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Behavioral Neuroscience

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The NO/sGC/PKG Signaling Pathway in the NAc Shell Is Necessary for the Acquisition of Morphine-Induced Place Preference

Fang Shen, Na Wang, Chong Qi, and Yi-Jing Li ●●●●

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There is evidence that the nitric oxide (NO)/soluble guanylyl cyclase (sGC)/cGMP-dependent protein kinase (PKG) signaling pathway in the basal lateral amygdala and hippocampus plays a key role in memory processing, but it is not known if this NO signaling pathway in the nucleus accumbens (Gomes et al., 2006), a known pivotal region in reward memory, is essential for drug-associated reward memory. We therefore investigated the effect of the NO/sGC/PKG signaling pathway in the nucleus accumbens (NAc) on morphine-induced conditioned place preference (CPP). Results showed that a preconditioning microinjection of the NO synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) into the NAc shell, but not into the core, significantly blocked the acquisition of morphine CPP. The blockage effect of L-NAME on the acquisition of CPP was imitated by the neuronal NOS inhibitor 7-nitroindazole, 3-bromo-, sodium salt (7-NI), the sGC inhibitor 1H-[1,2,4] oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ), and the PKG inhibitor Rp-8Br-PET-cGMPS. The 7-NI- or ODQ-induced effect was reversed by premicroinjection of the sGC activator YC-1 or the PKG activator 8-Br-cGMP in the NAc shell. However, microinfusion of 7-NI, ODQ, or Rp-8Br-PET-cGMPS into the NAc shell or the core had no effect on the expression of morphine CPP. These findings indicate that the NO/sGC/PKG signaling pathway in the NAc shell is critical for the acquisition of morphine-induced place preference, whereas the same signaling pathway in the NAc shell or core is not involved in the retrieval of morphine-induced place preference.

Keywords: nitric oxide signaling pathway, morphine, conditioned place preference, nucleus accumbens

Opiate addiction is a chronic relapsing disorder characterized by compulsive drug seeking/taking and a high risk of relapse even after long periods of abstinence. Relapse is often precipitated by drug-associated cues (De Vries & Shippenberg, 2002). Current preclinical studies have shown that changes occur in the nervous system during the development of addiction at molecular, cellular, and circuit levels. These changes are similar to those that occur during physiological learning and thus may play an essential role in the development and persistence of drug addiction. Research on the learning and memory processes induced by addictive drugs is therefore important for understanding the development and persistence of addiction and for finding ways to disrupt drug-associated memory.

The nucleus accumbens (NAc; Gomes et al., 2006), a major target and central component of the mesolimbic reward system, receives a dopaminergic projection from the ventral tegmental area and sends projections to the limbic system and the orbitofrontal cortex. This is a common neuronal pathway for physiological learning and memory and for drug addiction (Lisman & Grace, 2005). The core and shell share heterogeneous structures with distinct immunohistochemical characteristics, neuronal morphologies, and afferent and efferent connections (Brog, Salyapongse, Deutch, & Zahm, 1993; Heimer, Zahm, Churchill, Kalivas, & Wohltmann, 1991; Jongen-Rêlo, Voorn, & Groenewegen, 1994; Meredith, Agolia, Arts, Groenewegen, & Zahm, 1992). There are also differences between the NAc core and shell in modulating opiate- and psychostimulant drug−induced conditioned behavioral responses (Di Ciano & Everitt, 2001; Parkinson, Willoughby, Robbins, & Everitt, 2000). The conditioned association between environmental stimuli and the effects of an addictive drug is known to play an important role in the development of drug-induced reward memory and in relapse of drug use, even after long periods of abstinence. Pharmacotherapeutic severing or weakening of this association might improve the treatment of drug addiction.

Nitric oxide (NO) is a labile and highly diffusible gas synthesized from L-arginine by NO synthase (NOS). NO is primarily mediated by soluble guanylyl cyclase (sGC; Arnold, Mittal, Katsuki, & Murad, 1977; Marsault & Frelin, 1992). The binding of NO to the heme group of sGC increases the activity of this enzyme several 100-fold to produce a second messenger, cGMP (Murad, 2004), which then stimulates the cGMP-dependent protein kinase (PKG). In addition to activating sGC, NO can lead to S-nitrosylation of many target proteins (Nakamura et al., 2013;

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Selvakumar et al., 2013). cGMP can also be increased without NO involvement by activation of membrane-bound particulate guanylyl cyclases and by natriuretic peptides (Kuhn, 2004). Several studies have shown that NO is involved in the reward memory that is induced by addictive drugs. For example, administration of the neuronal NOS (nNOS) inhibitor 7-nitroindazole, 3-bromo-, sodium salt (7-NI; 25 mg/kg, intraperitoneal) blocked the acquisition of the conditioned place preference (CPP) that is induced by nicotine (Martin & Itzhak, 2000), alcohol (Itzhak & Martin, 2000), and cocaine (Itzhak, Martin, Black, & Huang, 1998) in mice. Additionally, mice without the nNOS gene were resistant to cocaine-induced CPP (Itzhak et al., 1998). Similarly, 7-NI (12.5–50 mg/kg, intraperitoneal) suppressed the acquisition, expression, and reinstatement of D-methamphetamine−induced CPP in rats (Li, Ren, & Zheng, 2002; Li, Yin, Shi, Lin, & Zheng, 2002). With regard to the role of NO in opiate-induced CPP, systemic injection of 25 mg/kg of 7-NI or 20 mg/kg of the nonselective NOS inhibitor L-N^G-nitroarginine blocked the acquisition of morphine CPP in male mice and in rats (Kivastik, Rutkauskaite, & Zharkovsky, 1996; Manzanedo, Aguilar, Rodriguez-Arias, Navarro, & Minarro, 2004). Earlier studies showed that intra-NAc injection with the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) blocked the acquisition of morphine CPP, and that the NO donor L-arginine with an ineffective dose of morphine (0.5 mg/kg) elicited significant CPP (Gholami, Haeri-Rohani, Sahraie, & Zarrindast, 2002). Few studies of NO in the NAc have examined its downstream molecular pathway. We recently reported that blockage of the NO/sGC/PKG signaling pathway in the CA1 region of the hippocampus hindered consolidation of morphine-induced CPP and that NO was most likely involved in the reward memory that is induced by morphine in the sGC and PKG (Shen, Li, Shou, & Cui, 2012). However, we do not know whether the effects of this signaling pathway on morphine-induced reward memory occur in the NAc shell and/or the NAc core. The present study was designed to determine the role of the NO/sGC/PKG signaling pathway in the NAc shell and core in the acquisition and expression of morphine CPP.

AQ: 7

Method

Subjects

We used male Sprague–Dawley rats weighing 220–250 g at the time of surgery. The rats were obtained from the Laboratory Animal Center of the Peking University Health Science Center, and housed four per cage in a 12:12 hr light:dark cycle (lights on at 7:00 p.m.) with food and water available at all times. Room temperature was maintained at 23° (*SD* = 2° C), and relative humidity was maintained at 45%–55%. Rats were handled for 5 days prior to the experiments. The behavioral experiments were conducted during the dark cycle. All experimental procedures were performed in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local committee for animal use and protection. Every effort was made to minimize animal suffering and to reduce the number of animals used.

AQ: 8

Drugs

Morphine hydrochloride was purchased from the First Pharmaceutical Factory of Qinghai, China, and dissolved in sterile saline to its final concentrations. L-NAME; 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 8-Br-cGMP were obtained from Sigma-Aldrich (St. Louis, MO). L-NAME was dissolved in 0.9% isotonic saline to a stock concentration of 1 μg/μl. Rp-8-Br-PET-cGMPS and 8-Br-cGMP were dissolved in distilled water for a stock concentration of either 2 μg/μl (Rp-8-Br-PET-cGMPS) or 20 μg/μl (8-Br-cGMP). 7-NI and ODQ were dissolved in 100% dimethyl sulfoxide (DMSO) to stock concentrations of 4 μg/μl and 0.748 μg/μl, which were then diluted 1:1 in artificial cerebrospinal fluid (ACSF) prior to infusion. YC-1 was dissolved in ACSF for a stock concentration of 0.304 μg/μl. The composition of ACSF was: 115 mM of sodium chloride, 3.3 mM of potassium chloride, 1 mM of magnesium sulfate, 2 mM of calcium chloride, 25.5 mM of sodium bicarbonate, 1.2 mM of sodium phosphate, and 10 mM of glucose (Ota, Monsey, Wu, & Schafe, 2010).

AQ: 9

AQ: 10

Place Preference Apparatus

Conditioning was conducted in black rectangular PVC boxes (795 × 230 × 250 mm^3) containing three chambers separated by guillotine doors (Shi et al., 2004). The two large black conditioning chambers (A and C; 280 × 220 × 225 mm^3) were separated by a small gray center choice chamber (B; 135 × 220 × 225 mm^3). Chamber A had four light-emitting diodes (LEDs), forming a square on the wall, and a stainless steel mesh floor (225 × 225 mm^2); Chamber C had four 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, spaced 47.5 mm from each other, were placed across the chambers. A computer interface recorded infrared-beam crossings to calculate the time that a rat spent in each chamber.

Cannula Implantation 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 the flat skull position. Stainless steel guide cannulas (outer diameter, 0.67 mm) were bilaterally implanted 1.5 mm above the NAc shell or the core. The NAc shell coordinates (Paxinos & Watson, 2005) were: anteroposterior, +1.6 mm; mediolateral, ±0.9 mm; and dorsoventral, −6.5 mm. The coordinates for the NAc core were: anteroposterior, +1.6 mm; mediolateral, ±2.0 mm; and dorsoventral, −6.0 mm. The cannulas were fixed to screws in the skull with dental cement. Internal cannulas were replaced with dummy cannulas, which were 0.5 mm longer than the guide 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 intranuclear 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

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syringes using PE 20 tubing. The tubing was backfilled with saline, with a small air bubble separating the saline from the drug solution. Drugs were administered with an infusion pump at a rate 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. The dummy cannula was then replaced, and the rat was returned to its home cage.

Conditioned Place Preference

Preconditioning 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. Data from preconditioning 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 (approximately 5%) were excluded from the experiments.

Conditioning phase. Animals were allowed two training sessions per day (at 8:30 a.m. and 3:30 p.m.) for 4 days (Days 1–4). Rats received morphine (4 mg/kg, intraperitoneal) and saline in one lateral chamber before being confined in the other lateral chamber for 45 min. Animals in control groups received their saline injections before training sessions in both lateral chambers. To counterbalance the treatment of the morphine-conditioned groups, half of the animals received morphine training in Compartment A and saline training in Compartment C, and half received morphine training in Compartment C and saline training in Compartment A. Further, half of the rats were conditioned with morphine in the morning session and saline in the afternoon, and the timing of treatment was reversed for the other half.

AQ: 11

Postconditioning phase. On Day 5, each animal was placed in the center choice chamber with the guillotine door removed to allow access to the entire apparatus for 15 min, and the time it 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 CPP tests was estimated by counting the total number of crossings between any two adjacent compartments.

Histological Verification

Each rat was killed by decapitation and its brain removed after the behavioral trials were completed. The brains were cut on a cryostat into 30-μm-thick sections and mounted on glass slides coated with gelatin to allow histological examination of the placement of cannulas and needles in the NAc shell and core. Cannula placements were assessed by Nissl staining using light microscopy. Figure 1 shows the location of representative cannula tips in the NAc shell and core. Only the data from rats that received histologically verified injections were included for analyses.

F1

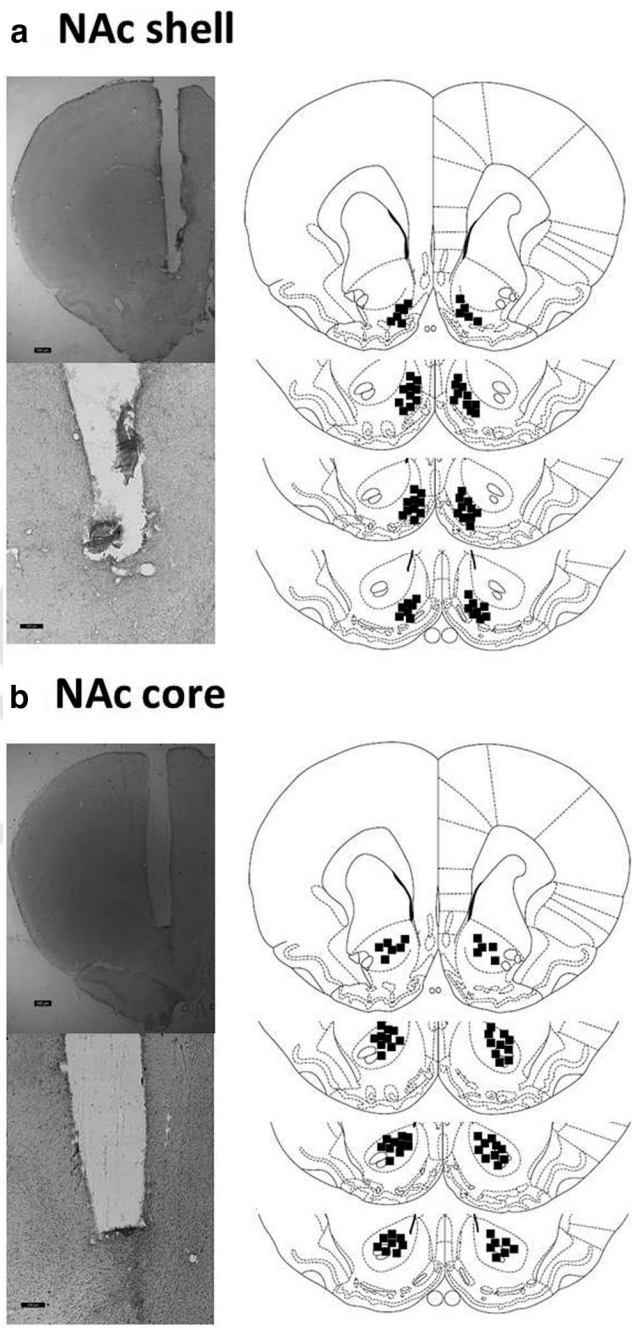

Figure 1. Representative cannula placements and microinjection sites in the nucleus accumbens (NAc). The NAc shell (a) and the core (b) of rats with conditioned place preference, microinjected with the reagents and their respective vehicles. The left charts are a representative photomicrograph of the infusion site in the NAc; the right are the distribution of microinjection sites in the NAc (gray circle).

Statistical Analysis

The 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. The results shown in Figure 2–5 were analyzed with two-way analysis of variance (Chen et al., 1999), followed with Bonferroni post hoc tests. The results shown in the Table were analyzed with a one-way analysis of variance (ANOVA), followed with

F2-5

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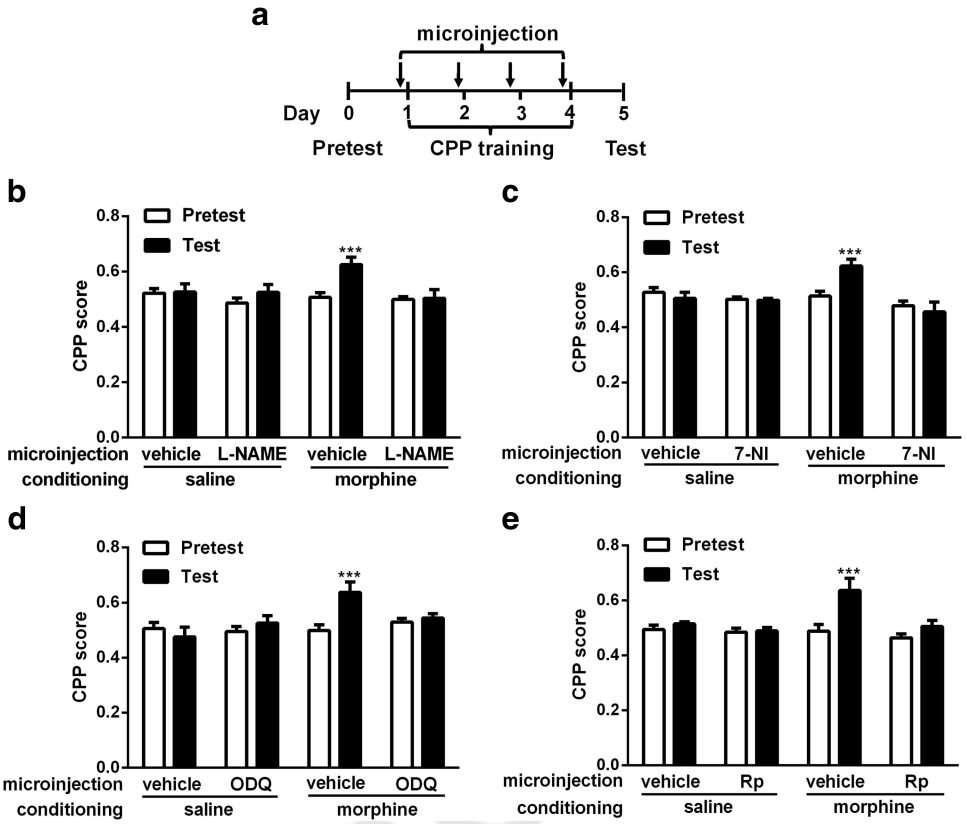

Figure 2. Effects of preconditioning microinjections of L-NAME, 7-NI, ODQ or Rp-8-Br-PET-cGMPS into the nucleus accumbens (NAc) shell on the acquisition of morphine CPP. Diagram outlining the behavioral procedures (a). Microinjection of L-NAME (b), 7-NI (c), ODQ (d), or Rp-8-Br-PET-cGMPS (e) into the NAc shell blocked the acquisition of morphine CPP. Data are mean (*SEM*) (n = 7–12). Blank and solid columns represent the data from the pre- and postconditioning tests, respectively. L-NAME = N^{ω}-nitro-L-arginine methyl ester; 7-NI = 7-nitroindazole, 3-bromo-, sodium salt; ODQ = 1H-[1,2,4] oxadiazolo-[4,3-a]quinoxalin-1-one; Rp = Rp-8-Br-PET-cGMPS; CPP = conditioned place preference. *** $p < .001$, pretest compared with test (two-way analysis of variance, Bonferroni post hoc test).

Newman–Keuls post hoc tests. Data were processed using Graph Pad Prism, Version 5.0. Statistical significance was set at $p < .05$.

Results

The Effect of L-NAME, 7-NI, ODQ, and Rp-8-Br-PET-cGMPS Microinjection Into the NAc Shell on the Acquisition of Morphine CPP

We performed four batch experiments to measure the effect of the NO/sGC/PKG signaling pathway in the NAc shell on the acquisition of morphine CPP. During the conditioning phase, rats that were conditioned with 4 mg/kg of morphine received a bilateral intra-NAc shell infusion of the NOS inhibitor L-NAME (0.5 μg/side/0.5 μl) (Gholami et al., 2002), 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), or the respective vehicle. The infusions were given 20 min before each morphine conditioning. The saline-conditioned control rats received the same intra-NAc shell infusions as the morphine groups. The CPP test was run on Day 5, and the CPP score with shuttle times were calculated (see Figure 2a).

In Batch 1, rats in the two morphine-conditioned groups were injected in the NAc shell with either the NOS inhibitor L-NAME or a 0.9% isotonic saline vehicle. Rats in the other two saline-conditioned control groups received the same intra-NAc shell infusions as the morphine-conditioned groups. A two-way ANOVA displayed significantly different effects of the four treatments, $F(3, 74) = 3.562$, $p < .05$, the pretest compared with the test, $F(1, 74) = 6.000$, $p < .05$, and the interaction of these two factors, $F(3, 74) = 2.886$, $p < .05$. The subsequent Bonferroni post hoc test showed that there was no significant increase in the CPP score in the expression test of the morphine-conditioned rats that had been infused in the NAc shell with the NOS inhibitor L-NAME ($t = 0.126$, $p > .05$), but there was a significant increase in the CPP score in the expression test of the morphine-conditioned rats that had been infused in the NAc shell with the 0.9% isotonic saline vehicle ($t = 3.870$, $p < .001$). In addition, there was no significant difference in the CPP score in the expression test of the saline-conditioned rat intra-NAc shells infused with

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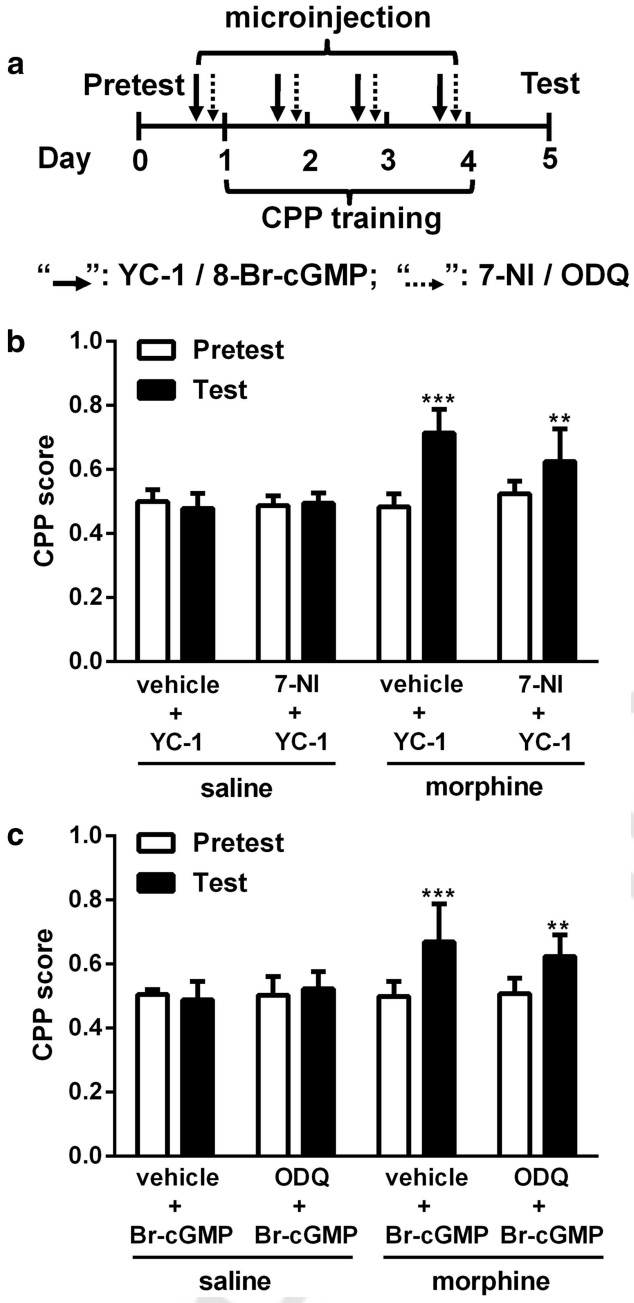

Figure 3. Effects of preconditioning microinjections of 7-NI plus YC-1 or ODQ plus 8-Br-cGMP into the nucleus accumbens (NAc) shell on the acquisition of morphine CPP. Diagram outlining the behavioral procedures (a). Solid arrows represent microinjection of YC-1 or 8-Br-cGMP, and dotted arrows represent microinjection of 7-NI or ODQ. Microinjection of YC-1 into the NAc shell reversed the blockage effect of 7-NI on the acquisition of morphine CPP (b). Microinjection of 8-Br-cGMP into the NAc shell reversed the blockage effect of ODQ on the acquisition of morphine CPP (c). Data are mean (*SEM*) (n = 7–12). Blank and solid columns represent the data from pre- and postconditioning tests, respectively. 7-NI = 7-nitroindazole, 3-bromo-, sodium salt; YC-1 = 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole; ODQ = 1H-[1,2,4] oxadiazolo-[4,3-a]quinoxalin-1-one; CPP = conditioned place preference. ** $p < .01$. *** $p < .001$, pretest compared with test (two-way analysis of variance, Bonferroni post hoc test).

either the NOS inhibitor L-NAME (t = 1.133, $p > .05$, pretest vs. test) or the 0.9% isotonic saline vehicle (t = 0.097, $p > .05$, pretest vs. test) (see Figure 2b).

In Batch 2, rats in the two morphine-conditioned groups were injected in the NAc shell with either the nNOS inhibitor 7-NI or a 50% DMSO ACSF vehicle. Rats in the other two saline-conditioned control groups received the same intra-NAc shell infusions as the morphine-conditioned groups. A two-way ANOVA revealed that there was a significant effect of the four treatments, $F(3, 58)$ = 9.230, $p < .001$. Although the pretest compared with the test did not show a significant effect, $F(1, 58)$ = 1.119, $p > .05$, there was a significant Test/Pretest × Treatment interaction effect, $F(3, 58)$ = 5.511, $p < .01$. The subsequent Bonferroni post hoc test showed that there was no significant increase in the CPP score in the expression test of the morphine-conditioned rats that had been infused in the NAc shell with the nNOS inhibitor 7-NI (t = 0.724, $p > .05$), but there was a significant increase in the CPP score in the expression test of the morphine-conditioned rats that had been infused in the NAc shell with the 50% DMSO ACSF vehicle (t = 4.242, $p < .001$). In addition, there was no significant difference in the CPP score in the expression test of the saline-conditioned rat intra-NAc shells infused with either the nNOS inhibitor 7-NI (t = 0.139, $p > .05$, pretest vs. test) or the 50% DMSO ACSF vehicle (t = 0.729, $p > .05$, pretest vs. test) (see Figure 2c).

In Batch 3, rats in the two morphine-conditioned groups were injected in the NAc shell with either the sGC inhibitor ODQ or a 50% DMSO ACSF vehicle. Rats in the other two saline-conditioned control groups received the same intra-NAc shell infusions as the morphine-conditioned groups. A two-way ANOVA revealed that there was a significant effect of the four treatments, $F(3, 60)$ = 3.493, $p < .05$, the pretest compared with the test, F (1, 60) = 4.472, $p < .05$, and the interaction of these two factors, $F(3, 60)$ = 3.948, $p < .05$. The subsequent Bonferroni post hoc test showed that there was no significant increase in the CPP score in the expression test of the morphine-conditioned rats that had been infused in the NAc shell with the sGC inhibitor ODQ (t = 0.396, $p > .05$), but there was a significant increase in the CPP score in the expression test of the morphine-conditioned rats that had been infused in the NAc shell with the 50% DMSO ACSF vehicle (t = 3.922, $p < .001$). In addition, there was no significant difference in the CPP score in the expression test of the saline-conditioned rat intra-NAc shells infused with either the sGC inhibitor ODQ (t = 0.884, $p > .05$, pretest vs. test) or the 50% DMSO ACSF vehicle (t = 0.818, $p > .05$, pretest vs. test) (see Figure 2d).

In Batch 4, rats in the two morphine-conditioned groups were injected in the NAc shell with either the PKG inhibitor Rp-8-Br-PET-cGMPS or a distilled water vehicle. Rats in the other two saline-conditioned control groups received the same intra-NAc shell infusions as the morphine-conditioned groups. A two-way ANOVA revealed that there was a significant effect of the four treatments, F (3, 58) = 5.468, $p < .01$, the pretest compared with the test, F (1, 58) = 12.07, $p < .001$, and the interaction of these two factors, F (3, 58) = 4.351, $p < .01$. The subsequent Bonferroni post hoc test showed that there was no significant increase in the CPP score in the expression test of the morphine-conditioned rats that had been infused in the NAc shell with the PKG inhibitor Rp-8-Br-PET-cGMPS (t = 1.319, $p > .05$), but there was a significant increase in the CPP score in the expression test of the

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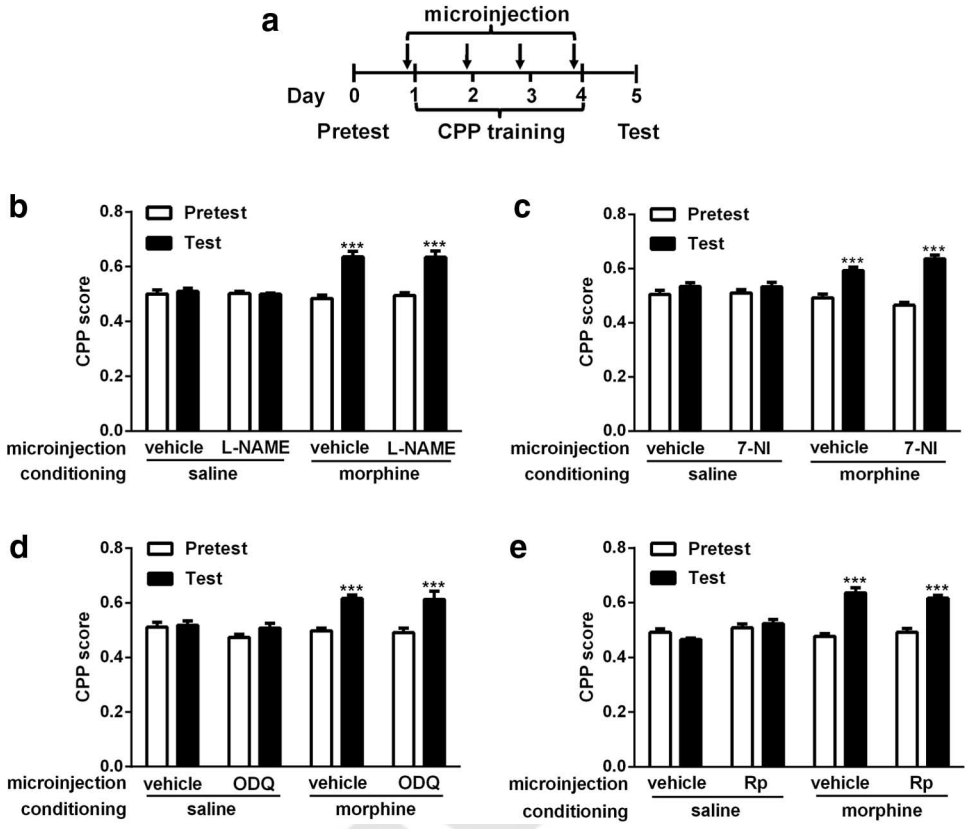

Figure 4. Effects of preconditioning microinjections of L-NAME, 7-NI, ODQ, or Rp-8-Br-PET-cGMPS into the nucleus accumbens (NAc) core on the acquisition of morphine CPP. Diagram outlining the behavioral procedures (a). Microinjection of L-NAME (b), 7-NI (c), ODQ (d), or Rp-8-Br-PET-cGMPS (e) into the NAc core had no effect on the acquisition of morphine CPP. Data are mean (*SEM*) (n = 8–10). Blank and solid columns represent the data from pre- and postconditioning tests, respectively. L-NAME = N^{ω}-nitro-L-arginine methyl ester; 7-NI = 7-nitroindazole, 3-bromo-, sodium salt; ODQ = 1H-[1,2,4] oxadiazolo-[4,3-a]quinoxalin-1-one; Rp = Rp-8-Br-PET-cGMPS; CPP = conditioned place preference. *** $p < .001$, pretest compared with test (two-way analysis of variance, Bonferroni post hoc test).

morphine-conditioned rats that had been infused in the NAc shell with the distilled water vehicle ($t = 4.719$, $p < .001$). In addition, there was no significant difference in the CPP score in the expression test of the saline-conditioned rat intra-NAc shells infused with either the PKG inhibitor Rp-8-Br-PET-cGMPS ($t = 0.145$, $p > .05$, pretest vs. test) or the distilled water vehicle ($t = 0.675$, $p > .05$, pretest vs. test) (see Figure 2e).

To describe intuitively the effect of L-NAME, 7-NI, ODQ, and Rp-8-Br-PET-cGMPS administered alone on the acquisition of saline-conditioned place preference, we compared the pre- and posttest scores in the saline-conditioned rats after the microinjection of drugs used in the Figure 2, and the results were shown in Table 1. A paired t test indicated that there was no significant difference in CPP scores (pre- vs. posttest) for each drug and the respective vehicle (see Table 1). These results suggested that intra-NAc shell microinjection of the L-NAME, 7-NI, ODQ, and Rp-8-Br-PET-cGMPS had no effect on the saline-systemic rats during the conditioned training, indicating that the drugs themselves had no effect of conditional preference or aversion.

The data shown in Figure 2 demonstrate that intra-NAc shell infusion with the nNOS inhibitor 7-NI, the sGC inhibitor ODQ, or the PKG inhibitor Rp-8-Br-PET-cGMPS blocked the acquisition of morphine CPP.

Premicroinjection of YC-1 and 8-Br-cGMP Into the NAc Shell Reversed the Blockage Effect of 7-NI and ODQ on the Acquisition of Morphine CPP

We performed two batch experiments before each morphine conditioning to measure whether NO was involved in the acquisition of morphine CPP through activation of sGC and PKG. The rats that were conditioned with 4 mg/kg of morphine received bilateral intra-NAc shell injections of either the sGC activator YC-1 (0.152 μg/side/0.5 μl) (Chan, Chan, & Chang, 2004) or the PKG activator 8-Br-cGMP (10 μg/side/0.5 μl) (Ota et al., 2008). After 5 min, they were infused with the nNOS inhibitor 7-NI or the sGC inhibitor ODQ. The saline-conditioned control rats received the same intra-NAc shell infusions as the morphine groups. The CPP expression test was run on Day 5, and the CPP score and shuttle times were calculated (see Figure 3a).

In Batch 1, rats in the two morphine-conditioned groups were first microinjected with the sGC activator YC-1, and 5 min later

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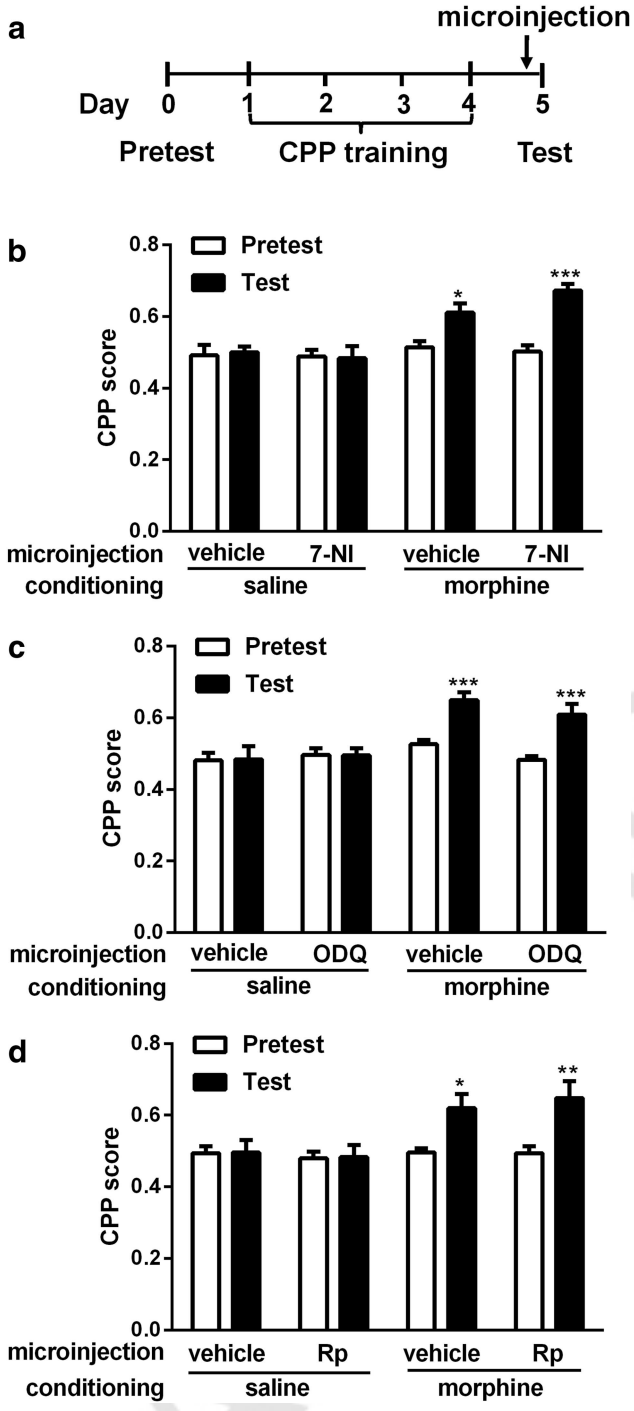

Figure 5. Effects of pretest microinjections of 7-NI, ODQ, or Rp-8-Br-PET-cGMPS into the nucleus accumbens (NAc) shell on the expression of morphine CPP. Diagram outlining the behavioral procedures (a). Microinjection of 7-NI (b), ODQ (c), or Rp-8-Br-PET-cGMPS (d) into the NAc shell had no effect on the expression of morphine CPP. Data are mean (*SEM*) (n = 7–9). Blank and solid columns represent the data from the pre- and postconditioning tests, respectively. 7-NI = 7-nitroindazole, 3-bromo-, sodium salt; ODQ = 1H-[1,2,4] oxadiazolo-[4,3-a]quinoxalin-1-one; Rp = Rp-8-Br-PET-cGMPS; CPP = conditioned place preference. * $p < .05$. ** $p < .01$. $^{***}p < .001$, pretest compared with test (two-way analysis of variance, Bonferroni post hoc test).

were injected in the NAc shell with either the nNOS inhibitor 7-NI or a 50% DMSO ACSF vehicle. Rats in the other two saline-conditioned control groups received the same intra-NAc shell infusions as the morphine-conditioned groups. A two-way ANOVA displayed significant effects of the four treatments, $F(3, 56) = 15.79$, $p < .001$, the pretest compared with the test, $F(1, 56) = 23.12$, $p < .001$, and the interaction of these two factors, $F(3, 56) = 15.23$, $p < .001$. The subsequent Bonferroni post hoc test showed that there were no significant increases in the CPP scores of the saline-conditioned rats that had been infused in the NAc shell with the 50% DMSO ACSF vehicle plus YC-1 ($t = 0.734$, $p > .05$) and those that had been infused in the NAc shell with 7-NI plus YC-1 ($t = 0.255$, $p > .05$). However, there was a significant increase in the CPP score in the morphine-conditioned rats that had been infused in the NAc shell with the ACSF vehicle plus YC-1 ($t = 7.723$, $p < .001$) compared with those that had been infused in the NAc shell with 7-NI plus YC-1 ($t = 3.821$, $p < .01$) (see Figure 3b).

In Batch 2, rats in the two morphine-conditioned groups were first microinjected with the PKG activator 8-Br-cGMP, and 5 min later were injected in the NAc shell with either the sGC inhibitor ODQ or a 50% DMSO ACSF vehicle. Rats in the other two saline-conditioned control groups received the same intra-NAc shell infusions as the morphine-conditioned groups. A two-way ANOVA showed significant effects of the four treatments, $F(3, 62) = 6.792$, $p < .001$, the pretest compared with the test, $F(1, 62) = 18.81$, $p < .001$, and the interaction of these two factors, $F(3, 62) = 7.441$, $p < .001$. The subsequent Bonferroni post hoc test showed that there were no significant increases in the CPP scores of the saline-conditioned rats that had been infused in the NAc shell with the 50% DMSO ACSF vehicle plus 8-Br-cGMP ($t = 0.500$, $p > .05$) or those that had been infused in the NAc shell with ODQ plus 8-Br-cGMP ($t = 0.542$, $p > .05$). However, there were significant increases in the CPP scores of the morphine-conditioned rats that had been infused in the NAc shell with the 50% DMSO ACSF vehicle plus 8-Br-cGMP ($t = 6.111$, $p < .001$) and those that had been infused in the NAc shell with ODQ plus 8-Br-cGMP ($t = 3.395$, $p < .01$) (see Figure 3c).

These data shown in Figure 3 demonstrate that intra-NAc shell preinfusion with the sGC activator YC-1 or the PKG activator 8-Br-cGMP reversed the blockage effect of the nNOS inhibitor 7-NI and the sGC inhibitor ODQ on the acquisition of morphine CPP.

The Effect of L-NAME, 7-NI, ODQ, and Rp-8-Br-PET-cGMPS Microinjection Into the NAc Core on the Acquisition of Morphine CPP

We performed four additional batch experiments to measure the effect of the NO/sGC/PKG signaling pathway in the NAc core on the acquisition of morphine-induced CPP. During the conditioning phase, rats that were conditioned with 4 mg/kg of morphine received a bilateral intra-NAc core infusion of the NOS inhibitor L-NAME, the nNOS inhibitor 7-NI, the sGC inhibitor ODQ, the PKG inhibitor Rp-8-Br-PET-cGMPS, or the respective vehicle 20 min before each morphine conditioning. The saline-conditioned control rats received the same intra-NAc core infusions as the morphine groups. The CPP test was executed on Day 5, and the CPP score and shuttle times were calculated (see Figure 4a).

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Table 1
Effects of the L-NAME, 7-NI, ODQ, or Rp on the Acquisition of Saline Conditioned Place Preference

Drugs	Vehicle	L-NAME	Vehicle	7-NI	Vehicle	ODQ	Vehicle	Rp
Pretest	0.53 (0.02)	0.49 (0.02)	0.53 (0.02)	0.50 (0.01)	0.51 (0.02)	0.49 (0.02)	0.51 (0.02)	0.51 (0.02)
Test	0.53 (0.03)	0.52 (0.03)	0.50 (0.02)	0.50 (0.01)	0.48 (0.04)	0.53 (0.03)	0.51 (0.02)	0.51 (0.02)
P value	0.903	0.306	0.163	0.777	0.321	0.163	0.263	0.845

Note. Values are *M* (*SEM*) of conditioned place preference (CPP) score (n = 7–10 in each group). During the conditioning days, the drug (L-NAME, 7-NI, ODQ, or Rp) was administered in the nucleus accumbens (NAc) shell prior to the confinement of the saline-systemic rats in one chamber of the apparatus and its respective vehicle was administered in the NAc shell prior to confinement in the opposite chamber. A paired t test indicated no significant difference between pretest and test CPP score for the drugs and their respective vehicle. L-NAME = N^{ω}-nitro-L-arginine methyl ester; 7-NI = 7-nitroindazole, 3-bromo-, sodium salt; ODQ = 1H-[1,2,4] oxadiazolo-[4,3-a]quinoxalin-1-one; Rp = Rp-8-Br-PET-cGMPS; Pretest = CPP score on the pretest day; Test = CPP score on the test day.

In Batch 1, rats in the two morphine-conditioned groups were injected in the NAc core with either the NOS inhibitor L-NAME or the 0.9% isotonic saline vehicle. Rats in the other two saline-conditioned control groups received the same intra-NAc core infusions as the morphine-conditioned groups. A two-way ANOVA revealed significant effects of the four treatments, $F(3, 68) = 11.74$, $p < .001$, the pretest compared with the test, $F(1, 68) = 55.25$, $p < .001$, and the interaction of these two factors, $F(3, 68) = 17.18$, $p < .001$. The subsequent Bonferroni post hoc tests showed that morphine-induced CPP was observed in rats treated by an intra-NAc core injection of either the NOS inhibitor L-NAME ($t = 6.789$, $p < .001$) or the 0.9% isotonic saline vehicle ($t = 7.786$, $p < .001$) (see Figure 4b).

In Batch 2, rats in the two morphine-conditioned groups were injected in the NAc core with either the nNOS inhibitor 7-NI or a 50% DMSO ACSF vehicle. Rats in the other two saline-conditioned control groups received the same intra-NAc core infusions as the morphine-conditioned groups. A two-way ANOVA revealed that there was a significant effect of the pretest compared with the test, $F(1, 62) = 67.78$, $p < .001$. Although there was no significant effect among the four treatments, $F(3, 62) = 2.590$, $p > .05$, there was a Test/Pretest × Treatments interaction effect, $F(3, 62) = 13.31$, $p < .001$. The subsequent Bonferroni post hoc tests showed that morphine-induced CPP was observed in rats treated with an intra-NAc core injection of either the nNOS inhibitor 7-NI ($t = 9.392$, $p < .001$) or the 50% DMSO ACSF vehicle ($t = 5.188$, $p < .001$) (see Figure 4c).

In Batch 3, rats in the two morphine-conditioned groups were injected in the NAc core with either the sGC inhibitor ODQ or a 50% DMSO ACSF vehicle. Rats in the other two saline-conditioned control groups received the same intra-NAc core infusions as the morphine-conditioned groups. A two-way ANOVA revealed a significant effect of the four treatments, $F(3, 62) = 5.712$, $p < .01$, the pretest compared with the test, $F(1, 62) = 28.82$, $p < .001$, and the interaction of these two factors, $F(3, 62) = 4.983$, $p < .01$. The subsequent Bonferroni post hoc tests showed that morphine-induced CPP was observed in rats treated with an intra-NAc core injection of either the sGC inhibitor ODQ ($t = 5.001$, $p < .001$) or the 50% DMSO ACSF vehicle ($t = 4.592$, $p < .001$) (see Figure 4d).

In Batch 4, rats in the two morphine-conditioned groups were injected in the NAc core with either the PKG inhibitor Rp-8-Br-PET-cGMPS or a distilled water vehicle. Rats in the other two saline-conditioned control groups received the same intra-NAc core infusions as the morphine-conditioned groups. A two-way ANOVA revealed that there was a significant effect of the four treatments, $F(3, 64) = 15.68$, $p < .001$, the pretest compared with the test, $F(1, 64) = 51.79$, $p < .001$, and the interaction of these two factors, $F(3, 64) = 21.88$, $p < .001$. The subsequent Bonferroni post hoc tests showed that morphine-induced CPP was observed in rats treated with an intra-NAc core injection of either the PKG inhibitor Rp-8-Br-PET-cGMPS ($t = 6.977$, $p < .001$) or a distilled water vehicle ($t = 8.442$, $p < .001$) (see Figure 4e).

The data shown in Figure 4 demonstrate that intra-NAc core infusion of the NOS inhibitor L-NAME, 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 CPP.

The Effect of 7-NI, ODQ, and Rp-8-Br-PET-cGMPS Microinjection Into the NAc Shell on the Expression of Morphine CPP

We performed three batch experiments to determine the effect of the NO/sGC/PKG signaling pathway on the expression of morphine CPP in the NAc shell. After 4 days of CPP conditioning, rats received a bilateral intra-NAc shell infusion of the nNOS inhibitor 7-NI, the sGC inhibitor ODQ, the PKG inhibitor Rp-8-Br-PET-cGMPS, or the respective vehicle. These infusions were given 20 min before the expression test on Day 5 (see Figure 5a).

In Batch 1, rats in the two morphine-conditioned groups were injected in the NAc shell with either the nNOS inhibitor 7-NI or a 50% DMSO ACSF vehicle. Rats in the other two saline-conditioned control groups received the same intra-NAc shell infusions as the morphine-conditioned groups. A two-way ANOVA revealed that there was a significant effect of the four treatments, $F(3, 54) = 9.660$, $p < .001$, the pretest compared with the test, $F(1, 54) = 17.91$, $p < .001$, and the interaction of these two factors, $F(3, 54) = 6.586$, $p < .001$. The subsequent Bonferroni post hoc tests showed that morphine-induced CPP was observed in rats treated with an intra-NAc shell injection of either the nNOS inhibitor 7-NI ($t = 5.428$, $p < .001$) or the 50% DMSO ACSF vehicle ($t = 3.072$, $p < .05$) (see Figure 5b).

In Batch 2, rats in the two morphine-conditioned groups were injected in the NAc shell with either the sGC inhibitor ODQ or a 50% DMSO ACSF vehicle. Rats in the other two saline-conditioned control groups received the same intra-NAc shell infusions as the morphine-conditioned groups. A two-way ANOVA revealed that there was a significant effect of the four treatments, $F(3, 60) = 9.307$, $p < .001$), the pretest compared with the test, $F(1, 60) = 15.51$, $p < .001$, and the interaction of these

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two factors, $F(3, 60) = 5.055$, $p < .01$. The subsequent Bonferroni post hoc tests showed that morphine-induced CPP was observed in rats treated with an intra-NAc shell injection of either the sGC inhibitor ODQ ($t = 4.070$, $p < .001$) or the 50% DMSO ACSF vehicle ($t = 4.002$, $p < .001$) (see Figure 5c).

In Batch 3, rats in the two morphine-conditioned groups were injected in the NAc shell with either the PKG inhibitor Rp-8-Br-PET-cGMPS or a distilled water vehicle. Rats in the other two saline-conditioned control groups received the same intra-NAc shell infusions as the morphine-conditioned groups. A two-way ANOVA revealed that there was a significant effect of the four treatments, $F(3, 55) = 4.486$, $p < .01$, the pretest compared with the test, $F(1, 55) = 11.43$, $p < .01$, and the interaction of these two factors, $F(3, 55) = 3.557$, $p < .05$. The subsequent Bonferroni post hoc tests showed that morphine-induced CPP was observed in rats treated with an intra-NAc shell injection of either the PKG inhibitor Rp-8-Br-PET-cGMPS ($t = 3.579$, $p < .01$) or the distilled water vehicle ($t = 2.973$, $p < .001$) (see Figure 5d).

The data shown in Figure 5 demonstrate that intra-NAc shell 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 expression of morphine CPP.

The Effect of 7-NI, ODQ, and Rp-8-Br-PET-cGMPS Microinjection Into the NAc Core on the Expression of Morphine CPP

We performed three more batch experiments to determine the effect of the NO/sGC/PKG signaling pathway on the expression of morphine CPP in the NAc core. After 4 days of CPP conditioning, rats received a bilateral intra-NAc core infusion of the nNOS inhibitor 7-NI, the sGC inhibitor ODQ, the PKG inhibitor Rp-8-Br-PET-cGMPS, or the respective vehicle. These infusions were given 20 min before the expression test on Day 5 (Figure 6a).

In Batch 1, rats in the two morphine-conditioned groups were injected in the NAc core with either the nNOS inhibitor 7-NI or a 50% DMSO ACSF vehicle. Rats in the other two saline-conditioned control groups received the same intra-NAc core infusions as the morphine-conditioned groups. A two-way ANOVA revealed that there was a significant effect of the four treatments, $F(3, 56) = 5.840$, $p < .01$, the pretest compared with the test, $F(1, 56) = 4.338$, $p < .001$, and the interaction of these two factors, $F(3, 56) = 4.582$, $p < .01$. The subsequent Bonferroni post hoc tests showed that morphine-induced CPP was observed in rats treated with an intra-NAc core injection of either the nNOS inhibitor 7-NI ($t = 3.676$, $p < .01$) or the 50% DMSO ACSF vehicle ($t = 2.698$, $p < .05$) (see Figure 6b).

In Batch 2, rats in the two morphine-conditioned groups were injected in the NAc core with either the sGC inhibitor ODQ or a 50% DMSO ACSF vehicle. Rats in the other two saline-conditioned control groups received the same intra-NAc core infusions as the morphine-conditioned groups. A two-way ANOVA revealed that there was a significant effect of the four treatments, $F(3, 62) = 5.564$, $p < .01$, the pretest compared with the test, $F(1, 62) = 14.71$, $p < .001$, and the interaction of these two factors, $F(3, 62) = 8.097$, $p < .001$. The subsequent Bonferroni post hoc tests showed that morphine-induced CPP was observed in rats treated with an intra-NAc core injection of either the sGC inhibitor

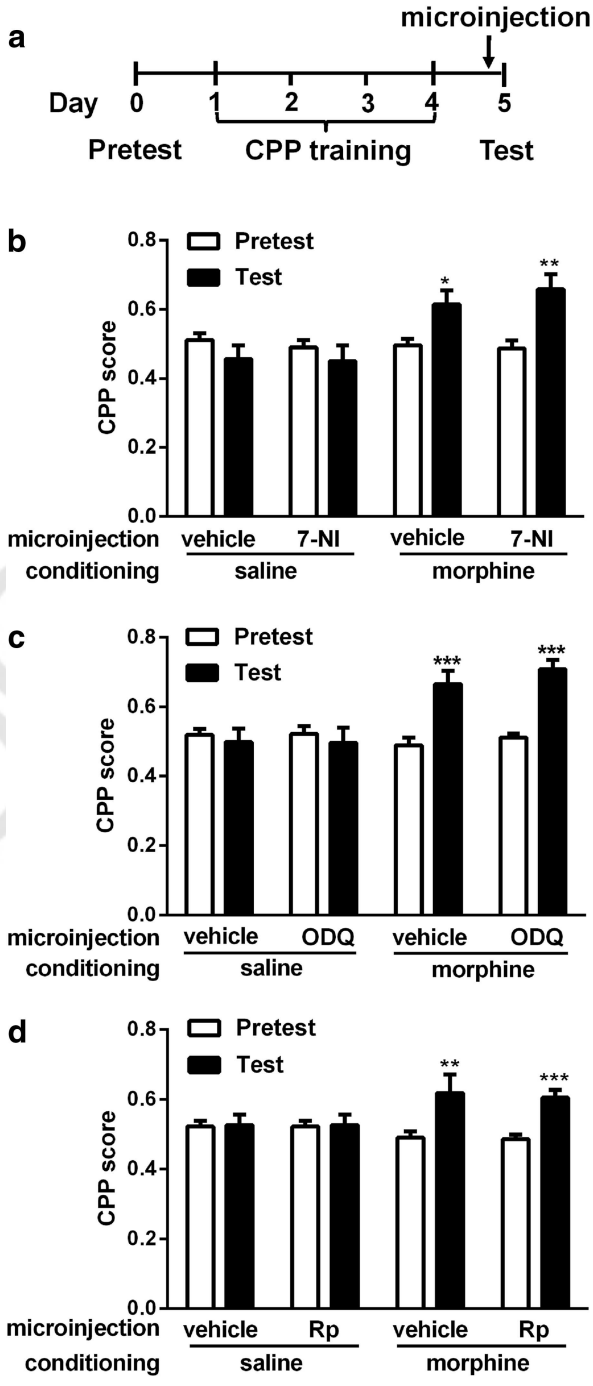

Figure 6. Effects of pretest microinjections of 7-NI, ODQ, or Rp-8-Br-PET-cGMPS into the nucleus accumbens (NAc) core on the expression of morphine CPP. Diagram outlining the behavioral procedures (a). Microinjection of 7-NI (b), ODQ (c), or Rp-8-Br-PET-cGMPS (d) into the NAc core had no effect on the expression of morphine CPP. Data are mean (*SEM*) ($n = 7$–12). Blank and solid columns represent the data from the pre- and postconditioning tests, respectively. 7-NI = 7-nitroindazole, 3-bromo-, sodium salt; ODQ = 1H-[1,2,4] oxadiazolo-[4,3-a]quinoxalin-1-one; Rp = Rp-8-Br-PET-cGMPS; CPP = conditioned place preference. $^{*}p < .05$. $^{**}p < .01$. $^{***}p < .001$, pretest compared with test (two-way analysis of variance, Bonferroni post hoc test).

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ODG ($t = 4.438$, $p < .001$) or the 50% DMSO ACSF vehicle ($t = 4.209$, $p < .001$) (see Figure 6c).

In Batch 3, rats in the two morphine-conditioned groups were injected in the NAc core with either the PKG inhibitor Rp-8-Br-PET-cGMPS or a distilled water vehicle. Rats in the other two saline-conditioned control groups received the same intra-NAc core infusions as the morphine-conditioned groups. A two-way ANOVA revealed that there was a significant effect of the pretest compared with the test, $F(1, 62) = 12.74$, $p < .001$, and the interaction of this factor and the treatment factor, $F(3, 62) = 3.694$, $p < .05$, but the differences among the four treatments were not significant, $F(3, 62) = 0.657$, $p > .05$. The subsequent Bonferroni post hoc tests showed that morphine-induced CPP was observed in rats treated with an intra-NAc core injection of either the PKG inhibitor Rp-8-Br-PET-cGMPS ($t = 3.926$, $p < .001$) or the distilled water vehicle ($t = 3.207$, $p < .01$) (see Figure 6d).

The data shown in Figure 6 demonstrate that intra-NAc core 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 expression of morphine CPP.

Discussion

NO/sGC/PKG Signaling Is Necessary for the Acquisition of Morphine-Induced Place Preference in the NAc Shell But Not in the NAc Core

Intra-NAc administration of the inhibitor L-NAME is known to decrease the CPP score of morphine-conditioned rats, which indicates that NO/NOS are critical for morphine CPP (Gholami et al., 2002). However, the mechanism underlying NO/NOS involvement in morphine CPP in the NAc subregions has not been investigated. We demonstrate for the first time that microinjection of the NOS inhibitor L-NAME into the NAc shell, but not in the NAc core, blocked the acquisition of morphine place preference. The results are consistent with previous data that have shown blockage of NO synthesis with L-NAME reduced the reinforcing properties of cocaine (Pulvirenti, Balducci, & Koob, 1996). Additionally, injection of 7-NI, which is more selective for nNOS than L-NAME, into the NAc shell blocked the acquisition of morphine-induced place preference. This result is consistent with the results of previous studies that have shown that 7-NI blocks CPP that is induced by cocaine (Itzhak et al., 1998), nicotine (Martin & Itzhak, 2000), and alcohol (Itzhak & Martin, 2000). Importantly, our finding that NOS was critical for the acquisition of morphine-induced place preference in only the NAc shell extended the findings of earlier studies that did not differentiate between the NAc subregions.

A growing body of evidence has shown that NO-dependent upregulation of sGC and PKG is involved in synaptic plasticity and memory formation. For example, long-term potentiation and long-term depression, which are processes involved in 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). Several other behavioral and pharmacological studies (Domek-Lopacinska & Strosznajder, 2008; Wang et al., 2008) have suggested that there is a causal link between NO and the activity of PKG. In our work, microinjection of the sGC inhibitor ODQ or the PKG inhibitor Rp-8-Br-PET-cGMPS into the NAc shell before morphine-conditioned training selectively blocked the acquisition of morphine CPP. This is similar to the effects of the nNOS inhibitor 7-NI. Our results also show that premicroinjection of the sGC activator YC-1 or the PKG activator 8-Br-cGMP in the NAc shell reversed the blockage effect on morphine CPP acquisition that is induced by the nNOS inhibitor 7-NI or the sGC inhibitor ODQ, which indicates involvement of NO in morphine CPP acquisition that occurs by means of the activation of sGC and PKG. These findings suggest that the sGC/PKG pathway is the downstream effector pathway of NO in this context, that is, NO is involved in the acquisition of morphine CPP through the activation of the NO/sGC/PKG signaling pathway. These data are similar to data showing that intra-CA1 administration of the inhibitors of NO, sGC, and PKG immediately after conditioned training blocked the consolidation of morphine-induced CPP (Shen et al., 2012). There are also several lines of evidence, consistent with our results, that implicate the NO/sGC/PKG signaling pathway in such different learning and memory tasks as fear memory (Ota et al., 2010; Ota et al., 2008; Paul et al., 2008), object recognition (Furini et al., 2010), and spatial learning (Böhme et al., 1993; Hölscher, McGlinchey, Anwyl, & Rowan, 1996; Prendergast, Buccafusco, & Terry, 1997; Qiang, Chen, Wang, Wu, & Qiao, 1997; Yamada et al., 1995). Inhibition of the NO/sGC/PKG signaling pathway in these experiments has been reported to disrupt memory processes in animals.

To investigate whether L-NAME, 7-NI, ODQ, or Rp-8-Br-PET-cGMPS have the preference or the aversion property, the saline-systemic rats were divided into two subgroups; the subgroups received intra-NAc shell injection of the drug and their vehicle, respectively. That is, during the conditioning days, the drug was administered prior to the confinement of the saline-systemic rats in one chamber of apparatus and their respective vehicle was administered prior to confinement in the opposite chamber. The CPP score was defined as the time spent in the drug-/vehicle-paired chamber divided by the total time on the test day. Results in our study showed that (a) compared with the pretest, there was no significant difference in the CPP score in the test of saline-systemic rat intra-NAc shells infused with either the drug or their respective vehicle (see Figure 2); and (b) the saline-systemic rats, which received microinjection L-NAME, 7-NI, ODQ, or Rp-8-Br-PET-cGMPS into the NAc shell, spent almost an equal amount of time in the drug-paired chamber and in their vehicle-paired chamber on the pretest/test day (see Table 1). These data show that intra-NAc shell microinjection of L-NAME, 7-NI, ODQ, or Rp-8-Br-PET-cGMPS had no effect on the saline-systemic rats, indicating that L-NAME, 7-NI, ODQ, or Rp-8-Br-PET-cGMPS had no CPP or conditioned place aversion effect. The data in Tables 2 and 3 demonstrate that injection of these reagents into the NAc shell or the core did not influence rats' shuttle times on the expression test day, that is, the reagents did not significantly affect rats' locomotor activity. We therefore conclude that intra-NAc shell injection of these reagents blocked the acquisition of morphine-induced place preference only because they had changed the activity of the NO/sGC/PKG signaling pathway, which indicates that the NAc

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Table 2
Locomotor Activity in Conditioned Place Preference Tests[a]

Group	Intraperitoneal	Locomotor activity	*n*
Intra-NAc shell			
Vehicle	Saline	463.5 (14.03)	8
L-NAME	Saline	425.8 (18.06)	10
Vehicle	Morphine	420.9 (21.99)	12
L-NAME	Morphine	415.1 (19.09)	11
Vehicle	Saline	410.3 (21.07)	7
7-NI	Saline	395.9 (12.23)	9
Vehicle	Morphine	415.1 (16.10)	10
7-NI	Morphine	428.4 (29.94)	7
Vehicle	Saline	438.9 (25.79)	8
ODQ	Saline	425.9 (22.38)	9
Vehicle	Morphine	447.3 (21.58)	9
ODQ	Morphine	459.1 (27.72)	8
Vehicle	Saline	445.3 (24.48)	8
Rp	Saline	435.8 (22.19)	9
Vehicle	Morphine	410.3 (12.11)	8
Rp	Morphine	452.6 (11.54)	8
Vehicle + YC-1	Saline	517.1 (26.44)	8
7-NI + YC-1	Saline	476.3 (30.87	8
Vehicle + YC-1	Morphine	538.4 (23.69)	7
7-NI + YC-1	Morphine	546.0 (21.19)	9
Vehicle + Br-cGMP	Saline	543.8 (19.95)	8
ODQ + Br-cGMP	Saline	513.7 (30.61)	7
Vehicle + Br-cGMP	Morphine	484.3 (18.91)	12
ODQ + Br-cGMP	Morphine	481.9 (19.27)	8
Intra-NAc core			
Vehicle	Saline	459.3 (26.03)	9
L-NAME	Saline	416.7 (23.46)	10
Vehicle	Morphine	426.9 (29.64)	10
L-NAME	Morphine	416.4 (26.64)	9
Vehicle	Saline	477.0 (18.54)	8
7-NI	Saline	478.6 (33.22)	8
Vehicle	Morphine	453.0 (27.10)	9
7-NI	Morphine	416.1 (25.40)	10
Vehicle	Saline	411.8 (25.55)	8
ODQ	Saline	442.0 (29.41)	8
Vehicle	Morphine	403.1 (20.12)	9
ODQ	Morphine	423.8 (27.99)	10
Vehicle	Saline	420.2 (22.87)	9
Rp	Saline	468.0 (17.99)	8
Vehicle	Morphine	469.0 (20.86)	9
Rp	Morphine	427.1 (19.80)	10

Note. Values are *M* (*SEM*). No significant differences in locomotor activity were detected by one-way analysis of variance. NAc = nucleus accumbens; L-NAME = N$^{\omega}$-nitro-L-arginine methyl ester; 7-NI = 7-nitroindazole, 3-bromo-, sodium salt; ODQ = 1H-[1,2,4] oxadiazolo-[4,3-a]quinoxalin-1-one; Rp = Rp-8-Br-PET-cGMPS; YC-1 = 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole.
[a] Locomotor activity of conditioned place preference tests in Figures 2–4.

shell NO/sGC/PKG signaling pathway might be involved in the acquisition of morphine-induced place preference.

The NO/sGC/PKG Signaling Pathway in the NAc Had No Effect on the Retrieval of Morphine-Induced Place Preference

Memory formation can be divided experimentally into acquisition and retrieval stages. During the acquisition phase, individuals receiving an addictive drug can build a strong link between the rewarding effects of the drug and relevant environment cues. In the retrieval phase, the relevant environmental cues can activate the individual's craving for the rewarding effects of the addictive drug and then drive drug-seeking behavior. We found in the present study that infusion of the nNOS inhibitor 7-NI, the sGC inhibitor ODQ, or the PKG inhibitor Rp-8-Br-PET-cGMPS into the NAc shell or the NAc core did not influence the retrieval of morphine CPP. The morphine-conditioned rats that had been microinjected with 7-NI, ODQ, or Rp-8-Br-PET-cGMPS showed a significant preference for the morphine-paired chamber. These findings imply that the NO/sGC/PKG signaling pathway in the NAc shell or the NAc core is not involved in the retrieval of morphine-related reinforcing effects. Our present findings are consistent with a previous report that administration of the NOS inhibitor L-NAME in the NAc before testing had no effect on the expression of morphine-induced place preference (Gholami et al., 2002).

The NO/sGC/PKG Signaling Pathway in the NAc Subregions Has Different Effects on the Different Stages of Morphine CPP

Before place conditioning, approximately equal proportions of NAc neurons showed excitation or inhibition when the rat was in any of the three rooms in the apparatus (morphine-paired, center, or saline-paired), but place conditioning increased the proportion of (a) neurons that were inhibited while the rat was in the morphine-paired room and (b) neurons that were excited in the saline-paired room. This change in population responses of NAc AQ: 19

Table 3
Locomotor Activity in Conditioned Place Preference Tests[a]

Group	Intraperitoneal	Locomotor activity	*n*
Intra-NAc shell			
Vehicle	Saline	471.8 (32.38)	8
7-NI	Saline	448.1 (30.69)	7
Vehicle	Morphine	442.6 (33.26)	8
7-NI	Morphine	454.3 (25.45)	8
Vehicle	Saline	337.0 (14.59)	8
ODQ	Saline	451.0 (26.18)	8
Vehicle	Morphine	418.8 (22.28)	9
ODQ	Morphine	425.2 (24.71)	9
Vehicle	Saline	413.5 (18.46)	8
Rp	Saline	422.8 (17.15)	8
Vehicle	Morphine	446.9 (26.48)	8
Rp	Morphine	451.9 (22.92)	8
Intra-NAc core			
Vehicle	Saline	479.5 (19.95)	8
7-NI	Saline	412.6 (34.08)	7
Vehicle	Morphine	453.8 (16.50)	9
7-NI	Morphine	447.8 (24.73)	8
Vehicle	Saline	440.0 (24.40)	10
ODQ	Saline	441.5 (21.46)	8
Vehicle	Morphine	393.6 (33.32)	9
ODQ	Morphine	441.5 (21.46)	8
Vehicle	Saline	428.6 (17.16)	8
Rp	Saline	446.0 (26.69)	8
Vehicle	Morphine	457.4 (27.10)	7
Rp	Morphine	454.8 (14.99)	12

Note. Values are *M* (*SEM*). No significant differences in locomotor activity were detected by one-way analysis of variance. NAc = nucleus accumbens; L-NAME = N$^{\omega}$-nitro-L-arginine methyl ester; 7-NI = 7-nitroindazole, 3-bromo-, sodium salt; ODQ = 1H-[1,2,4] oxadiazolo-[4,3-a]quinoxalin-1-one; Rp = Rp-8-Br-PET-cGMPS.
[a] Locomotor activity of conditioned place preference tests in Figures 5–6.

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neurons is a neural correlate of the change in location preference that is manifested as morphine CPP (German & Fields, 2007). The shell and the core of the NAc have anatomically different afferent and efferent connections, and these different connections may play different roles in drug-induced motivated behavior (Heimer et al., 1991; Voorn, Gerfen, & Groenewegen, 1989). Our present results show that the NO/sGC/PKG signaling pathway in the NAc shell, but not in the NAc core, is essential for the acquisition of morphine-induced place preference. Consistent with these results, intra-NAc shell infusions of the D1 receptor antagonist SCH 39166 and the D2 receptor antagonist L-sulpiride impaired the acquisition but not the expression of morphine CPP (Fenu, Spina, Rivas, Longoni, & Di Chiara, 2006).

It is widely accepted that the NAc receives dopaminergic afferents from the ventral tegmental area and is one of the regions in which the diffusible gas NO has been implicated in the regulation of dopamine release (Gracy & Pickel, 1997). Pogun, Baumann, and Kuhar (1994) reported that sodium nitroprusside, a generator of NO, decreases [^{3}H]dopamine uptake in synaptosomal preparations in the NAc of rats, which suggests that NO-evoked inhibition of the dopamine transporter function contributes to the increase in dopamine efflux. Previous studies of the neuroanatomical and neurochemical features of the NAc shell and core have also revealed that the basal level of extracellular dopamine is about 3 times greater in the core than in the shell. The neurochemical milieu of the shell is therefore more diverse and more sensitive to a variety of pharmacological and physiological stimuli than that of the core (Zahm, 1999). From the above finding, we can speculate that inhibition of the NO signaling pathway in the NAc shell, but not in the core, impairs the acquisition of morphine-induced place preference by regu-
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Conclusion

The present study demonstrates that blockage of the NO/sGC/PKG signaling pathway in the NAc shell, but not the core, disrupts the acquisition of morphine-conditioned place preference. Additionally, this signaling pathway in either the NAc shell or the NAc core had no effect on the retrieval of morphine-
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AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES 1

AQau—Please confirm the given-names and surnames are identified properly by the colors. ■= Given-Name, ■= Surname
The colors are for proofing purposes only. The colors will not appear online or in print.

AQ1—Author: running head acceptable? If not, revise to no more than 50 total character spaces. Note that not all letters in running are in upper case.

AQ2—Author: provide institutional affiliation(s) for coauthors Shen, Wang, Qi, and Li. None provided in documentation.

AQ3—Author: APA style permits two affiliations. Suggest choosing between Ministries.

AQ4—Author: review abstract carefully for insertion of abbreviations used, per APA style.

AQ5—Author: nucleus accumbens abbreviated correctly? See abstract, tables, and figure as well.

AQ6—Author: revisions to sentence accurate? "Hopefully" used incorrectly.

AQ7—Author: is the last part of this sentence, after the comma, accurate as written (there were no edits).

AQ8—Author: the temperature value is SD, correct?

AQ9—Author: ACSF defined correctly?

AQ10—Author: chemical compounds spelled out correctly?

AQ11—Author: should compartment be chamber, as per the description in **Place Preference Apparatus**?

AQ12—Author: which table? or did you mean tables?

AQ13—Author: revisions to sentence accurate?

AQ14—Author: note APA lettering style for panels in figures (i.e., a not A) here and throughout.

AQ15—Author: see query about reporting data to more than 3 decimal places. Note that, where reported to 4 places, the values have been rounded.

AQ16—Author: note revision to the phrase "in the expression test of the saline-conditioned rat intra-NAc shells infused" here and in the identical passages in the next three sections.

AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES 2

AQ17—Author: revisions to sentence accurate?

AQ18—Author: revisions to sentence accurate?

AQ19—Author: revisions to sentence accurate?

AQ20—Author: isn't this sentence saying the same thing as the first sentence of your conclusion? If so, delete here.

AQ21—Author: provide departmental and institutional affiliations for Shen, Wang, Qi, and Li.

AQ22—Author: APA style permits two affiliations. See previous note on selecting two

AQ23—Author: as per APA and statistical manuals, report p values to no more than 3 decimal placed. Note rounding p values in Table 1.