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Role of the NO/sGC/PKG signaling pathway of hippocampal CA1 in morphine-induced reward memory

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ABSTRACT

Evidence suggests that the nitric oxide (NO)/soluble guanylyl cyclase (sGC)/cGMP dependent protein kinase (PKG) signaling pathway plays a key role in memory processing, but the actual participation of this signaling cascade in the hippocampal CA1 during morphine-induced reward memory remains unknown. In this study, we investigated the role of the NO/sGC/PKG signaling pathway in the CA1 on morphine-induced reward memory using a conditioned place preference (CPP) paradigm. We found that rats receiving an intraperitoneal (i.p.) injection of 4 mg/kg morphine exhibited CPP, whereas rats treated with only 0.2 mg/kg morphine failed to produce CPP. Intra-CA1 injection of the neuronal NO synthase (nNOS) inhibitor 7-NI, the sGC inhibitor ODQ or the PKG inhibitor Rp-8-Br-PET-cGMPS had no effect on the acquisition of CPP by 4 mg/kg morphine. Intra-CA1 injection of 7-NI blocked the consolidation of CPP induced by 4 mg/kg morphine, and this amnesic effect of 7-NI was mimicked by ODQ and Rp-8-Br-PET-cGMPS. Intra-CA1 injection of the NOS substrate L-arg or the sGC activator YC-1 with an ineffective dose of morphine (0.2 mg/kg, i.p.) elicited CPP. This response induced by L-arg or YC-1 was reversed by pre-microinjection of Rp-8-Br-PET-cGMPS in the CA1. These results indicated that the activation of the NO/sGC/PKG signaling pathway in the CA1 is necessary for the consolidation of morphine-related reward memory.

1. Introduction

Drug addiction can be understood as a pathological subversion of normal brain learning and memory processes and is strengthened by the motivational impact of drug-associated stimuli leading to the development of compulsive drug-seeking habits. Conditioned association between the environmental stimuli and effects of an addictive drug plays an important role in the maintenance of drug-induced reward memory and in the relapse of drug addicts, even after long periods of abstinence (Markou et al., 1993; Robbins & Everitt, 1999; See, Fuchs, Ledford, & McLaughlin, 2003). Breaking or weakening this association by pharmacotherapy would greatly improve the treatment of drug addiction.

In addition to the nucleus accumbens, the hippocampus also receives dopaminergic projections from the ventral tegmental area (VTA) (Scatton, Simon, Lemoal, & Bischoff, 1980) and plays a critical role in drug-induced associative reward memory (Berke & Eichenbaum, 2001). For instance, hippocampal lesions disturbed the acquisition and expression of cocaine-induced conditioned place preference (CPP) (Meyers, Zavala, & Neisewander, 2003). In parallel, intra-hippocampal infusion of morphine induced the acquisition of morphine CPP, indicating that the hippocampus is involved in the development of morphine CPP (Corrigall & Linseman, 1988; Dong et al., 2006). Therefore, the addictive drug may initiate a process that involves hippocampus-based learning to produce the CPP. However, the molecular mechanisms of addictive-induced CPP in the hippocampus remain unclear.

Nitric oxide (NO) is a labile and highly diffusible gas synthesized from L-arg by NO synthase (NOS). NO is mostly mediated by soluble guanylyl cyclase (sGC) (Arnold, Mittal, Katsuki, & Murad, 1977; Marsault & Frelin, 1992). Binding of NO to the heme group of sGC increases the activity of this enzyme several hundredfold to produce the second messenger cGMP (Murad, 2004), which then stimulates cGMP dependent protein kinase (PKG). Several studies have shown that NO is involved in the reward memory induced by addictive drugs, e.g., the administration of the neuronal NOS (nNOS) inhibitor 7-NI (25 mg/kg, i.p.) blocked the acquisition of CPP induced by nicotine (Martin & Itzhak, 2000), alcohol (Itzhak & Martin, 2000) and cocaine (Itzhak, Martin, Black, & Huang, 1998) in mice. Moreover, mice without the nNOS gene were resistant to cocaine-induced CPP (Itzhak et al., 1998). Similarly, 7-NI (12.5–50 mg/kg, i.p.) suppressed the acquisition, expression and

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reinstatement of D-methamphetamine induced CPP in rats (Li, Ren, & Zheng, 2002; Li, Yin, Shi, Lin, & Zheng, 2002). With respect to the role of NO in opiate-induced CPP, systemic injection of 7-NI and the non-selective NOS inhibitor L-N^G-nitroarginine (L-NOARG) blocked morphine CPP in mice and rats (Kivastik, Rutkauskaite, & Zharkovsky, 1996; Manzanedo, Aguilar, Rodriguez-Arias, Navarro, & Minarro, 2004).

Note that nearly all of the previous studies in this area focused on NO without examining its downstream molecular pathway and mostly utilized systemic injections of components that affected the activity of NO. Only one report showed that intra-hippocampal CA1 administration of the NO substrate L-arg enhanced the acquisition and expression of morphine CPP, which can be blocked by pre-administration of the NOS inhibitor L-NAME (Karami, Zarrindast, Sepehri, & Sahraei, 2002). This report did not investigate whether NO is involved in the consolidation of reward memory. In the present study, we explored whether the activation of the NO/sGC/PKG signaling pathway in the hippocampal CA1 is necessary for the acquisition and consolidation of morphine-induced CPP.

2. Materials and methods

2.1. Subjects

This study used male Sprague Dawley rats weighing 220–250 g at the time of surgery. These rats were obtained from the Laboratory Animal Center of the Peking University Health Science Center. The rats were housed 4 per cage in a 12:12 h light:dark cycle (lights on at 7 p.m.) with food and water available at all times. The room temperature was maintained at 23 ± 2 °C, and the relative humidity was maintained at 45–55%. Behavioral experiments were conducted during the dark cycle, and the rats were handled for 5 days prior to the experiments. All of the 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. Every effort was made to minimize animal suffering and to reduce the number of animals used.

2.2. Drugs

Morphine hydrochloride was purchased from the First Pharmaceutical Factory of Qinghai, China, and 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-Nitroindazole, 3-Bromo-, sodium salt (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-arginine (L-arg) were obtained from Sigma-Aldrich (St. LOUIS, MO, USA). Rp-8-Br-PET-cGMPS was dissolved in distilled water to a stock concentration of 2 μg/μl. 7-NI and ODQ were dissolved in 100% DMSO to stock concentrations of 4 μg/μl and 0.748 μg/μl, which were then diluted 1:1 in ACSF prior to infusion. YC-1 and L-arg were dissolved in ACSF for a stock concentration of 0.304 μg/μl and 0.05 μg/μl. The composition of ACSF is (mM): 115 NaCl, 3.3 KCl, 1 $MgSO_4$, 2 $CaCl_2$, 25.5 $NaHCO_3$, 1.2 NaH_2PO_4, and 10 glucose (Ota, Monsey, Wu, & Schafe, 2010).

2.3. Place preference apparatus

Conditioning was conducted in black colored rectangular PVC boxes ($795 \times 230 \times 250\ mm^3$) containing three chambers separated by guillotine doors (Shi et al., 2004). The 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 4 light-emitting diodes (LEDs) forming a square on the wall and a stainless-steel mesh floor ($225 \times 225\ mm^2$), chamber C had 4 LEDs forming a triangle on the wall and a stainless-steel rod floor (15 mm apart), and chamber B had a flat stainless steel floor. Fourteen photo beams were placed across the chambers at a distance of 47.5 mm. A computer interface was used to record the time that the rat spent in each chamber by means of infrared beam crossings.

2.4. Cannula implantation and microinjections

The rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and secured in a Kopf stereotaxic apparatus (Kopf Instruments, Tujunga, CA). The incisor bar was lowered 3.3 mm below horizontal zero to achieve the flat skull position. Stainless steel guide cannulas (0.67 mm outer diameter) were bilaterally implanted 0.5 mm above the CA1 region of the hippocampus. The CA1 region coordinates were 3.8 mm posterior to the bregma, ±2.2 mm lateral to the midline and −2.4 mm ventral to the dorsal surface of the skull (Paxinos & Watson, 1998). The cannulas were fixed to screws in the skull with dental cement. Internal cannulas were replaced with dummy cannulas to keep the cannulas patent and prevent infection. The rats were given at least 5–7 days to recover before the conditioning procedures.

In studies involving intra-CA1 infusions, the dummy cannulas were removed, and infusion cannulas (0.3 mm in outer diameter) were inserted. The hip of the injection needle was protruded 0.5 mm beyond the guide cannula tip. The cannulas were connected to 1.0-μl Hamilton syringes via PE 20 tubing. The tubing was back-filled with saline, with a small air bubble separating the saline from the drug solution. Drugs were infused with an infusion pump at a speed of 0.25 μl/min. After infusion, the cannula was left in place for 1 min to allow the drugs to diffuse from the needle.

2.5. Conditioned place preference

2.5.1. Pre-conditioning test phase

On day 0, the rats were allowed to freely explore the entire apparatus for 15 min to assess the unconditioned chamber preference. The time (in seconds) spent in each compartment and the shuttle times were recorded. The CPP apparatus was considered to be unbiased in its assessment of the chamber preferences of untreated rats. The chambers selected for pairing with morphine were counterbalanced within each group. The data from pre-conditioning tests were used to separate animals into groups with approximately equal biases for each chamber. Rats with a bias for either of the lateral chambers were excluded (approximately 5%) from the experiments.

2.5.2. Conditioning phase

The animals were allowed twice daily training sessions (8:30 and 15:30) for 4 days (days 1–4). Before being confined into one lateral chamber for 45 min, rats were received morphine (i.p., 0.2 or 4 mg/kg), and saline in the other lateral chamber. Animals in control groups received saline injections before both training sessions in lateral chambers. In the morphine-conditioned groups, half of the animals received morphine training in compartment A and saline training in compartment C, while the rest received morphine and saline training in compartments C and A, respectively, as a counterbalance. Moreover, half of the rats were conditioned with morphine in the morning session and saline in the afternoon, while the other half were treated in the reverse order.

2.5.3. Post-conditioning phase

On day 5, all of the animals were placed in the center choice chamber with the guillotine door removed to allow access to the entire apparatus for 15 min and the time spent in each side was recorded. The CPP score was defined as the time spent in the morphine-paired chamber divided by the total time spent in both the morphine and the saline-paired chambers during CPP testing. The locomotor activity during all of CPP tests was estimated by counting the total number of crossings between any two adjacent compartments.

2.6. Histological verification

After completion of behavioral trials, each rat was killed by decapitation and the brains were removed. To enable histological examination of the placement of cannulas and needles in the CA1 region, the brains were cut on a cryostat into 30-μm-thick sections and mounted on glass slides coated with gelatin. Cannula placements were assessed by Nissl staining using light microscopy. Fig. 1 showed the location of the representative cannula tips in the CA1 of the hippocampus. Only the data from rats that received histologically verified injections were included for analyses.

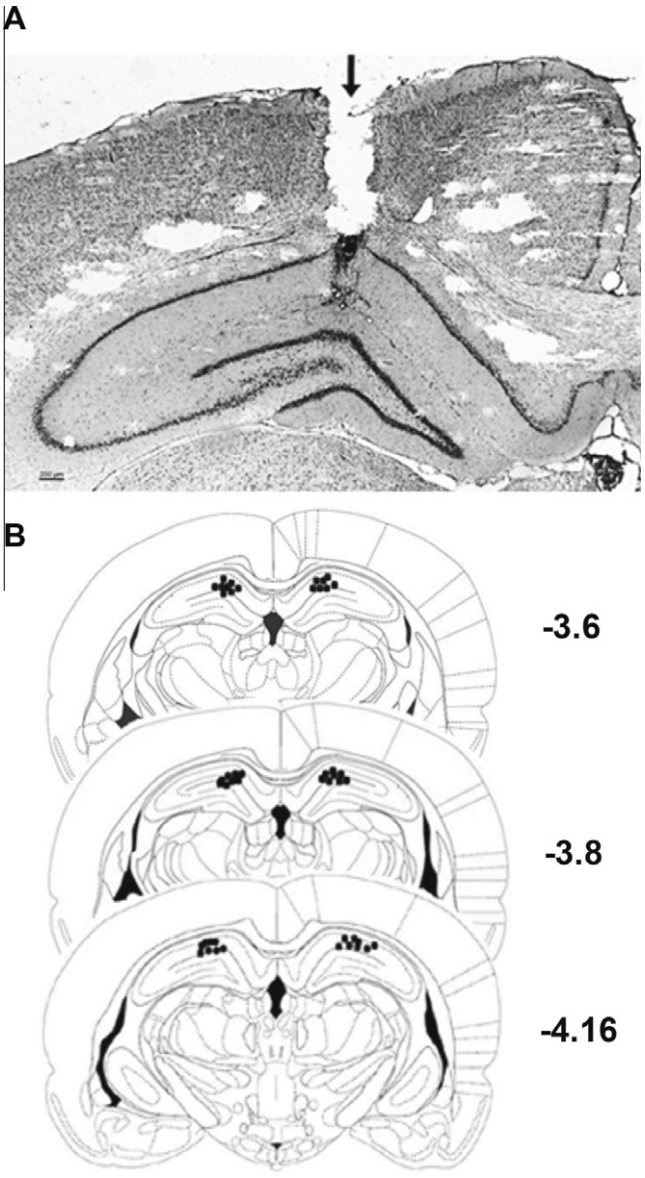

Fig. 1. Representative cannula placements and microinjection sites in the CA1. (A) Representative photomicrograph of the infusion site in the CA1 where the arrowhead points to the infusion cannula tract, scale bar = 200 μm. (B) Distribution of microinjection sites in the CA1 (gray circle) plotted on drawings of coronal sections from the atlas of Paxinos and Watson (1998).

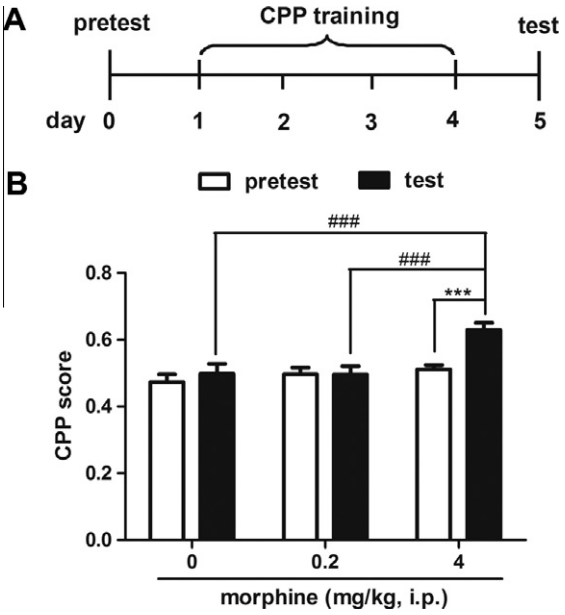

Fig. 2. Effect of different doses of morphine (0.2, 4 mg/kg, i.p.) on the induction of condition place preference. (A) Diagram outlining the behavioral procedures. (B) Rats that received alternating injections of 4 mg/kg morphine and saline (2 ml/kg) showed a significant preference for the morphine-paired chamber. Blank and solid columns represent data from pre- and post-conditioning tests, respectively. $^{***}p < 0.001$, pretest vs. test. $^{###}p < 0.001$, compared the groups with different dose of morphine. The data are expressed as means ± SEM, and analyzed using two-way ANOVA followed by Bonferroni *post hoc* test, $n = 7–9$.

2.7. Statistical analysis

CPP score represents the index of place preference for each rat, calculated by dividing the time spent in the drug-paired compartment by the time spent in both conditioning compartments. Results from Figs. 2–6 were analyzed with two-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* tests, results form Table were analyzed with one-way ANOVA followed by Newman–Keuls *post hoc* tests. Data were processed by the commercially available software Graph Pad Prism 5.0. The accepted level of statistical significance is $p < 0.05$.

3. Results

3.1. Effect of different doses of morphine on place preference conditioning

To establish a morphine-induced CPP model, the rats were randomly divided into morphine and saline groups. The pre-conditioning test showed that the animals spent almost an equal amount of time in the two lateral chambers (A: 334.5 ± 10.30 s, C: 317.3 ± 7.022 s), with no significant difference ($t = 1.385$, $p > 0.05$). After 4 days of alternative morphine (0.2 or 4 mg/kg, i.p.) and saline (2 ml/kg, i.p.) treatments or saline conditioning alone (controls), rats underwent the CPP expression test on day 5 (Fig. 2A).

Two-way ANOVA showed significant effects of the dose of morphine ($F_{2,42} = 9.102$, $p < 0.001$), the pretest vs. the test ($F_{1,42} = 6.831$, $p < 0.05$) and interaction of these two factors ($F_{2,42} = 4.284$,

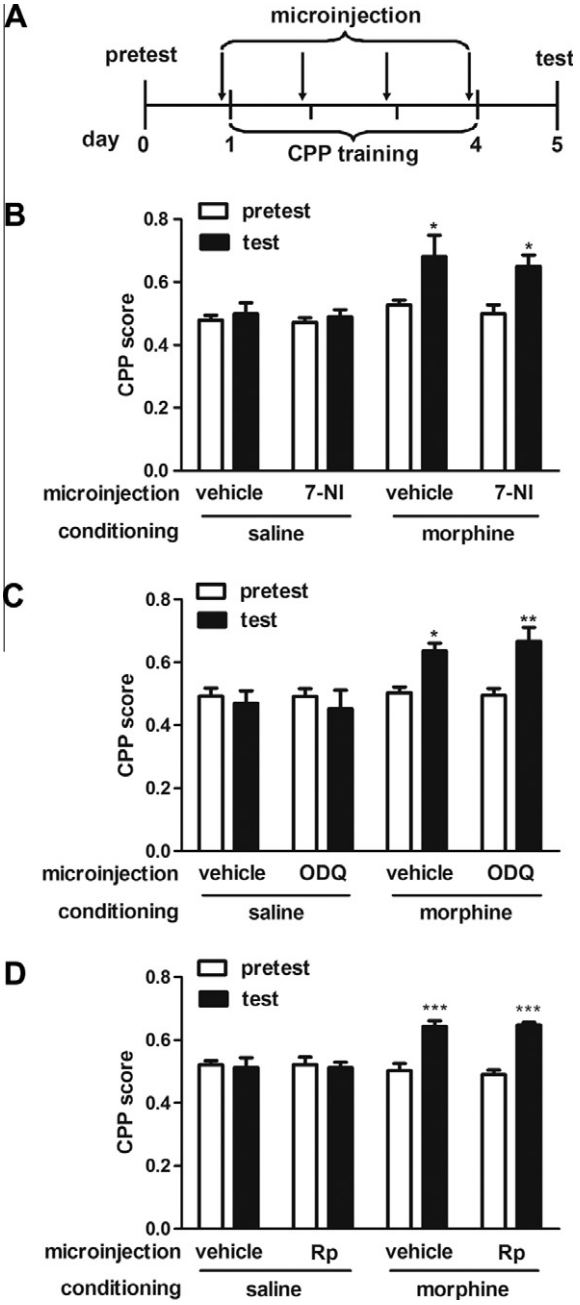

Fig. 3. Effect of 7-NI, ODQ or Rp-8-Br-PET-cGMPS pre-conditioning by microinjection into the CA1 on the acquisition of CPP in response to 4 mg/kg morphine. (A) Diagram outlining the behavioral procedures. (B) Microinjection of 7-NI or its vehicle into the CA1 had no effect on the acquisition of morphine CPP. (C) Microinjection of ODQ or its vehicle into the CA1 had no effect on the acquisition of morphine CPP. D. Microinjection of Rp-8-Br-PET-cGMPS or its vehicle into the CA1 had no effect on the acquisition of morphine CPP. The data are expressed as means ± SEM, $n = 6–10$. Blank and solid columns represent the data from the pre- and post-conditioning tests, respectively. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, pretest vs. test (two way ANOVA, Bonferroni *post hoc* test). Rp, Rp-8-Br-PET-cGMPS.

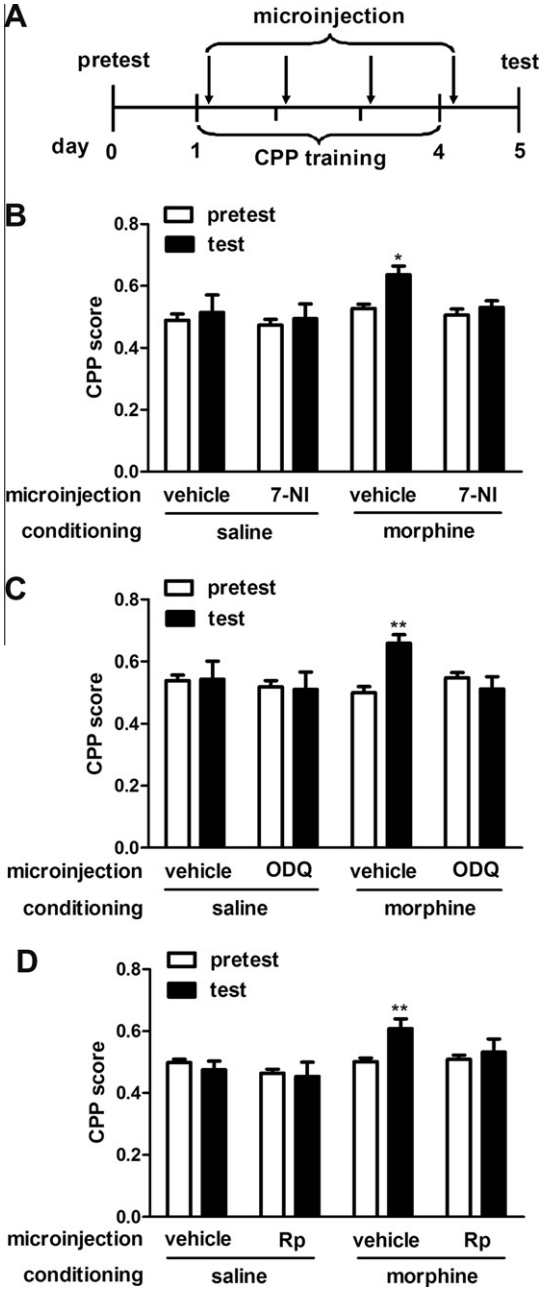

Fig. 4. Effect of 7-NI, ODQ or Rp-8-Br-PET-cGMPS post-conditioning by microinjection into the CA1 on the consolidation of CPP induced by 4 mg/kg morphine. (A) Diagram outlining the behavioral procedures. (B) Microinjection of 7-NI (but not vehicle) into the CA1 blocked the consolidation of morphine CPP. (C) Microinjection of ODQ (but not vehicle) into the CA1 blocked the consolidation of morphine CPP. D. Microinjection of Rp-8-Br-PET-cGMPS (but not vehicle) into the CA1 blocked the consolidation of morphine CPP. Data are expressed as means ± SEM, $n = 7–13$. Blank and solid columns represent the data from pre- and post-conditioning tests, respectively. $^{*}p < 0.05$ and $^{**}p < 0.01$, pretest vs. test (two way ANOVA, Bonferroni *post hoc* test). Rp, Rp-8-Br-PET-cGMPS.

$p < 0.05$). The followed Bonferroni *post hoc* tests showed that significant place preference was only observed in the group treated with morphine at dose of 4 mg/kg ($t = 4.016$, $p < 0.001$). Furthermore, there were significant effects of 0 mg/kg vs. 4 mg/kg and 0.2 mg/kg vs. 4 mg/kg ($p < 0.001$ respectively, two-way ANOVA followed by Bonferroni *post hoc* tests) (Fig. 2B).

The above results showed that 4 mg/kg morphine successfully induced CPP, whereas 0.2 mg/kg morphine failed to produce CPP. Therefore, in the following experiments 4 mg/kg can be used as an effective dose of morphine and 0.2 mg/kg can be used as an ineffective dose.

3.2. Intra-CA1 infusion of 7-NI, ODQ or Rp-8-Br-PET-cGMPS had no effect on the acquisition of morphine CPP

To measure the effect of NO, sGC and PKG on the acquisition of morphine-induced CPP, we performed three batch experiments. During the conditioning phase, rats conditioning by 4 mg/kg

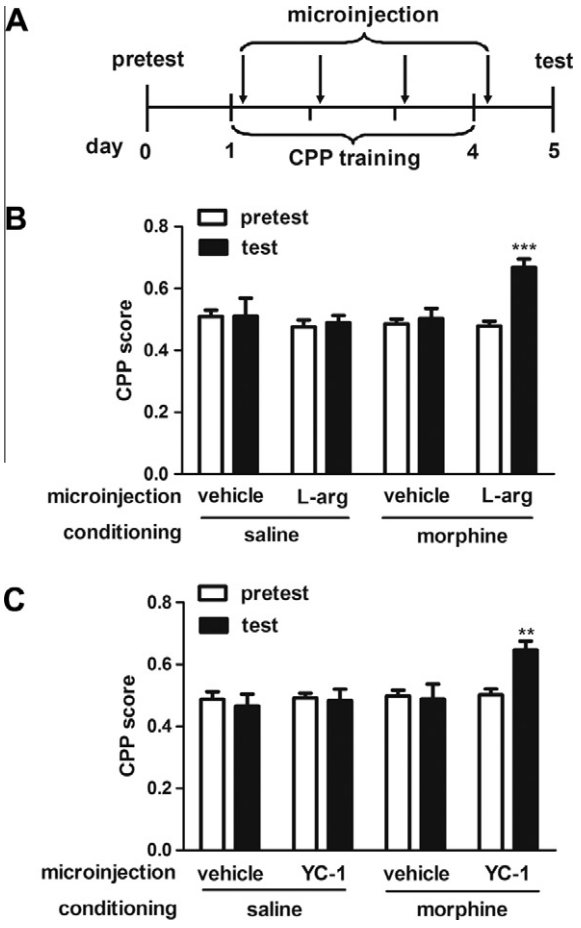

Fig. 5. Effect of L-arg or YC-1 post-conditioning by microinjection into the CA1 on the consolidation of CPP induced by 0.2 mg/kg morphine. (A) Diagram outlining the behavioral procedures. (B) Microinjection of L-arg (but not vehicle) into the CA1 with 0.2 mg/kg morphine successfully induced CPP. C. Microinjection of YC-1 (but not vehicle) into the CA1 with 0.2 mg/kg morphine successfully induced CPP. The data are expressed as the means ± SEM, $n = 8–9$. Blank and solid columns represent the data from the pre- and post-conditioning tests, respectively. $^{**}p < 0.01$ and $^{***}p < 0.001$, pretest vs. test (two way ANOVA, Bonferroni *post hoc* test). Rp, Rp-8-Br-PET-cGMPS.

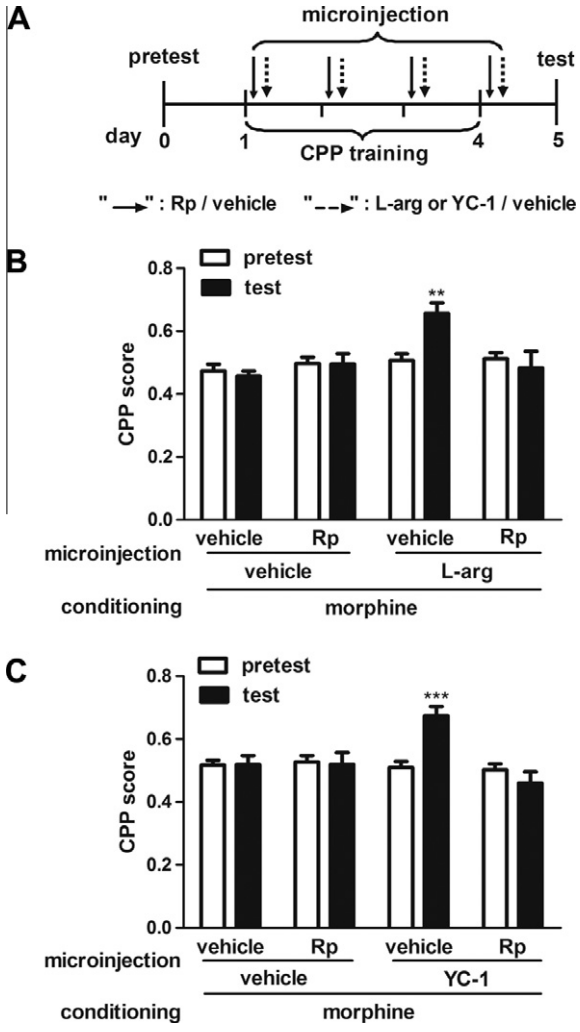

Fig. 6. Effect of Rp-8-Br-PET-cGMPS post-conditioning pre-microinjected into the CA1 on the consolidation of CPP induced by 0.2 mg/kg morphine plus L-arg or YC-1. (A) Diagram outlining the behavioral procedures. Solid arrows represent microinjection of Rp or vehicle, and dotted arrows represent microinjection of L-arg (YC-1) or vehicle. (B) Microinjection of Rp-8-Br-PET-cGMPS (but not vehicle) into the CA1 inhibited the consolidation of CPP induced by 0.2 mg/kg morphine plus L-arg. C. Microinjection of Rp-8-Br-PET-cGMPS (but not vehicle) into the CA1 inhibited the consolidation of CPP induced by 0.2 mg/kg morphine plus YC-1. The data are expressed as means ± SEM, $n = 8–10$. Blank and solid columns represent the data from the pre- and post-conditioning tests, respectively. $^{**}p < 0.01$ and $^{***}p < 0.001$, pretest vs. test (two way ANOVA, Bonferroni *post hoc* test). Rp, Rp-8-Br-PET-cGMPS.

morphine received a bilateral intra-CA1 infusion of the nNOS inhibitor 7-NI (1 μg/side/0.5 μl) (Ota et al., 2010), the sGC inhibitor ODQ (0.187 μg/side/0.5 μl) (Chianca, Lin, Dragon, & Talman, 2004), the PKG inhibitor Rp-8-Br-PET-cGMPS (1 μg/side/0.5 μl) (Ota, Pierre, Ploski, Queen, & Schafe, 2008; Ota et al., 2010), or the respective vehicle 20 min before each morphine conditioning, and the saline-conditioned control rats received the same intra-CA1 infusions as the morphine group. The CPP expression test was executed on day 5, and the CPP score and shuttle times were calculated (Fig. 3A).

In the batch 1, rats in the two morphine-conditioned groups were intra-CA1 injected of either the nNOS inhibitor 7-NI or 50% DMSO ACSF vehicle. Rats in the other two saline-conditioned control groups received the same intra-CA1 infusions as the morphine-conditioned groups. Two-way ANOVA displayed significant effects of the four treatments ($F_{3,54} = 7.539$, $p < 0.001$), the pretest vs. the test ($F_{1,54} = 14.76$, $p < 0.001$) and the interaction of these two factors ($F_{3,54} = 3.015$, $p < 0.05$). The followed Bonferroni *post hoc* tests showed 4 mg/kg morphine-induced CPP was observed in rats treated by intra-CA1 injection of either the nNOS inhibitor 7-NI ($t = 3.051$, $p < 0.05$, pretest vs. test) or 50%DMSO ACSF vehicle ($t = 3.137$, $p < 0.05$, pretest vs. test) (Fig. 3B).

In the batch 2, rats in the two morphine-conditioned groups were intra-CA1 injected of either the sGC inhibitor ODQ or 50% DMSO ACSF vehicle. Rats in the other two saline-conditioned control groups received the same intra-CA1 infusions as the morphine-conditioned groups. Two-way ANOVA displayed significant effects of the four treatments ($F_{3,52} = 5.623$, $p < 0.01$), the pretest vs. the test ($F_{1,52} = 6.240$, $p < 0.05$) and the interaction of these two factors ($F_{3,52} = 4.960$, $p < 0.01$). The followed Bonferroni *post hoc* tests showed 4 mg/kg morphine-induced CPP was observed in rats treated by intra-CA1 injection of either the sGC inhibitor ODQ ($t = 3.681$, $p < 0.01$, pretest vs. test) or 50%DMSO ACSF vehicle ($t = 2.842$, $p < 0.05$, pretest vs. test) (Fig. 3C).

In the batch 3, rats in the two morphine-conditioned groups were intra-CA1 injected of either the PKG inhibitor Rp-8-Br-PET-cGMPS or distilled water vehicle. Rats in the other two saline-conditioned control groups received the same intra-CA1 infusions as

the morphine-conditioned groups. Two-way ANOVA displayed significant effects of the four treatments ($F_{3,56} = 4.747$, $p < 0.01$), the pretest vs. the test ($F_{1,52} = 23.92$, $p < 0.001$) and the interaction of these two factors ($F_{3,56} = 10.08$, $p < 0.001$). The followed Bonferroni *post hoc* tests showed 4 mg/kg morphine-induced CPP was observed in rats treated by intra-CA1 injection of either the PKG inhibitor Rp-8-Br-PET-cGMPS ($t = 5.567$, $p < 0.001$, pretest vs. test) or distilled water vehicle ($t = 5.518$, $p < 0.001$, pretest vs. test) (Fig. 3D).

These data in Fig. 3 showed that intra-CA1 infusion of the nNOS inhibitor 7-NI, the sGC inhibitor ODQ or the PKG inhibitor Rp-8-Br-PET-cGMPS had no effect on the acquisition of morphine-induced CPP.

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

To measure the effect of NO, sGC and PKG on the consolidation of morphine-induced CPP, we performed three batch experiments. During the conditioning phase, rats conditioning by 4 mg/kg morphine received a bilateral intra-CA1 infusion of the nNOS inhibitor 7-NI (1 μg/side/0.5 μl), the sGC inhibitor ODQ (0.187 μg/side/0.5 μl), the PKG inhibitor Rp-8-Br-PET-cGMPS (1 μg/side/0.5 μl), or the respective vehicle immediately after each conditioning session, and the saline-conditioned control rats received the same intra-CA1 infusions as the morphine group. The CPP expression test was executed on day 5, and the CPP score and shuttle times were calculated (Fig. 4A).

In the batch 1, rats in the two morphine-conditioned groups were intra-CA1 injected of either the nNOS inhibitor 7-NI or 50% DMSO ACSF vehicle. Rats in the other two saline-conditioned control groups received the same intra-CA1 infusions as the morphine-conditioned groups. Two-way ANOVA displayed that significant effects of the four treatments ($F_{3,52} = 3.996$, $p < 0.05$) and the pretest vs. the test ($F_{1,52} = 4.290$, $p < 0.05$), but the interaction of these two factors was not significant ($F_{1,52} = 1.017$, $p > 0.05$). The followed Bonferroni *post hoc* test showed that there was no significant increase in the CPP score in the expression test of morphine-conditioned rats intra-CA1 infused with the nNOS inhibitor 7-NI ($t = 0.5907$, $p > 0.05$, pretest vs. test), whereas a significant increase in the CPP score was found in the expression test of rats intra-CA1 infused with 50% DMSO ACSF vehicle ($t = 2.600$, $p < 0.05$) (Fig. 4B).

In the batch 2, rats in the two morphine-conditioned groups were intra-CA1 injected of either the sGC inhibitor ODQ or 50% DMSO ACSF vehicle. Rats in the other two saline-conditioned control groups received the same intra-CA1 infusions as the morphine-conditioned groups. Though two-way ANOVA did not reveal significant effects of the four treatments ($F_{3,52} = 1.806$, $p > 0.05$) and the pretest vs. the test ($F_{1,52} = 3.366$, $p > 0.05$), there was an interaction effect of these two factors ($F_{3,52} = 3.838$, $p < 0.05$). The followed Bonferroni *post hoc* test showed that there was no significant increase in the CPP score in the expression test of morphine-conditioned rats intra-CA1 infused with the sGC inhibitor ODQ ($t = 0.02995$, $p > 0.05$, pretest vs. test), whereas a significant increase in the CPP score was found in the expression test of rats intra-CA1 infused with 50% DMSO ACSF vehicle ($t = 3.789$, $p < 0.01$, pretest vs. test) (Fig. 4C).

In the batch 3, rats in the two morphine-conditioned groups were intra-CA1 injected of either the PKG inhibitor Rp-8-Br-PET-cGMPS or distilled water vehicle. Rats in the other two saline-conditioned control groups received the same intra-CA1 infusions as the morphine-conditioned groups. Two-way ANOVA revealed that there was a significant effect of the four treatments ($F_{3,78} = 4.938$, $p < 0.01$). Though the pretest vs. the test did not showed a significant effect ($F_{1,78} = 1.638$, $p > 0.05$), there was an interaction effect of these two factors ($F_{3,78} = 2.802$, $p < 0.05$). The followed Bonferroni *post hoc* test showed that there was no significant increase in the CPP score in the expression test of morphine-conditioned rats intra-CA1 infused with the PKG inhibitor Rp-8-Br-PET-cGMPS ($t = 0.5847$, $p > 0.05$, pretest vs. test), whereas a significant increase in the CPP score was found in the expression test of rats intra-CA1 infused with distilled water vehicle ($t = 3.148$, $p < 0.01$, pretest vs. test) (Fig. 4D).

These data in Fig. 4 showed that the intra-CA1 infusion of the nNOS inhibitor 7-NI, the sGC inhibitor ODQ or the PKG inhibitor Rp-8-Br-PET-cGMPS blocked the consolidation of morphine CPP.

3.4. Intra-CA1 infusion of L-arg or YC-1 led to CPP induction by an ineffective dose of morphine (0.2 mg/kg)

To further measure the effect of NO and sGC on the consolidation of morphine-induced CPP, we performed two batch experiments. During the conditioning phase, rats conditioning by 0.2 mg/kg morphine received a bilateral intra-CA1 infusion of the NOS substrate L-arg (0.025 μg/side/0.5 μl) (Gholami, Haeri-Rohani, Sahraie, & Zarrindast, 2002), the sGC activator YC-1 (0.152 μg/side/0.5 μl) (Chan, Chan, & Chang, 2004), or the corresponding vehicle immediately after each conditioning session, and the saline-conditioned control rats received the same intra-CA1 infusions as the morphine group. The CPP expression test was executed on day 5, and the CPP score and the shuttle times were calculated (Fig. 5A).

In the batch 1, rats in the two morphine-conditioned groups were intra-CA1 injected of either the NOS substrate L-arg or ACSF vehicle. Rats in the other two saline-conditioned control groups received the same intra-CA1 infusions as the morphine-conditioned groups. Two-way ANOVA displayed significant effects of the four treatments ($F_{3,60} = 3.945$, $p < 0.05$), the pretest vs. the test ($F_{1,60} = 7.106$, $p < 0.01$) and the interaction of these two factors ($F_{3,60} = 4.830$, $p < 0.01$). The followed Bonferroni *post hoc* test showed that there was no significant increases in the CPP score in the morphine-conditioned rats intra-CA1 infused with ACSF vehicle ($t = 0.4118$, $p > 0.05$, pretest vs. test), whereas a significant increase in the CPP score was found in the morphine-conditioned rats intra-CA1 infused with the NOS substrate L-arg ($t = 4.699$, $p < 0.001$, pretest vs. test) (Fig. 5B).

In the batch 2, rats in the two morphine-conditioned groups were intra-CA1 injected of either the sGC activator YC-1 or ACSF vehicle. Rats in the other two saline-conditioned control groups received the same intra-CA1 infusions as the morphine-conditioned groups. Two-way ANOVA revealed that there was a significant effect of the four treatments ($F_{3,60} = 4.038$, $p < 0.05$). Though the pretest vs. the test did not showed a significant effect ($F_{1,60} = 1.441$, $p > 0.05$), there was an interaction effect of these two factors ($F_{3,60} = 3.191$, $p < 0.05$). The followed Bonferroni *post hoc* test showed that there was no significant increase in the CPP score in the morphine-conditioned rats intra-CA1 infused with ACSF vehicle ($t = 0.2239$, $p > 0.05$, pretest vs. test), whereas a significant increase in the CPP score was observed in the morphine-conditioned rats intra-CA1 infused with the sGC activator YC-1 ($t = 3.225$, $p < 0.01$, pretest vs. test) (Fig. 5C).

These data in Fig. 5 showed that when the NOS substrate L-arg or the sGC activator YC-1 was micro-infused in the CA1, the ineffective dose of morphine successfully induced CPP.

3.5. Pre-microinjection of Rp-8-Br-PET-cGMPS blocked the consolidation of CPP induced by 0.2 mg/kg morphine plus L-arg or YC-1

To measure whether NO and sGC involved in the consolidation of morphine-induced CPP through activation of PKG, we performed two batch experiments. During the conditioning phase, all rats were conditioned by 0.2 mg/kg morphine. Immediately after each conditioning session, the rats primarily received intra-CA1

injection of either the PKG inhibitor Rp-8-Br-PET-cGMPS (1 μg/side/0.5 μl) or distilled water vehicle and, 20 min later, were intra-CA1 infused of the NOS substrate L-arg (0.025 μg/side/0.5 μl), the sGC activator YC-1 (0.152 μg/side/0.5 μl), or the respective vehicle. The CPP expression test was executed on day 5, and the CPP score and shuttle times were calculated (Fig. 6A).

In the batch 1, immediately after each conditioning session, four groups of rats were intra-CA1 infused the NOS substrate L-arg or ACSF vehicle 20 min after pre-microinjection of either the PKG inhibitor Rp-8-Br-PET-cGMPS or distilled water vehicle. Two-way ANOVA revealed that there was a significant effect of the four treatments ($F_{3,62} = 6.074$, $p < 0.01$). Though the pretest vs. the test did not showed a significant effect ($F_{1,62} = 1.556$, $p > 0.05$), there was an interaction effect of these two factors ($F_{3,62} = 4.190$, $p < 0.01$). The followed Bonferroni *post hoc* test showed that only the group intra-CA1 receiving distilled water vehicle plus the NOS substrate L-arg exhibited a significant preference for the morphine-paired compartments ($t = 3.701$, $p < 0.01$, pretest vs. test). No significant differences were observed in the groups intra-CA1 receiving distilled water vehicle plus ACSF vehicle ($t = 0.4062$, $p > 0.05$, pretest vs. test), intra-CA1 receiving the PKG inhibitor Rp-8-Br-PET-cGMPS plus ACSF vehicle ($t = 0.04399$, $p > 0.05$, pretest vs. test) and intra-CA1 receiving the PKG inhibitor Rp-8-Br-PET-cGMPS plus the NOS substrate L-arg ($t = 0.04399$, $p > 0.05$, pretest vs. test) (Fig. 6B).

In the batch 2, immediately after each conditioning session, four groups of rats were intra-CA1 infused the sGC activator YC-1 or ACSF vehicle 20 min after pre-microinjection of either the PKG inhibitor Rp-8-Br-PET-cGMPS or distilled water vehicle. Two-way ANOVA revealed that there was a significant effect of the four treatments ($F_{3,60} = 6.375$, $p < 0.001$). Though the pretest vs. the test did not showed a significant effect ($F_{1,60} = 2.358$, $p > 0.05$), there was an interaction effect of these two factors ($F_{3,60} = 6.450$, $p < 0.001$). The followed Bonferroni *post hoc* test showed that only the group intra-CA1 receiving distilled water vehicle plus the sGC activator YC-1 exhibited a significant preference for the morphine-paired compartments ($t = 4.691$, $p < 0.001$, pretest vs. test). No significant differences were observed in the groups intra-CA1 receiving distilled water vehicle plus ACSF vehicle ($t = 0.05374$, $p > 0.05$, pretest vs. test), intra-CA1 receiving the PKG inhibitor Rp-8-Br-PET-cGMPS plus ACSF vehicle ($t = 0.1803$, $p > 0.05$, pretest vs. test) and intra-CA1 receiving the PKG inhibitor Rp-8-Br-PET-cGMPS plus the sGC activator YC-1 ($t = 1.075$, $p > 0.05$, pretest vs. test) (Fig. 6C).

These data in Fig. 6 showed that intra-CA1 pre-infusion of the PKG inhibitor Rp-8-Br-PET-cGMPS disrupted the CPP induced by 0.2 mg/kg morphine plus the NOS substrate L-arg or the sGC activator YC-1.

4. Discussion

To our knowledge, no previous studies have reported the involvement of the NO/sGC/PKG signaling pathway in the consolidation of morphine reward memory. In this study, we demonstrated for the first time that intra-CA1 microinjection of the nNOS inhibitor 7-NI immediately after conditioning blocked the consolidation of morphine CPP and that 7-NI alone could not elicit a preference or aversion. All of these findings implied that NO in the CA1 is essential for the consolidation of morphine-related reinforcing effects.

A growing body of evidence shows that NO-dependent upregulation of sGC and PKG is involved in synaptic plasticity and enhanced memory formation. For example, it is known that long-term potentiation (LTP) and long-term depression (LTD), the cellular mechanisms of learning and memory, require the activation of sGC and PKG in the hippocampus (Arancio, Kandel, & Hawkins, 1995; Bon & Garthwaite, 2003; Boulton, Southam, & Garthwaite, 1995; Chetkovich, Klann, & Sweatt, 1993; Haley, Wilcox, & Chapman, 1992; Stanton et al., 2003; Zhuo, Kandel, & Hawkins, 1994). More importantly, several behavioral and pharmacological studies (Domek-Lopacinska & Strosznajder, 2008; Wang et al., 2008) point to the existence of a causal link between NO and the activity of PKG. To investigate the molecular mechanism by which NO is involved in the consolidation of morphine CPP, we observed the effect of intra-CA1 infusion of the sGC inhibitor ODQ or the PKG inhibitor Rp-8-Br-PET-cGMPS on the consolidation of morphine CPP. The morphine CPP was blocked by administering ODQ or Rp-8-Br-PET-cGMPS into the CA1 after conditioning and ODQ or Rp-8-Br-PET-cGMPS alone did not produce a preference or aversion. These results suggested that sGC and PKG also played an important role in the consolidation of morphine CPP. Our results are similar to that the inhibition of the NO/sGC/PKG signaling pathway hinders the consolidation of long-term object recognition memory and the inhibitory avoidance memory (Furini et al., 2010; Zinn et al., 2009). However, our findings are inconsistent with the observation by Kleppisch et al that mice with a hippocampus-specific deletion of PKG show normal performance in a discriminatory water maze and have intact contextual fear conditioning (Kleppisch et al., 2003). These differences may result from some variations in the experimental technology used to knock down the PKG level in the hippocampus.

Additionally, our results further showed that administration of L-arg or YC-1 in the CA1 after conditioning produced significant CPP with the ineffective morphine dose of 0.2 mg/kg, suggesting that the activation of NO and sGC was required for the consolidation of morphine-induced reward memory. This finding is consistent with previous studies that the injection of L-arg into the lateral brain ventricle immediately following the training trail improves the consolidation of passive avoidance learning (Telegdy & Kokavszky, 1997). Our further experiments revealed that pre-microinjection of Rp-8-Br-PET-cGMPS in the CA1 impaired the consolidation of morphine CPP induced by L-arg or YC-1 plus the ineffective morphine dose of 0.2 mg/kg, indicating that the involvement of NO in morphine reward memory occurred through the activation of sGC and PKG. These results are supported by a previous study that the enhancement of learning behavior by YC-1 was antagonized by intracerebroventricular injection of the PKG inhibitor Rp-8-Br-PET-cGMPS (Chien, Liang, & Fu, 2008a). The above findings suggested that the sGC/PKG pathway is the downstream effector pathway of NO in this context, i.e., that NO is involved in the consolidation of morphine-related memory through the activation of the NO/sGC/PKG signaling pathway.

Importantly, intra-CA1 administration of inhibitors of the NO/sGC/PKG signaling pathway significantly blocked CPP only when given immediately after conditioning, suggesting that protein kinases (such as PKG) are transiently activated during a specific time window to allow for memory consolidation. These findings are analogous to those by Cervo, Mukherjee, Bertaglia, and Samanin (1997) that the inhibitors of PKA or PKC only significantly reduced the time spent by rats in the cocaine compartment when given immediately after each conditioning session, with no effect observed for administration before cocaine during the conditioning phase (Cervo et al., 1997). These results agree with the previous finding that the optimal time to interfere with memory consolidation is the immediate post-training period (Barondes & Cohen, 1968; Bourtchouladze et al., 1998; Cohen & Barondes, 1968).

Memory formation can be experimentally divided into the two stages of acquisition and consolidation, so we also explored the effect of the NO/sGC/PKG signaling pathway on the acquisition of morphine CPP. Our data revealed that pre-conditioning inhibition of NO, sGC and PKG did not affect the morphine CPP score,

indicating that this signaling pathway was not involved in the acquisition of morphine-related memory. This was consistent with the previous finding that pre-conditioning administration of the NOS inhibitor L-NAME in the hippocampal CA1 area has no effect on the acquisition of morphine CPP in rats (Karami et al., 2002). The NO/sGC/PKG signaling pathway induces the activation of the neuronal transcription machinery (Lu, Kandel, & Hawkins, 1999) as well as the assembly of the spliceosome (Wang et al., 1999), which explains why the critical role of this signaling pathway in the consolidation requires gene expression and protein synthesis (Davis & Squire, 1984), but not the acquisition of morphine-induced reward memory.

In the control groups, intra-CA1 microinjection of 7-NI, ODQ and Rp-8-Br-PET-cGMPS had no effect on the acquisition and consolidation of saline-induced CPP (Figs. 3 and 4). Similarly, intra-CA1 micro-infusion of L-arg and YC-1 did not impact the consolidation of saline-induced CPP (Fig. 5). These results suggest that in our experiments the reagents themselves could not result in rats' preference or aversion response to the A or the C chamber of CPP equipment. Data in the Supplemental table showed that intra-CA1 injection of these reagents did not influence rats' locomotor activity. Therefore, intra-CA1 injection of these reagents could blocked or improved the consolidation of morphine-induced CPP only because they had changed the activity of the NO/sGC/PKG signaling pathway.

It is widely accepted that the hippocampal CA1 region receives a dopaminergic input originating predominantly in the VTA (Scatton et al., 1980) and that all five types of dopamine receptors (Meador-Woodruff, 1994) are involved in reward-related memory (Rezayof, Zarrindast, Sahraei, & Haeri-Rohani, 2003). Furthermore, the dopaminergic actions of cocaine CPP may be mediated via the activation of the NO/sGC/PKG signaling pathway (Kim & Park, 1995). NO is a retrograde transmitter that signals presynaptic neurons, causing an increase in the release of dopamine (Pudiak & Bozarth, 1993). Therefore, the NO/sGC/PKG signaling pathway might be a neuronal messenger mediating the release of dopamine in the CA1, which could explain the finding that inhibition of this signaling pathway impaired the consolidation of morphine-related memory.

The hippocampus is well-known as a pivotal region involved in the reward of drug abuse. In our previous work, we demonstrated that the level of NR2B-containing *N*-methyl-D-aspartate receptor (NMDAR) in the hippocampus elevated in morphine CPP rats, while inhibition of NR2B-containing NMDAR in the dorsal hippocampus blocked morphine CPP (Ma et al., 2006). NOS is tethered the NMDAR by the scaffolding protein postsynaptic density-95 (PSD-95). In this complex, NMDAR-mediated Ca^{2+} influxes regulate nNOS activity and NO production (Bredt & Snyder, 1989; Christopherson, Hillier, Lim, & Bredt, 1999; Garthwaite, Garthwaite, Palmer, & Moncada, 1989). Gerald A. Rameau (2007) demonstrated that NMDAR can affect the activity of NOS by phosphorylation of NOS at S1412 and S847. In addition, the NMDAR antagonist MK801 blocked the increase in cGMP induced by glutamate (Rameau et al., 2007). Therefore, it is reasonable to speculate that the NO/sGC/PKG signaling pathway may be the downstream cascade of NMDAR response to morphine-associated memory.

Although the downstream pathway to NO signaling pathway during memory processing is not known, recent findings showed that pharmacological activation of sGC during two-way shuttle-avoidance training increased the expression of ERK1/2 and CREB, which has been widely implicated in long-term memory (Chien, Liang, & Fu, 2008b; Ota et al., 2008). In accordance with this study, Ota et al. (2008) found that YC-1-induced enhancement of LTP in the lateral amygdala was reversed by concurrent application of the MEK inhibitor U0126 (Ota et al., 2008). These results suggest that the NO/sGC/PKG signaling pathway participate in memory via the activation of ERK-mediated of CREB-regulated transcription.

In conclusion, our results demonstrate that blockade of the NO/sGC/PKG signaling pathway in the CA1 specifically disrupts the consolidation of morphine-induced CPP. This furthers our understanding of the molecular mechanisms of morphine-associated memory and provides a new potential pathway for pharmacotherapy of opiate abuse.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nlm.2012.07.005.

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