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mGluR5 in the nucleus accumbens shell regulates morphine-associated contextual memory through reactive oxygen species signaling

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ABSTRACT

Emerging evidence indicates that metabotropic glutamate receptor 5 (mGluR5) critically modulates drug and drug-related behaviors. However, the role of mGluR5 in the opiate-induced contextual memory remains unclear. Here, we found that microinfusion of the mGluR5 antagonist 3-((2-Methyl-1,3-thiazol-4-yl)ethynyl)pyridine (MTEP) into the nucleus accumbens (NAc) shell, but not into the core, significantly attenuated the expression of morphine conditioned place preference (CPP) in rats. Following the expression of morphine CPP, the protein level of membrane mGluR5 was selectively increased in the NAc shell. In primary striatal neurons, we observed that treatment with the mGluR5 agonist CHPG increased the phosphorylation level of extracellular signal-regulated kinase (ERK), which was dependent on the mGluR5-inositol-1,4,5-trisphosphate-reactive oxygen species (ROS) pathway. Moreover, the microinjection of the ROS scavenger Tempol into the NAc shell of rats blocked the expression of morphine CPP. Further, the administration of t-BOOH, a ROS donor, into the NAc shell rescued the retrieval impairment of morphine CPP produced by MTEP. Our previous study demonstrated that the expression of morphine CPP increased the phosphorylation of ERK selectively in the NAc shell. Thus, results of the present study suggest that mGluR5 in the NAc shell, but not in the core, is essential for the retrieval of morphine contextual memory, which is mediated at least in part, through the ROS/ERK signaling pathway. Uncovering the molecular basis of opiate contextual memory will benefit the development of new therapeutic approaches for the treatment of opiate addiction.

Keywords Conditioned place preference (CPP), metabotropic glutamate receptor 5 (mGluR5), morphine, nucleus accumbens (NAc), reactive oxygen species (ROS), retrieval.

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INTRODUCTION

Clinical findings have shown that the environment associated with drug use could provoke relapse in addicts (O'Brien *et al.* 1992). Notably, the nucleus accumbens (NAc) has been identified in the execution of conditioned responses to drug-associated contextual cues in laboratory animals (Bossert *et al.* 2006). As the neural substrate of drug addiction shares common circuit underlying normal learning and memory (Robbins, Ersche & Everitt 2008), the NAc is obviously of concern in drug contextual memory processes.

The metabotropic glutamate receptor 5 (mGluR5) has drawn increasing attention in addiction due to its involvement in drug-seeking behavior and extinction learning in rats after cocaine self-administration (Cleva & Olive 2012). Chiamulera *et al.* reported that mGluR5 null-mutant mice did not exhibit increased locomotor activity after acute cocaine injection nor did they self-administer cocaine (Chiamulera *et al.* 2001). The systemic administration of mGluR5 antagonists can attenuate self-administration and the reinstatement of cocaine, nicotine, alcohol, methamphetamine and heroin (Paterson & Markou 2005; van der Kam, de Vry & Tzschentke 2007; Gass *et al.* 2009; Kumaresan *et al.* 2009). Considering the abundant mGluR5 expression in the shell and core subregions of the NAc, a recent report showed that mGluR5 blockade in the NAc core of rats significantly attenuated context-induced reinstatement after cocaine abstinence (Knackstedt, Trantham-

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Davidson & Schwendt 2014); however, the contribution of mGluR5 in the NAc shell to drug contextual memory remains unknown.

It has been established that the stimulation of mGluR5 yields diacylglycerol, which activates protein kinase C (PKC) and inositol-1,4,5-trisphosphate (IP_3), which releases Ca^{2+} from intracellular stores. Li *et al.* demonstrated that mGluR5 can couple with IP_3 to trigger the production of reactive oxygen species (ROS) in nociceptive processing (Li, Ji & Neugebauer 2011). ROS signaling is widely recognized as a pathogenic factor in neurotoxicity and neurodegenerative diseases (Barnham, Masters & Bush 2004). And increase in ROS production or decrease in ROS removal could cause oxidative stress (OS), while low levels of ROS are emerging as versatile messengers indispensable to synaptic plasticity. Moreover, ROS have been shown to regulate synaptic plasticity-related signaling molecules and receptors, such as the extracellular signal-regulated kinase (ERK) and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors (Hongpaisan, Winters & Andrews 2004; Lee *et al.* 2012). ROS also have been demonstrated to modulate long-term potentiation (LTP), a cellular substrate for learning and memory (Knapp & Klann 2002). Additionally, studies have shown that addictive drugs can evoke enormous ROS production (Kovacic 2005) and ROS scavengers can prevent cocaine- and methamphetamine-induced behavior sensitization (Numa *et al.* 2008; Shiba *et al.* 2011). Thus, it is likely that ROS might be involved in the regulation of mGluR5 signaling in the NAc during drug contextual memory.

The present study was designed to investigate the role of mGluR5-ROS signaling in the NAc subregions in the retrieval of opiate contextual memory using the morphine-induced conditioned place preference (CPP) paradigm. First, we tested the effect of mGluR5 blockade with an mGluR5 antagonist in the NAc shell and core on the expression of morphine CPP in rats. We then explored the coupling mechanism between mGluR5 and ROS in primary striatal neurons. Finally, with intra-NAc microinjections, we determined whether mGluR5-ROS signaling was necessary for the expression of morphine CPP.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats were obtained from the Laboratory Animal Center of Peking University Health Science Center and housed with food and water supplied *ad libitum*. The room temperature was maintained at 23 ± 2°C, with relative humidity at 50 ± 5%. All experimental procedures were approved by the Animal Use Committee of Peking University Health Science Center.

Drugs

The following compounds were used for the *in vitro* study: (RS)-2-Chloro-5-hydroxyphenylglycine (CHPG, 1049, Tocris, Minneapolis, MN, USA), 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt, 1028, Tocris), Fluo-3 AM ester (50013, Biotum, San Francisco, CA, USA), MitoSOX™ (M36008, Invitrogen, Carlsbad, CA, USA), 4-Hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (Tempol, 176141, Sigma, St. Louis, MO, USA), thapsigargin (T9033, Sigma) and xestospongin C (X2628, Sigma). CHPG is a selective metabotropic glutamate receptor 5 (mGluR5) agonist. CPCCOEt is a selective non-competitive mGluR1 antagonist. Fluo-3 AM is a long wavelength fluorescent calcium indicator. MitoSOX™ (Molecular Probes, Carlsbad, CA, USA) is a dye used for the highly selective detection of superoxide in the mitochondria of live cells. Tempol is a superoxide dismutase mimetic. Thapsigargin is an inhibitor of the Ca^{2+} ion pump proteins of intracellular membranes located in the endoplasmic reticulum (ER). Xestospongin C is a selective and membrane-permeable inhibitor of the IP_3 receptor. Stock solutions of CHPG (10 mM), CPCCOEt (300 mM), fluo-3 AM (5 mM), MitoSOX™ (5 mM), Tempol (100 mM), thapsigargin (20 μM) and xestospongin C (2 μM) were prepared with 0.1% DMSO in PBS.

For the behavioral experiments, morphine hydrochloride was purchased from the First Pharmaceutical Factory of Qinghai, China and dissolved in sterile saline. 3-((2-Methyl-1,3-thiazol-4-yl)ethynyl)pyridine hydrochloride (MTEP, mGluR5 antagonist, 2921, Tocris) was dissolved in 20% DMSO in ACSF to a stock concentration of 20 μg/μl, and the tert-butyl hydroperoxide solution (t-BOOH, ROS donor, 458139, Sigma) was dissolved in ACSF to the final concentration on the day of the experiment.

Morphine-induced CPP

Conditioning was conducted as previously reported (Xu *et al.* 2012). 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). The CPP procedure consisted of three phases including the pretest, conditioning and test. On day 0, the rats were allowed to freely explore the entire apparatus for 15 minutes to assess the baseline preference. The time (in seconds) spent in each compartment and the shuttle times were recorded. On days 1–4, the rats were allowed two training sessions per day. Before being confined into one of the two lateral chambers for 45 minutes, the rats received either morphine (4 mg/kg, i.p.) or 0.9% saline (2 ml/kg, i.p.). The animals in the control groups received their saline injections before the training sessions in both

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lateral chambers. On day 5, the tests for the expression of CPP in a drug-free state (15 minutes) were performed. 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 the CPP test. The locomotor activity during all CPP tests was estimated by counting the total number of crossings between any two adjacent compartments. The animals not receiving expression test were remained in their home cages.

Cannula implantation and microinfusions

The rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and secured in a Kopf stereotaxic apparatus. Stainless steel guide cannulas (0.67 mm in outer diameter) were bilaterally implanted 1.5 mm above the NAc shell or core. The NAc shell coordinates (Lv *et al.* 2011) were anterior/posterior, +1.6 mm; medial/lateral, ± 0.9 mm; and dorsal/ventral, −6.5 mm, and the NAc core coordinates were anterior/posterior, +1.6 mm; medial/lateral, ±2.0 mm; and dorsal/ventral, −6.0 mm. Internal cannulas were replaced with dummy cannulas to keep it patent and prevent infection.

When the dummy cannulas were removed, infusion cannulas (0.3 mm in outer diameter) were inserted. Bilateral shell or core microinjections were completed with an infusion pump at the speed of 0.25 µl/minute and the microinjection volume for each side was 0.5 µl. The cannula was left in place for an additional 1 minute to allow for drug diffusion. MTEP (2 µg/µl, 0.5 µl/side), Tempol (100 µg/µl, 0.5 µl/side) and t-BOOH (5 µg/µl, 0.5 µl/side) were bilaterally microinjected into the nucleus 30, 15 and 20 minutes prior to the morphine CPP tests, respectively. The time and dosages were based on previous reports (Lu *et al.* 2004; Chan *et al.* 2009; Gwak, Hassler & Hulsebosch 2013; Wang *et al.* 2013).

Histological verification

The rat 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. 1b' and c' showed the location of representative cannula tips in the NAc shell and core. Rats with microinjection sites out of the target brain area were excluded.

Preparation of membrane fraction and immunoblot of mGluR5

The rats were decapitated immediately after the CPP tests and the brains were quickly removed and frozen in N-hexane (−70°C) for approximately 40 seconds. The preparation of the membrane fraction was conducted using procedures described previously (Tyrrell *et al.* 2001; Voulalas, Schetz & Undieh 2011). Bilateral tissue punches (12/16 gauge) of the NAc core and shell were homogenized in 10 volumes of homogenization buffer [10 mM HEPES (pH 7.4), 2 mM EDTA, 320 mM sucrose and 2% (v/v) protease inhibitor cocktail] using a Potter-Elvehjem tissue grinder with a Teflon pestle. The homogenate was centrifuged at $1000 \times g$ for 10 minutes to pellet nuclei and cellular debris. The supernatant was reserved and the pellet was washed with homogenization buffer and centrifuged again at $1000 \times g$ for 5 minutes. The pooled supernatants were centrifuged for 5 minutes at $1000 \times g$ to remove residual debris. This post-nuclear fraction was used as the total protein fraction and some was reserved. Then, the leftover post-nuclear fraction was centrifuged for 45 minutes at $200\,000 \times g$. The supernatant corresponded to a crude cytoplasmic fraction, and the crude membrane pellet was washed in membrane buffer [25 mM HEPES (pH 7.4), 2 mM EDTA and protease inhibitors]. Both the crude cytoplasmic and membrane fractions were centrifuged again at $200\,000 \times g$ for 30 minutes. The resulting pellet was saved as the membrane fraction. To solubilize the membrane fraction, 50 µl ice-cold RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 150 mM NaCl, 1% NP-40 and 0.1% SDS (Beijing Applygen Technologies Inc, Beijing, China) was added to the pellet, and the unsolubilized material was pelleted by centrifugation at $12\,000 \times g$ for 5 minutes at 4°C, while the soluble material in the supernatant was collected for additional processing.

The protein extracts (30 µg) were not boiled and then electrophoresed in 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The western blots were blocked in TBST buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.1% Tween 20] with 5% non-fat milk and then incubated with primary antibodies diluted in TBST (mGluR5, 1:1000, AB5675, Millipore, Billerica, MA, USA; Na-K ATPase, 1:3000, ab7671, Abcam, Cambridge, UK; β-actin, 1:2000, A2228, Sigma). The blots were then incubated with anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (1:2000, Zhongshan Biotechnology, Beijing, China) and developed using West Dura chemiluminescent substrate (Thermo, Rockford, IL Campus, USA). Densitometry was conducted using band intensities. To correct for the inconsistencies in loading, the membrane mGluR5 proteins were normalized to Na-K ATPase proteins and the total mGluR5 proteins were normalized to β-actin proteins.

Primary striatal neuronal culture

Striatal tissues isolated from the neonatal day 1 Sprague Dawley rats were dissected and then placed in 0.125%

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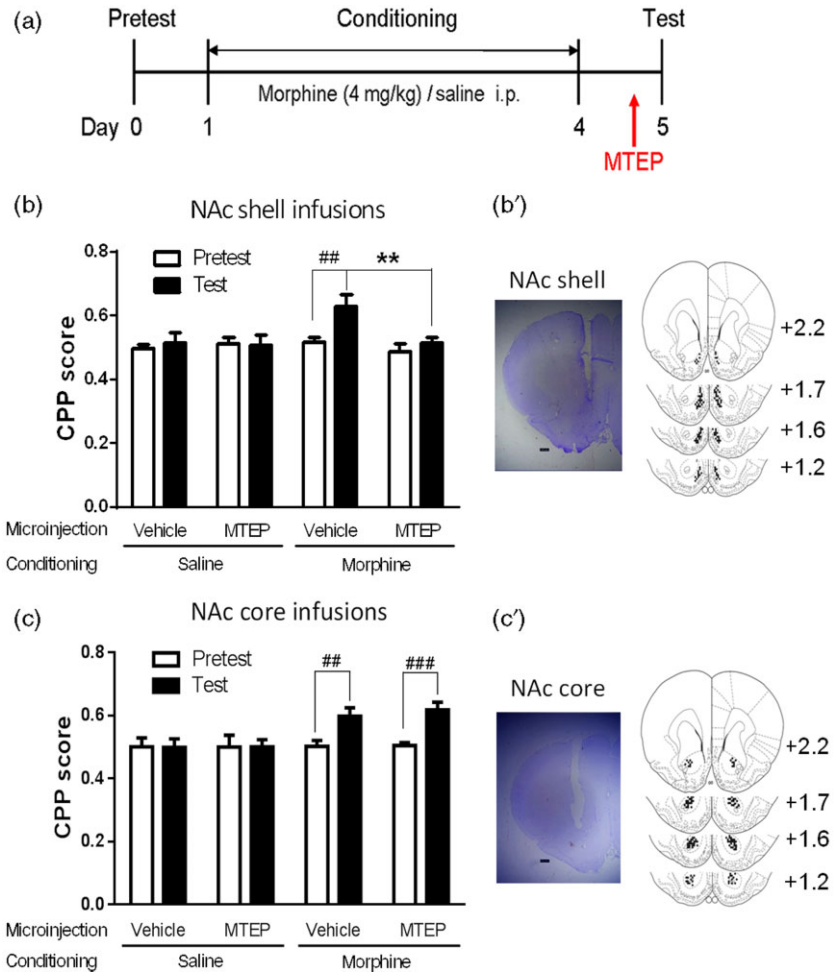

Figure 1 Effect of intra-NAc microinjection of the mGluR5 antagonist MTEP on the expression of morphine CPP. (a) Timeline of the experiment. (b) Bilateral microinjection of 2 μg of MTEP into the NAc shell blocked the expression of morphine CPP and had no effect on saline CPP. Data are the mean ± SEM of CPP scores. ##$P < 0.01$, pretest versus test; **$P < 0.01$, compared with morphine conditioning group with MTEP administration (two-way RM ANOVA, Bonferroni *post hoc* test). $n = 9–10$. (c) Bilateral microinjection of 2 μg of MTEP into the NAc core had no influence on the expression of morphine CPP or saline CPP. Data are the mean ± SEM of CPP scores. ##, ###$P < 0.01$, 0.001, respectively, pretest versus test (two-way RM ANOVA, Bonferroni *post hoc* test). $n = 9–11$. (b' and c') Left: represent histological sites of cannula placement in the NAc shell (b') and core (c'), respectively. Right: coronal sections depicting the injection sites (closed circles) in the NAc shell (b') and core (c') from rats included in the behavioral pharmacology experiment

trypsin (Gibco, Carlsbad, CA, USA) for 30 minutes at 37°C. The cells were then resuspended in DMEM (Gibco) medium plus 10% fetal bovine serum (HyClone, Logan, Utah, USA) and 2 mM GlutaMAX™ (Gibco). The dissociated neurons were plated onto 0.01% poly-D-Lysine-coated 35 mm petri dishes at a density of 1×10^6 cells/dish. After being cultured at 37°C in 5% CO_2 for 24 hours, the media was replaced with a fresh mixture of 98% neurobasal medium (Gibco), 2% B27 (Gibco) and 2 mM GlutaMAX™. Half of the medium was changed every 2–3 days, and 10 μM cytosine arabinofuranoside (Ara-c, Sigma) was added between the second and fourth day to control the proliferation of non-neuronal cells.

After the treatment, seventh day striatal neurons were sonicated in ice-cold RIPA buffer and centrifuged at 12 000 × *g* for 5 minutes. The resulting supernatant was reserved for immunoblotting of ERK1/2 using anti-phospho-ERK1/2 antibody (1:1000, # 4370, Cell Signaling, Danvers, MA, USA) and anti-ERK1/2 antibody (1:1000, # 4695, Cell Signaling).

Immunofluorescence

Cells in culture dishes were fixed with 4% paraformaldehyde for 20 minutes, permeabilized with 0.3% Triton X-100 for 30 minutes, and blocked from non-specific binding with 10% donkey serum in PBS for 1 hour at room temperature, before being incubated with anti-GAD67 antibody (1:1000, MAB5406, Millipore) overnight at 4°C. After incubating with a secondary antibody

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for 1 hour, the stained sections were examined with an Olympus FV1000 confocal microscope (Olympus Corporation, Tokyo, Japan) under a 40 × objective.

Ca^{2+} imaging and the detection of ROS

Changes in cytosolic-free Ca^{2+} were measured using the fluorescent membrane-permanent probe fluo-3 AM (Biotum, San Francisco, CA, USA). The cells were loaded with 2 μM fluo-3 AM for 45 minutes at 37°C, washed with external solution (ES) containing (in mM): 130 NaCl, 5 KCl, 2 KH_2PO_4, 1 $MgCl_2$, 2.5 $CaCl_2$, 10 glucose, 10 HEPES (pH adjusted to 7.2 with NaOH base and osmolality adjusted with sucrose to 300 mOsm). For Ca^{2+} free experiments, ES buffer (Ca^{2+}-free buffer) was prepared (Miglio *et al.* 2005). To determine the production of ROS, the cultures were loaded with the MitoSOX™ Red mitochondrial superoxide indicator (2.5 μM) in the dark at 37°C for 20 minutes. MitoSOX is chemically targeted to mitochondria, exhibiting red fluorescence when oxidized.

A fluorometric recording chamber was mounted on a Leica TCS SP8 confocal microscope. Fluo-3 and MitoSOX were excited at 488 and 514 nm respectively, and recorded for 400 seconds. Images were acquired every 5 seconds with a 40 × oil immersion objective. Ca^{2+} transients and ROS production were calculated as the ratio of the fluorescence intensity during drug application (F) over the average baseline fluorescence intensity 20 seconds before drug application (F/F0).

Statistical analysis

Data were expressed as the mean ± the standard error of the mean (SEM). The results of CPP and mGluR5 immunoblotting were analyzed with a two-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test. A one-way ANOVA followed by Newman–Keuls *post hoc* test was used for analyzing other data. The data were processed by the software Graph Pad Prism 5.0, and the statistical significance was set at $P < 0.05$.

RESULTS

Selective blockade of mGluR5 in the NAc shell prevented the expression of morphine CPP

To determine the effect of mGluR5 in the NAc on the retrieval of morphine-associated contextual memory, rats were divided into saline and morphine conditioning groups, and received bilateral microinjections of the mGluR5 antagonist MTEP or its vehicle into the NAc shell and core prior to the expression of morphine CPP (Fig. 1a). A two-way repeated-measures (RM) ANOVA was conducted to analyze the CPP score, with MTEP (0 and 2 μg/μl, 0.5 μl/side) as the between-subject factor and test (pretest and test) as the within-subject factor.

As shown in Fig. 1b, MTEP was microinfused into the NAc shell. In the saline conditioning groups, the ANOVA revealed no significant effects of MTEP ($F_{1, 17} = 0.0163$, $P = 0.8999$) or test ($F_{1, 17} = 0.05$, $P = 0.8257$), and there was no MTEP × test interaction ($F_{1, 17} = 0.2219$, $P = 0.6436$). These data suggested that the intra-NAc shell microinjection of MTEP did not elicit a preference or aversion on its own. In the morphine conditioning groups, the ANOVA revealed significant effects of MTEP ($F_{1, 17} = 7.366$, $P = 0.0147$) and test ($F_{1, 17} = 10.49$, $P = 0.0048$). The following Bonferroni *post hoc* test showed a significant difference of CPP test scores between the groups treated by vehicle versus MTEP ($t = 3.351$, $P < 0.01$), indicating the blockade of mGluR5 by MTEP in the NAc shell attenuated morphine CPP. Moreover, no significant differences were found in locomotor activity among these groups (data not shown).

As shown in Fig. 1c, MTEP was microinfused into the NAc core. In the saline conditioning groups, the ANOVA revealed no significant effects of MTEP ($F_{1, 16} = 0.0011$, $P = 0.9729$) or test ($F_{1, 16} = 0.0004$, $P = 0.9841$), and no MTEP × test interaction ($F_{1, 16} = 0.0021$, $P = 0.9636$). These data suggest that the intra-NAc core microinjection of MTEP did not elicit a preference or aversion on its own. In the morphine conditioning groups, the ANOVA revealed only a significant effect of test ($F_{1, 20} = 31.96$, $P < 0.0001$). The following Bonferroni *post hoc* test showed significant increases in the CPP score of morphine conditioning rats with both vehicle ($t = 3.662$, $P < 0.01$) and MTEP ($t = 4.333$, $P < 0.001$) pretreatment. These findings indicate that the intra-NAc core injections of MTEP did not attenuate morphine CPP. Moreover, no significant differences were found in locomotor activity among these groups (data not shown).

Overall, the behavioral pharmacology data above showed that mGluR5 in the NAc shell, but not the core, was involved in the expression of morphine CPP.

Membrane mGluR5 protein increased in the NAc shell following the expression of morphine CPP

Next, the alteration of the mGluR5 protein level in the NAc shell and core was measured after the expression of morphine CPP (Fig. 2a). A two-way ANOVA, with treatment (saline and morphine) as the between-subject factor and test (no-test and test) as the within-subject factor, was conducted. For the membrane level of mGluR5 in the NAc shell, the ANOVA revealed significant effects of treatment ($F_{1, 16} = 7.124$, $P = 0.0168$) and test ($F_{1, 16} = 11.76$, $P = 0.0034$) and a treatment × test interaction ($F_{1, 16} = 5.797$, $P = 0.0285$). Subsequent Bonferroni *post hoc* analyses showed that membrane mGluR5 protein

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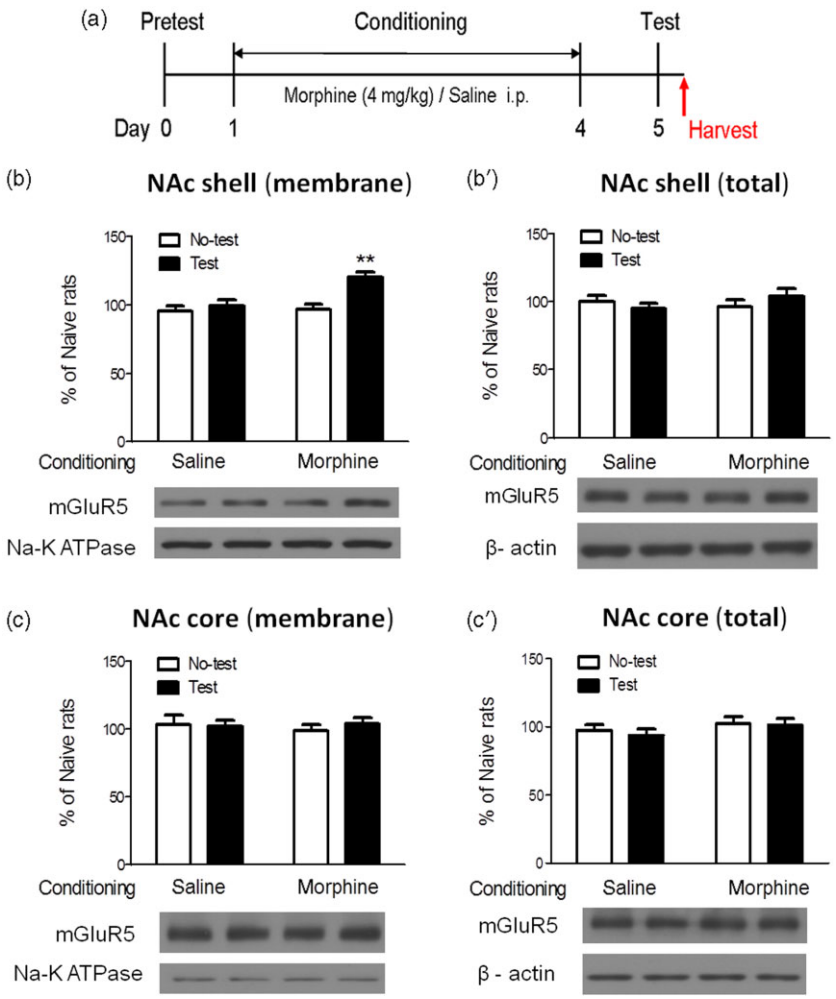

Figure 2 Changes in membrane and total protein levels of mGluR5 in the NAc shell and core in rats with or without a CPP test. (a) Timeline of the experiment. (b and b') Re-exposure to the morphine-paired context significantly increased the membrane (b), but not the total (b'), mGluR5 protein expression in the NAc shell of morphine conditioning rats. The data are normalized to the naive control and expressed as the mean ± SEM. $^{**}P < 0.01$, compared with morphine conditioning no-test group (two-way ANOVA, Bonferroni *post hoc* test). $n = 5$. (c and c') Re-exposure to the morphine-paired context had no influence on the membrane (c) and total (c') mGluR5 protein expression in the NAc core of rats among all groups. The data are expressed as the mean ± SEM of the normalized mGluR5 content and analyzed using two-way ANOVA followed by the Bonferroni *post hoc* test. $n = 5$

was significantly increased in the NAc shell following the expression of morphine CPP ($t = 4.127$, $P < 0.01$, Fig. 2b). In contrast, the detection of the total mGluR5 protein level showed no inter-group differences in the NAc shell (Fig. 2b'). Moreover, in the NAc core, both the membrane and total mGluR5 protein showed a relatively constant level among the groups (Fig. 2c and c'). Thus, the expression of morphine CPP induced an increase in the mGluR5 protein level in the membrane of the NAc shell, but not the core.

mGluR5 coupled with IP_3 to increase ROS production in primary striatal neurons

Evidence has emerged that a novel mGluR5-IP_3-mitochondrial ROS signaling cascade in the central amygdala could increase neuronal excitability (Li *et al.* 2011). To test whether a similar signaling cascade was engaged by gamma-aminobutyric acid-ergic (GABAergic) neurons in the NAc, we cultured primary striatal neurons. After 7 days in culture, small and medium-sized neurons with extended processes could be obtained, and the predominant number of neurons was identified as GABAergic by staining for glutamic acid decarboxylase-67 (GAD67), a phenotype-specific maker of GABAergic interneurons (Fig. 3).

First, we determined IP_3-mediated Ca^{2+} release induced by mGluR5 activation in primary striatal neurons. As shown in Fig. 4c, the application of the mGluR5 agonist CHPG (0.5 mM) evoked a rapid and sharp increase in the intracellular Ca^{2+} concentration in the neurons. In the Ca^{2+}-free medium, CHPG incubation

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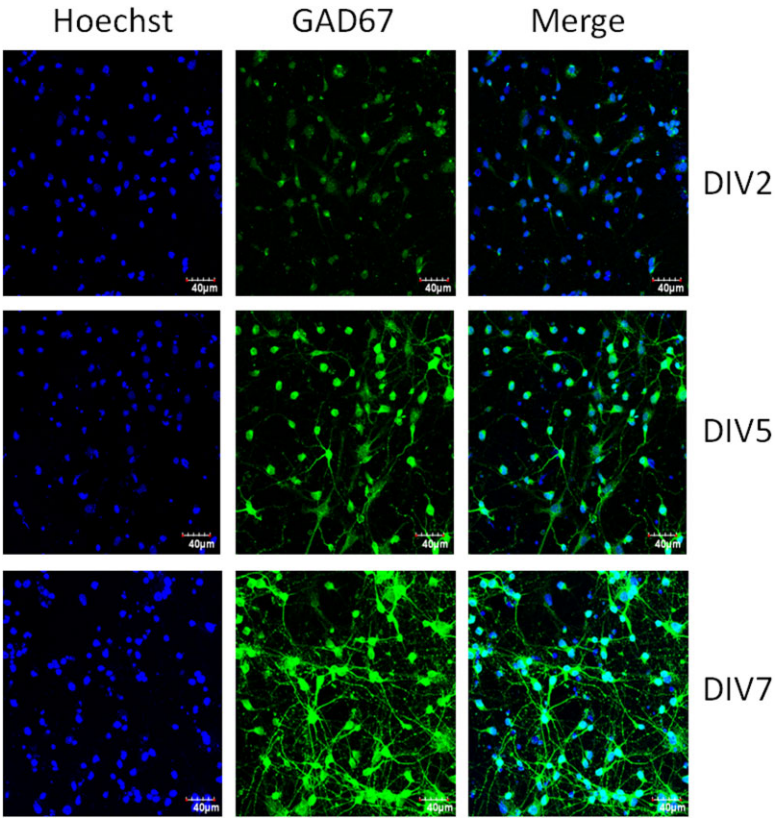

Figure 3 Identification of GABAergic neurons with the antibody for GAD67 from primary rat striatal neurons at the indicated time points (DIV 2, 5 and 7). DIV, day *in vitro*; GABAergic, gamma-aminobutyric acid-ergic; GAD67, glutamic acid decarboxylase-67. Scale bar, 40 μm

also produced an expected increase of cytoplasmic Ca^{2+} (Fig. 4d), indicating that the Ca^{2+} elevation was caused by intracellular Ca^{2+} release. Accordingly, the CHPG-induced Ca^{2+} increase was significantly reduced in cells pre-treated with the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin (1 μM, Fig. 4e) or the IP_3 receptor inhibitor xestospongin C (1 μM, Fig. 4f). Therefore, mGluR5 activation induced the release of intracellular Ca^{2+} via IP_3.

Next, we tested whether the release of intracellular Ca^{2+} could facilitate ROS formation in primary striatal neurons. MitoSOX, a fluorescent dye, has been used to detect ROS specifically (Robinson, Janes & Beckman 2008). As depicted in Fig. 5b, the activation of mGluR5 with CHPG (0.5 mM) significantly increased MitoSOX fluorescence intensity compared with either the baseline level or the PBS group. However, the enhanced fluorescent signal could be prevented in cells pre-treated with xestospongin C (1 μM) or MTEP (100 μM), but not CPCCOEt (100 μM; Fig. 5c–e), indicating that the CHPG-mediated increase of ROS-related fluorescence intensity was inhibited by IP_3R or mGluR5 blockade, but not by mGluR1 blockade. In summary, these data showed that the mGluR5-IP_3-mitochondrial ROS signaling pathway exists in striatal GABAergic neurons.

ROS scavenging prevented mGluR5 agonist-induced ERK activation in primary striatal neurons

This experiment was conducted to examine whether ROS were required for mGluR5 downstream synaptic plasticity-related proteins, e.g. ERK1/2. As shown in Fig. 6a, the level of phospho-ERK1/2 was obviously increased 10 minutes after bath-applied CHPG (0.1 and 1 mM, $F_{2,9} = 10.62$, $P = 0.0043$). Then, we determined whether the mGluR5-dependent ERK activation was redox modulated by ROS. After the co-application of 0.1 mM CHPG with Tempol (1, 5 and 10 mM), a stable membrane-permeable superoxide dismutase mimetic, in a bath, the CHPG-induced increase in phosphorylation of ERK1/2 was blocked by Tempol ($F_{4,15} = 11.25$, $P = 0.0002$, Fig. 6b). Furthermore, the effect of Tempol alone on phospho-ERK1/2 was also examined (Fig. 6c). Indeed, the bath application of 10 mM Tempol decreased the phosphorylation of ERK1/2 ($P < 0.05$), while lower concentrations of Tempol (1 and 5 mM) did not affect the baseline level of phospho-ERK1/2. Thus, 5 mM Tempol specifically suppressed 0.1 mM CHPG-induced ERK activation, without affecting baseline synaptic transmission. Altogether, these data indicated that the mGluR5-dependent activation of ERK required ROS in primary striatal neurons.

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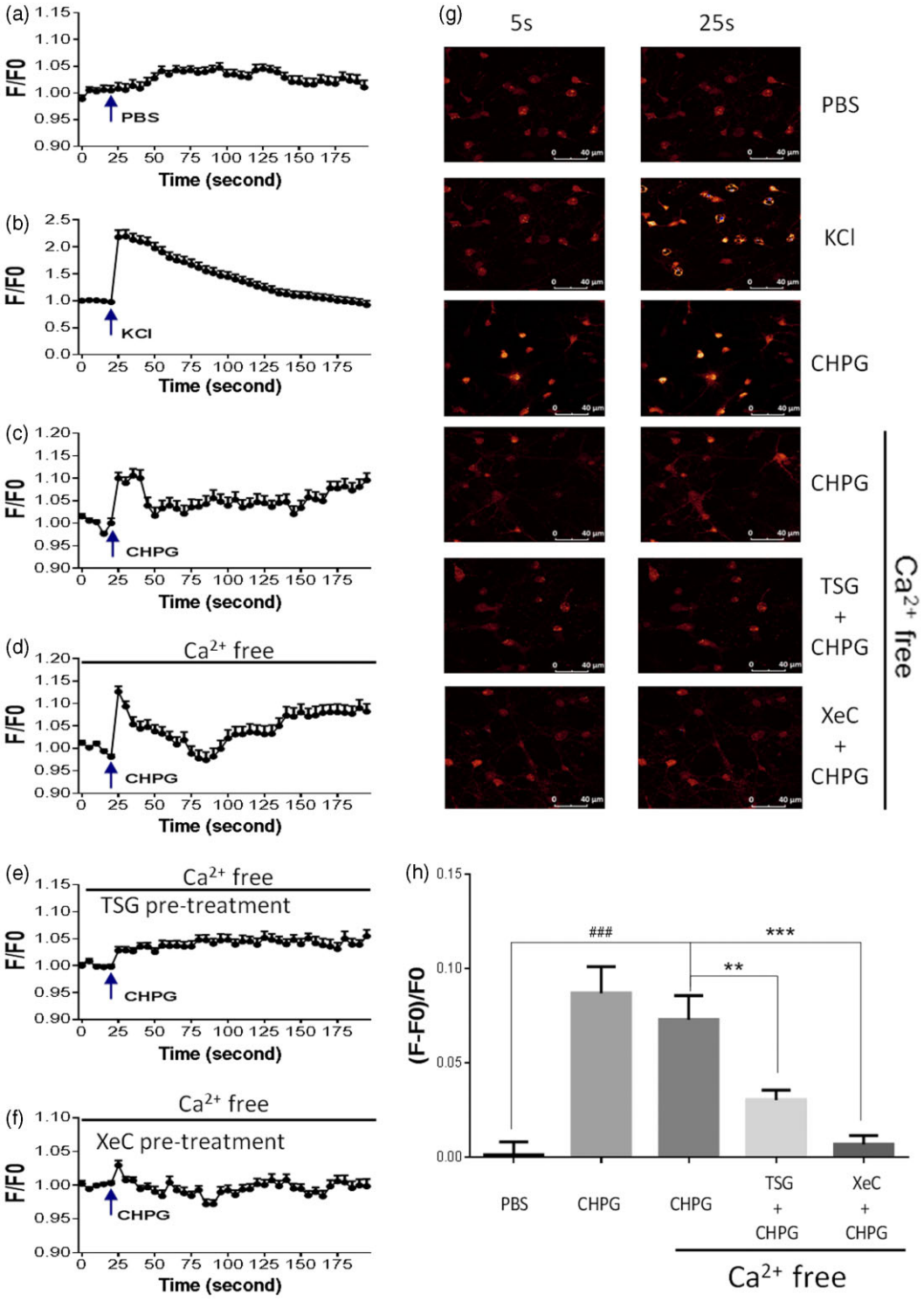

Figure 4 Inhibition of IP_3 receptor activity attenuated the mGluR5-mediated cytoplasmic Ca^{2+} increase in primary striatal neurons. (a–f) Transient Ca^{2+} traces in randomly selected neurons loaded with the Ca^{2+} indicator fluo-3 (2 μM, 45 minutes). In regular external solution (ES), cells were treated with PBS (a), KCl (60 mM, b) and CHPG (0.5 mM, mGluR5 agonist, c) at 20 seconds (indicated by the arrow). In Ca^{2+}-free ES, CHPG-applied cells were pre-treated with PBS (d), thapsigargin (1 μM, 15 minutes, endoplasmic reticulum Ca^{2+} ATPase inhibitor, e) and xestospongin C (1 μM, 15 minutes, IP_3 receptor inhibitor, f). (g) Representative images of fluo-3-loaded striatal neurons acquired at 5 and 25 seconds as treated in (a–f). Scale bar, 40 μm. (h) CHPG application significantly increased fluo-3 fluorescence intensity in the Ca^{2+} free environment and thapsigargin and xestospongin C significantly decreased the CHPG effect. Bar graphs expressed the highest values of the ΔF/F0 ratio of fluo-3 fluorescence. The data are expressed as the mean ± SEM. ###$P < 0.001$, compared with the PBS group; **, ***$P < 0.01$, 0.001, respectively, compared with the CHPG group in the Ca^{2+}-free environment (one-way ANOVA, Newman-Keuls *post hoc* test). $n = 35–39$ cells. Three or more independent experiments analyzing for each condition were performed. TSG, thapsigargin; XeC, xestospongin C

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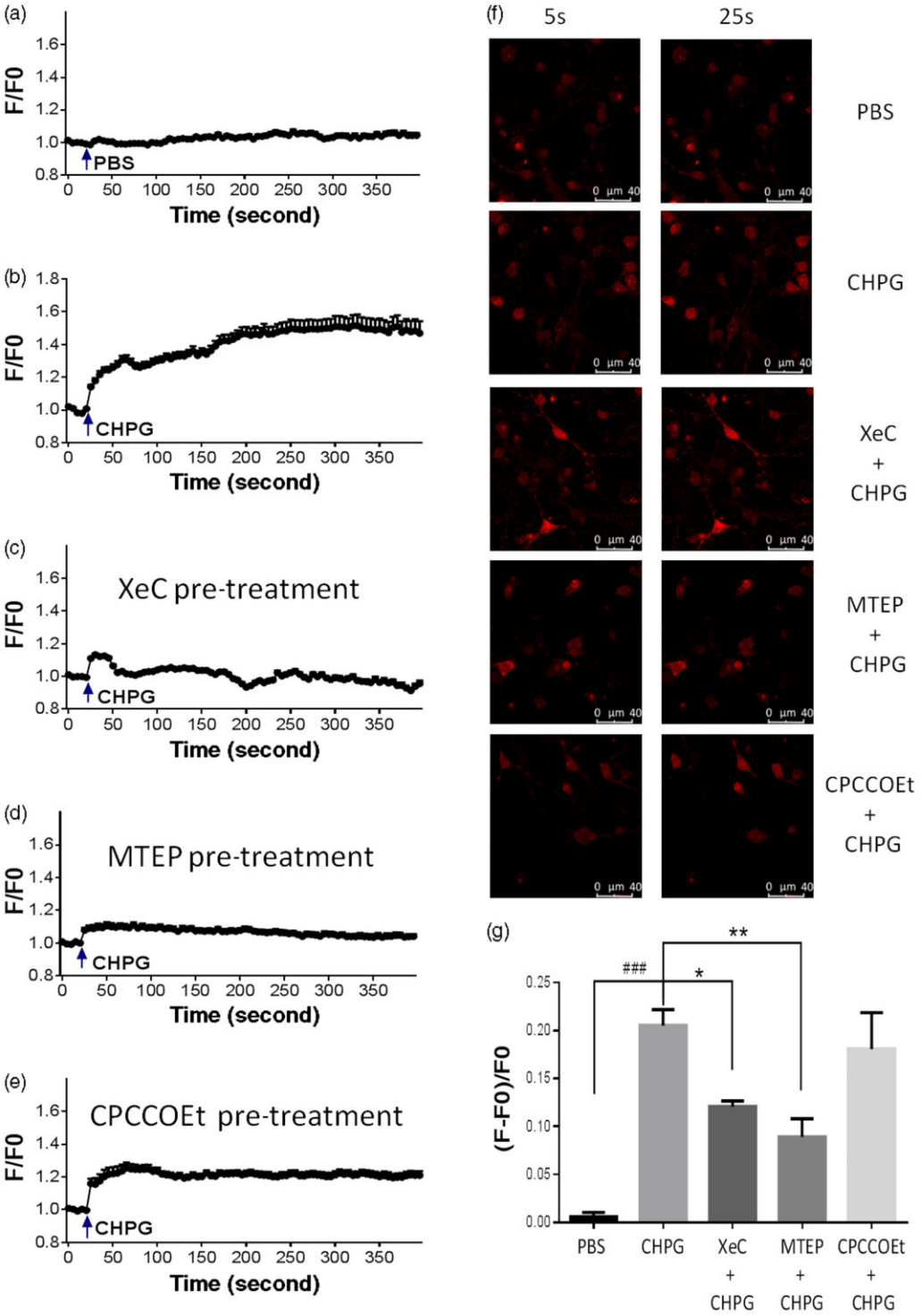

Figure 5 Activation of mGluR5, but not mGluR1, promoted ROS generation linked with IP_3 in primary striatal neurons. (a–e) Time courses data in randomly selected neurons after incubation in MitoSOX (2.5 μM, 20 minutes, ROS indicator). Cells were applied with PBS (a) and CHPG (0.5 mM, mGluR5 agonist, b–e) at 20 seconds (indicated by the arrow). CHPG-applied cells were pre-treated with xestospongin C (1 μM, 15 minutes, IP_3 receptor inhibitor, c), MTEP (100 μM, 15 minutes, mGluR5 antagonist, d) and CPCCOEt (100 μM, 15 minutes, mGluR1 antagonist, e). (f) Representative images of MitoSOX loaded striatal neurons acquired at 5 and 25 seconds as treated in (a–e). Scale bar, 40 μm. (g) Bath application of CHPG significantly increased MitoSOX fluorescence intensity. Both xestospongin C and MTEP, but not CPCCOEt, significantly decreased the CHPG effect. Bar graphs expressed the highest values of the ΔF/F0 ratio of MitoSOX fluorescence intensity. The data are expressed as the mean ± SEM. ###$P < 0.001$, compared with the PBS group; *, **$P < 0.05$, 0.01, respectively, compared with the CHPG alone group (one-way ANOVA, Newman–Keuls *post hoc* test). $n = 26$–30 cells. Three or more independent experiments analyzing for each condition were performed. XeC, xestospongin C

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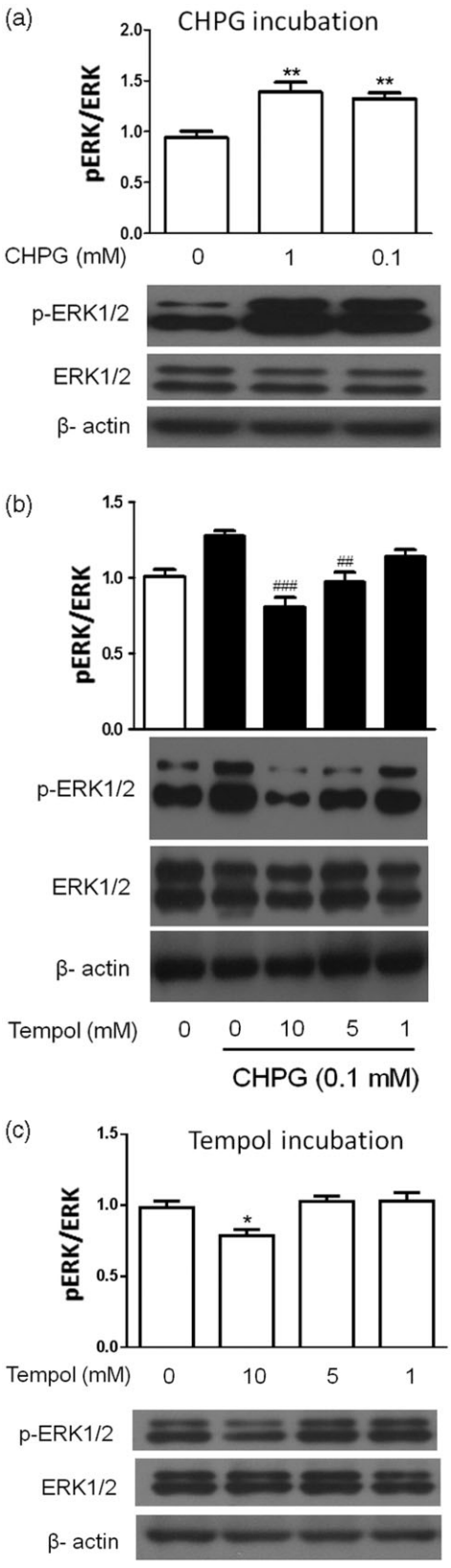

Figure 6 ROS were required for mGluR5-dependent activation of ERK in primary striatal neurons. (a) Increased p-ERK1/2 in primary striatal neurons exposed to CHPG (0.1 and 1 mM, mGluR5 agonist) for 10 minutes. The data are expressed as the mean ± SEM of the normalized p-ERK1/2 content; $^{**}P < 0.01$, compared with the control group (one-way ANOVA, Newman–Keuls *post hoc* test). $n = 4$. (b) Striatal neurons were pre-treated with 20 minutes Tempol (1, 5 and 10 mM, ROS scavenger) and then exposed to CHPG for 10 minutes. Tempol produced significant inhibition of ERK activation by CHPG. The data are expressed as the mean ± SEM of the normalized p-ERK1/2 content; ##, $^{###}P < 0.01$, 0.001, respectively, compared with the CHPG (0.1 mM) alone group (one-way ANOVA, Newman–Keuls *post hoc* test). $n = 4$. (c) Higher concentration of Tempol (10 mM, 30 minutes) treatment inhibited the basal ERK activation in primary striatal neurons, while lower concentrations of Tempol (1 and 5 mM, 30 minutes) had no effect. The data are expressed as the mean ± SEM of the normalized p-ERK1/2 content; $^{*}P < 0.05$, compared with the control group (one-way ANOVA, Newman–Keuls *post hoc* test). $n = 4$. p-ERK1/2, phosphorylated-ERK1/2; ERK1/2, total ERK1/2

ROS donor t-BOOH reversed the inhibition of morphine CPP expression caused by MTEP

Based on the above results in primary striatal neurons, we tested whether ROS in the NAc shell attributed to the neuroadaptive changes during the expression of morphine CPP in rats. Rats were divided into saline and morphine conditioning groups, and Tempol or vehicle was bilaterally microinjected into the NAc shell before the CPP test (Fig. 7a). A two-way RM ANOVA was conducted to analyze the CPP score, with Tempol (0 and 100 μg/μl, 0.5 μl/side) as the between-subject factor and test (pretest and test) as the within-subject factor.

As shown in Fig. 7b, the ANOVA revealed no significant effects of Tempol ($F_{1,16} = 0.2144$, $P = 0.6496$) or test ($F_{1,16} = 0.06365$, $P = 0.804$) and no Tempol × test interaction ($F_{1,16} = 0.006785$, $P = 0.9354$) in the saline conditioning groups. These data suggested that the intra-NAc shell microinjection of Tempol did not elicit a preference or aversion on its own. For the morphine conditioning groups, the ANOVA revealed a significant effect of Tempol ($F_{1,19} = 5.5$, $P = 0.03$) and a Tempol × test interaction ($F_{1,19} = 8.847$, $P = 0.0078$). The following Bonferroni *post hoc* test showed a significant difference in the CPP test scores between the groups treated by vehicle versus Tempol ($t = 3.718$, $P < 0.01$), indicating a significant inhibitory effect of Tempol on the expression of morphine CPP. Moreover, no significant differences were found in locomotor activity among these groups (data not shown).

Subsequently, we investigated whether the ROS donor t-BOOH could rescue the memory impairment produced by MTEP (Fig. 8a). Rats were given bilateral microinjections of MTEP (2 μg/rat) into the shell

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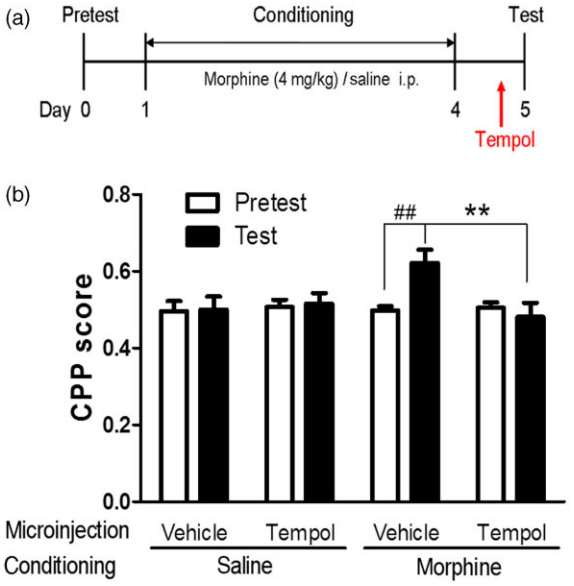

Figure 7 Effect of intra-NAc shell microinjection of the ROS scavenger Tempol on the expression of morphine CPP. (a) Timeline of the experiment. (b) Bilateral microinjection of Tempol into the NAc shell blocked the expression of morphine CPP and had no effect on saline CPP. The data are expressed as the mean ± SEM of CPP scores. ##$P < 0.01$, pretest versus test; **$P < 0.01$, compared with the morphine conditioning group with Tempol microinjection (two-way RM ANOVA, Bonferroni *post hoc* test). $n = 9$–11

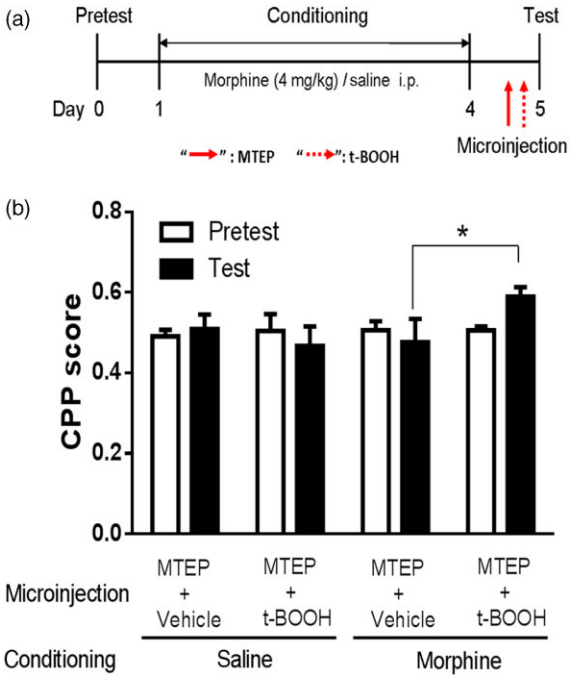

Figure 8 Effect of NAc shell MTEP pre-microinjection on the expression of morphine CPP plus the ROS donor t-BOOH. (a) Timeline of the experiment. (b) Co-infusions of t-BOOH and MTEP into the NAc shell restored morphine CPP. No significant differences in the CPP scores were found between the saline conditioning groups following intra-NAc shell administration. The data are expressed as the mean ± SEM of CPP scores. *$P < 0.05$, compared with the morphine conditioning group with MTEP+ Vehicle microinjection (two-way RM ANOVA, Bonferroni *post hoc* test). $n = 8$–9

10 minutes before t-BOOH (0 and 5 μg/μl, 0.5 μl/side) injections, and the MTEP-pre-treated rats were tested for CPP 20 minutes after the t-BOOH or vehicle injections. As depicted in Fig. 8b, the CPP scores from morphine conditioning rats were analyzed with a two-way RM ANOVA with treatment (vehicle and t-BOOH) as the between-subject factor and test (pretest and test) as the within-subject factor. Though the different treatments did not show a significant effect ($F_{1,15} = 2.18$, $P = 0.1605$), a significant effect of interaction between treatment and test was observed ($F_{1,15} = 5.318$, $P = 0.0358$). Specifically, a *post hoc* analysis revealed that there was a significant difference in the CPP test scores between the groups treated with vehicle and t-BOOH ($t = 2.491$, $P < 0.05$). Thus, t-BOOH distinctly rescued the attenuation of morphine CPP caused by MTEP, which suggests that ROS are important downstream factors of mGluR5.

DISCUSSION

In the present study, we first demonstrated that the expression of morphine CPP was associated with a specifically increase in membrane mGluR5 protein of the NAc shell but not the core, and the microinjection of the mGluR5 antagonist MTEP into the NAc shell prevented the expression of morphine CPP in rats. Second, the experiments conducted in primary striatal GABAergic neurons revealed that the mGluR5-dependent activation of ERK required the mGluR5-IP_3-ROS signaling pathway, implying a potential mechanism for the involvement of mGluR5 in the expression of morphine CPP. Finally, the application of the ROS scavenger Tempol into the NAc shell prevented the expression of morphine CPP in a manner similar to MTEP. And interestingly, the co-infusion of the ROS donor t-BOOH with MTEP reversed the memory impairment caused by the NAc shell pre-treatment with MTEP in rats. Collectively, these results suggested that the activation of mGluR5 in the NAc shell, but not the core, promoted the retrieval of morphine contextual memory, which, in part, occurs through ROS signaling.

It has been well established that mGluR5 plays a critical role in learning and memory. MGluR5 knockout mice had impairments in NMDAR-dependent LTP and spatial learning in the Morris water maze (MWM) and contextual fear conditioning (Lu *et al.* 1997). Moreover, the genetic ablation of mGluR5 in mice disrupted the process of fear extinction and spatial reversal learning (Xu *et al.* 2009). Furthermore, the mGluR5 antagonist MPEP induced deficits in a 24-hour retention in a Y-maze

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alternation test and significantly impaired acquisition in the MWM task (Balschun & Wetzel 2002; Steckler *et al.* 2005). The recall of extinction memory could be inhibited by the systemic administration or microinjection of MPEP into the medial prefrontal cortex (Fontanez-Nuin *et al.* 2011). Though the distribution of mGluR5 in the NAc shell and core is similar, the present study found that mGluR5 in the NAc shell, but not the core, was essential for the retrieval of morphine-associated contextual memory. This result could be explained by the fact that the NAc shell is mostly connected with limbic structures, such as the hippocampus, which might bring about its critical role on behavior in response to contextual cues (Crombag *et al.* 2008). Also, the density of basolateral amygdala projection to the shell is greater than those projecting to the core (Kelley, Domesick & Nauta 1982). And evidence has shown that the innervation of the basolateral amygdala and NAc shell (but not the core) is critically involved in the modulation of memory consolidation (LaLumiere, Nawar & McGaugh 2005).

The NAc core and shell are two functionally and anatomically dissociable subdivisions. Reversible inactivation of the NAc core attenuated cocaine seeking in cue-induced reinstatement, and NAc shell inactivation failed to alter the same behavior (Fuchs *et al.* 2004). Conversely, mGluR2/3 activation in the NAc shell, but not the core, decreased the context-induced reinstatement of heroin seeking (Bossert *et al.* 2006). Based on these studies, it appeared that the NAc core and shell were differentially involved in the reinstatement of drug seeking, depending on the type of drug-associated cues. That is, the NAc core was important in discrete cue-induced drug seeking, and the shell was necessary for context-mediated drug seeking, which supports our present findings. However, a recent report showed that blockade of mGluR5 in the NAc core of rats significantly attenuated context-induced reinstatement following cocaine abstinence (Knackstedt *et al.* 2014). One potential reason for this divergence may be some fundamental differences between opiate and psychostimulant addiction (Crombag *et al.* 2008; Badiani *et al.* 2011). Another reason may be related to the different animal models used. Knackstedt *et al.* conducted experiments with a drug self-administration procedure mediated by response-outcome operant-based memory, while our study utilized the CPP model modulated by Pavlovian-based memory (Shalev, Grimm & Shaham 2002; Milton & Everitt 2010).

The present results showed an increase in membrane mGluR5 protein of the NAc shell, but not the core, following the expression of morphine CPP, which implied the activation of mGluR5. In neurons, mGluR5 is predominantly located postsynaptically around the perisynaptic annulus of dendritic spines (Niswender & Conn 2010). This observation fits with the hypothesis that NAc glutamate spilt out of the synaptic cleft to activate extrasynaptic glutamate receptors in the reinstatement of drug seeking (Brown, Kupchik & Kalivas 2013). Additionally, the activation of mGluR5 can trigger multiple signaling pathways, including PKC and IP_3. Recent studies have demonstrated that phosphorylation of PKCγ in the NAc was increased following cocaine priming-induced reinstatement of drug seeking (Schmidt *et al.* 2014); however, a potential role for IP_3 in the behavioral response induced by addictive drugs has not been examined. As IP_3-mediated calcium release can increase mitochondrial calcium uptake and ROS production (Wu *et al.* 2007), we also observed that mitochondrial ROS formation was induced by the mGluR5 agonist CHPG in primary striatal GABAergic neurons. Even more noteworthy, biochemical studies in cultures have revealed that mGluR5-ROS signaling couples to the activation of ERK, and our previous study showed the activation of ERK in the NAc shell, but not the core, after the expression of morphine CPP in rats (Xu *et al.* 2012). Similar to studies in psychostimulant-induced behavioral sensitization models (Numa *et al.* 2008; Shiba *et al.* 2011), here, we showed that mGluR5-ROS signaling in the NAc shell contributed to the retrieval of morphine-associated contextual memory.

It is interesting to find the messenger property of ROS in addiction mechanisms. Low levels of ROS, such as superoxide and hydrogen peroxide, can increase neuronal excitability. In hippocampal or amygdala neurons, the activation of protein kinase A, calcium/calmodulin-dependent protein kinase II, PKC and ERK was enhanced by increased mitochondrial superoxide (Knapp & Klann 2000; Hongpaisan *et al.* 2004; Li *et al.* 2011), and these protein kinases in the NAc have been critically implicated in reward-based learning. Moreover, ROS were key factors in the activation-dependent phosphorylation of AMPA receptors at GluR1 (Serine 831 and 845) and GluR2 (Serine 880) subunits, which resulted in the rapid trafficking of AMPAR (Lee *et al.* 2012). As these phosphorylation sites also contributed to the reinstatement of drug seeking, whether changes in the AMPAR surface location in reinstatement models is ROS-dependent should be investigated in future work (Pierce & Wolf 2013).

At present, antioxidants have also drawn some attention in the research of addiction. In humans, it has been reported that N-acetylcysteine could reduce cigarette use, marijuana use and present effectiveness in cocaine dependence (Mardikian *et al.* 2007). In animals, the co-administration of the mitochondria-targeted antioxidant melatonin with morphine ameliorated morphine-induced behavioral sensitization in mice (Feng *et al.* 2013). Thus, a crucial role of antioxidants for the treatment of drug addiction is worth further exploration in the future.

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In conclusion, the present study provided the first evidence that mGluR5-ROS signaling in the NAc shell is selectively involved in the retrieval of morphine contextual memory. These findings imply that a potential pharmacological compound, on the basis of mGluR5 antagonists and some antioxidants, might be hopeful for the treatment of relapse of addicts to opiates triggered by environmental cues.

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Disclosure/Conflict of Interest

The authors declare that they have no conflicts of interest.

Authors Contribution

Cailian Cui and Chong Qi conceived and designed the research; Chong Qi, Xinjuan Wang and Feifei Ge performed the experiments; Feifei Ge, Fang Shen, Yijing Li and Junkai Wang provided technical support; Chong Qi drafted the manuscript; and Cailian Cui, Chong Qi and Yijing Li edited and revised the manuscript.

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