvement of GluR1 in the retrieval of morphine-associated memory may result from its regulation of hippocampal synaptic plasticity.

In conclusion, our study found that when morphine-conditioned rats were re-exposed to the drug-paired context, the up-regulation of the NR2B-NMDAR in the CA1 stimulated the activity of the NO/sGC/PKG signaling pathway by phosphorylating Akt^{Ser473}. Then, the activated NO/sGC/PKG signaling pathway might facilitate the trafficking of GluR1-AMPAR in the CA1, leading to the retrieval of morphine-associated memory (Fig. 6).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2015.11.008.

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