Neuropharmacology xxx (2011) 1–8

Contents lists available at SciVerse ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Essential role of protein kinase C in morphine-induced rewarding memory

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ARTICLE INFO

Article history:
Received 31 August 2010
Received in revised form
30 September 2011
Accepted 3 October 2011

Keywords:
Nucleus accumbens
Morphine-conditioned place preference
Protein kinase C
Protein kinase Cγ
γV5-3

ABSTRACT

Protein kinase C (PKC) is involved in intra-cellular signal transduction in various physiological and pathological processes including substance abuse. In the present study, the role of PKC in morphine-induced rewarding memory was investigated using the conditioned place preference (CPP) model. We found a significant translocation of PKCs from cytosol to membrane component in nucleus accumbens (NAc) of morphine-conditioned rats in a dose-dependent manner. The translocation was reduced gradually with the maintenance of morphine-induced CPP. Specifically, the protein level of PKCγ in membrane of the NAc was increased in morphine CPP rats, and decreased during the attenuation of morphine-induced CPP, while the protein level of PKCγ in cytosol of the NAc showed an opposite change. Furthermore, the PKC translocation inhibitor γV5-3 impaired the morphine-induced CPP when micro-injected into the NAc. These findings indicated that PKC, especially the γ isoform, is essential for the acquisition and maintenance of morphine-associated reward memory.

1. Introduction

The protein kinase C (PKC) family, which includes at least 10 isoforms, is divided into 3 groups: conventional PKCs (α, βI, βII, γ), novel PKCs (δ, ε, η, θ) and atypical PKCs (ξ, ι/λ) (Newton, 2003). The conventional PKCs are diacylglycerol (DAG) sensitive and Ca^{2+} responsive, the novel PKCs are sensitive to DAG but not to Ca^{2+}, while the atypical PKCs are insensitive to both DAG and Ca^{2+} (Rykx et al., 2003). One of the major signal transduction cascades that lead to PKC activation is the phosphoinositide (PI) signaling pathway (Fisher et al., 1992). Stimulation of certain G protein-coupled receptors activates phospholipase C, which hydrolyzes phosphatidylinositol-4, 5-bis-phosphate to form inositol triphosphate (IP_3) and DAG. IP_3 binds to intra-cellular receptors causing release of Ca^{2+} from the endoplasmic reticulum, whereas DAG binds to and activates most PKC isozymes (Nishizuka, 1995).

Chen and Huang (1991) demonstrated that PKC is involved in μ-opioid receptor-induced signals that promote sustained potentiation of *N*-methyl-D-aspartate receptor (NMDAR)-mediated glutamate responses. In addition, our previous work demonstrated that NR2B-containing NMDA receptors in the NAc are essential in morphine-induced reinforcing effects (Ma et al., 2006, 2007). In the present study, using the conditioned place preference (CPP), we investigated the importance of PKC in the NAc in morphine-related associative memory paradigm, and also by intra-NAc microinjection of γV5-3, a PKCγ translocation inhibitor, we explored if the translocation of PKCγ from cytosol to membrane is necessary for acquisition and maintenance of morphine-induced CPP.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats, 200–230 g at the beginning of the experiment, were housed 4 per cage in a 12:12 h light/dark cycle (lights on at 07:00 a.m.) with food and water supplied *ad libitum* except during behavioral training sessions. The room temperature was maintained at 22 ± 1 °C and relative humidity at 45–55%. Rats were handled twice a day during the first 5 days after arrival. All experimental procedures were approved by the Animal Use Committee of Peking University Health Science Center.

2.2. Apparatus

A three-chambered CPP apparatus (795 × 230 × 250 mm^3) with distinct visual and tactile cues was used to conduct the conditioning (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 4

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doi:10.1016/j.neuropharm.2011.10.001

light-emitting diodes (LEDs) forming a square on the wall and a stainless-steel mesh floor while chamber C had 4 LEDs forming a triangle on the wall and a stainless-steel rod floor, and chamber B had a plain wooden floor. Fourteen photobeams were placed across the chambers and were 47.5 mm apart. Through a computer interface, the time spent in each chamber was recorded by means of infrared beam crossings.

2.3. Morphine CPP conditioning procedure

2.3.1. Pre-conditioning test

On day 0, rats were placed in the center choice chamber B with the guillotine doors removed to allow access to the entire apparatus for 15 min and time spent in each chamber was recorded. The pre-conditioning test showed that animals spent almost an equal amount of time in the two end chambers (A: 315 ± 8.23 s, C: 308 ± 7.51 s) and less time in the small center choice chamber (B: 275 ± 10.01 s). There were no significant differences in the time spent in the two end chambers ($p > 0.05$). Thus, the CPP apparatus was considered as unbiased in terms of chamber preferences of untreated rats. The chambers selected for pairing with morphine were counterbalanced within each group. The data from pre-conditioning tests were used to separate animals into groups with approximately equal biases for each chamber. Rats with a bias for either of the lateral chambers were excluded (about 5%) from the experiments.

2.3.2. Conditioning

The animals were allowed for training sessions twice a day (08:30 and 15:30) for 4 days (day 1–4). Before being confined into one lateral chamber for 45 min, rats were received morphine (*i.p.*, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10 mg/kg for CPP expression studies and 3 mg/kg for CPP retention studies), and physical saline in the other lateral chamber. Animals in control groups received saline injections before both training sessions in alternate lateral chambers. In the morphine treated groups, half of the animals were received morphine training in compartment A and saline training in compartment C, while the rest were received morphine and saline training in compartment C and A respectively for counterbalance. Moreover, half of rats were conditioned with morphine in the morning session and saline in the afternoon, while the other half were received contrary sessions.

2.3.3. Post-conditioning test

On day 5, all animals were placed in chamber B with the guillotine doors removed to allow access to the entire apparatus for 15 min and time spent in each chamber was recorded.

2.3.4. Retention of morphine CPP

After the establishment of CPP, rats were randomly divided into 10 groups. At every test point of 1st, 8th, 16th, 32nd, 64th days after the last day of conditioning training, two corresponding groups of rats, conditioned with morphine and saline respectively, were tested in CPP chambers. The time spent in each chamber was recorded. CPP was not expressed any more on the 64th day.

The locomotor activity during all of the above mentioned CPP tests was estimated by counting the total number of crossings between any two adjacent compartments.

2.4. Implantation of microinjection cannulae

Rats were anesthetized with pentobarbital sodium solution (40 mg/kg, *i.p.*) and mounted on a Kopf stereotaxic apparatus (Kopf Instruments, Tujunga, CA). The scalp was incised and retracted, and the head position was adjusted to place bregma and lambda in the same horizontal plane. Small holes (1 mm in diameter) were drilled on the skull for bilateral placement of stainless-steel guide cannulae (0.8 mm in outer diameter) into the NAc shell (NAcS) (anteroposterior (AP) +1.5 mm, lateral (L) ±0.9 mm, dorsoventral (DV) −6.5 mm), 2.0 mm above the intended side of injection. Guide cannulae were fixed on the skull with sterile stainless-steel screws and the dental acrylic cement.

Stainless-steel obturators (0.4 mm in outer diameter) were inserted into the guide cannulae to avoid occlusion. The obturators were removed and reinserted every day during the recovery period after cannulation surgery to prevent them from adhering to the cannulae. Every animal was injected with penicillin (1.5×10^5 U/rat, *s.c.*) after surgery and the following 3 days.

2.5. Microinjections

Animals were microinjected 30 min before conditioning. Obturators were removed and bilateral infusion cannulae (0.4 mm in outer diameter) were inserted, extending 1.0 mm beyond the tip of guide cannulae in the NAcS. Bilateral microinjections of γV5-3 (1 μg or 10 μg/rat) or Tat (10 μg/rat) or vehicle (5 μl normal saline/rat) were administered into the NAcS using a 10-μl syringe (0.5 μl/min). The microinjectors were left in place for an additional minute to allow for drug diffusion. The obturators were then reinserted, and rats were placed back to their home cages.

2.6. Histology and histochemistry

Histological verification of cannulae location was performed after behavioral tests. Rats were anesthetized with chloral hydrate (40 mg/kg, i.p.) and perfused intracardially with 0.9% saline (300 ml) followed by 4% paraformaldehyde solution (350 ml). The brains were removed and post-fixed in 4% paraformaldehyde solution for 24 h, then immersed in 20% sucrose solution followed by 30% sucrose solution until sectioning. Coronal sections (30 μm thick) were cut on a cryostat (−22 °C) and wet-mounted on glass microscope slides (Fig. 6). Four animals whose cannulae were placed incorrectly were excluded from data analysis.

To verify proper diffusion of γV5-3, naïve rats were microinjected with biotin-labeled Tat-γV5-3 (10 μg/rat) and perfused with 0.9% saline followed by 4% paraformaldehyde solution 30 min after microinjection and coronal slices were made as described above. Sections containing the NAc shell were processed using a StreptAvidin Peroxidase-DAB reaction to visualize the extent of the distribution and cellular uptake of the Tat-γV5-3. For immunohistochemistry detection, slides were washed with phosphate-buffered saline (PBS) containing 0.5% Triton X-100 for 30 min, and incubated with peroxidase-labeled StreptAvidin (25 μg/μl) (Zhongshan Biotechnology, Beijing, China) for 2 h at room temperature. The sections were then washed with 0.5% Triton X-100 in PBS for 30 min again, and stained using DAB (Zhongshan Biotechnology, Beijing, China) reaction for 5 min. Slides were then dehydrated for 5 min in 80%, 5 min in 90%, 2 × 5 min in 100% ethanol and 2 × 5 min in xylol, and embedded in Neutral Balsam. Stained slides were viewed and photographed with a CCD camera attached to a microscope (DMIRB, Leica, Germany). As shown in Fig. 7, biotin-labeled Tat-γV5-3 diffused into the cytoplasm of cells surrounding the cannula track.

2.7. Tissue dissection and preparation

Rats were decapitated immediately after testing. The brains were removed and placed on an ice-cold plate for dissection of the NAc. The samples were frozen in

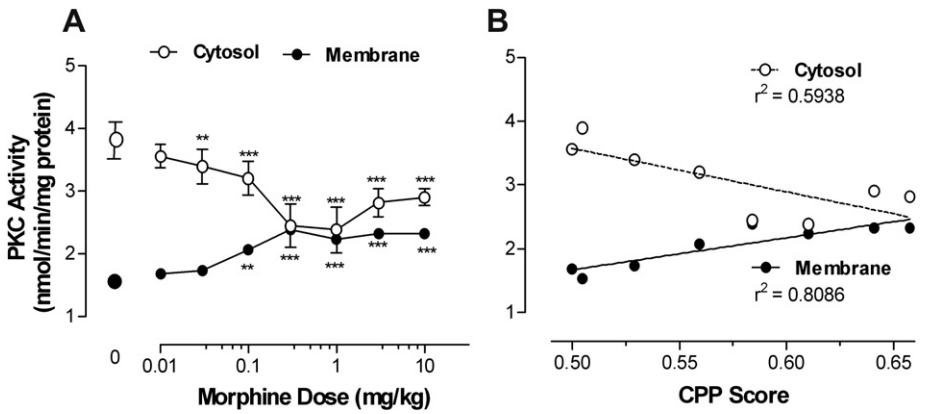

Fig. 1. Effects of morphine dose on PKC activity of NAc in CPP expression. A. PKC activity in the NAc membrane and cytosol fractions from rats conditioned by morphine (0–10 mg/kg). Data were expressed as mean ± S.E.M and analyzed using two-way repeated measures ANOVA followed by Bonferroni *post-hoc* test. **, ***, $p < 0.01$, 0.001, compared with saline (i.e. 0 mg/kg morphine) group. $n = 4$ in each group. B. Correlation of PKC activity in the NAc cytosol and membrane fractions with the CPP expression score. Data were expressed as the means of PKC activity and CPP scores, and analyzed by linear regression.

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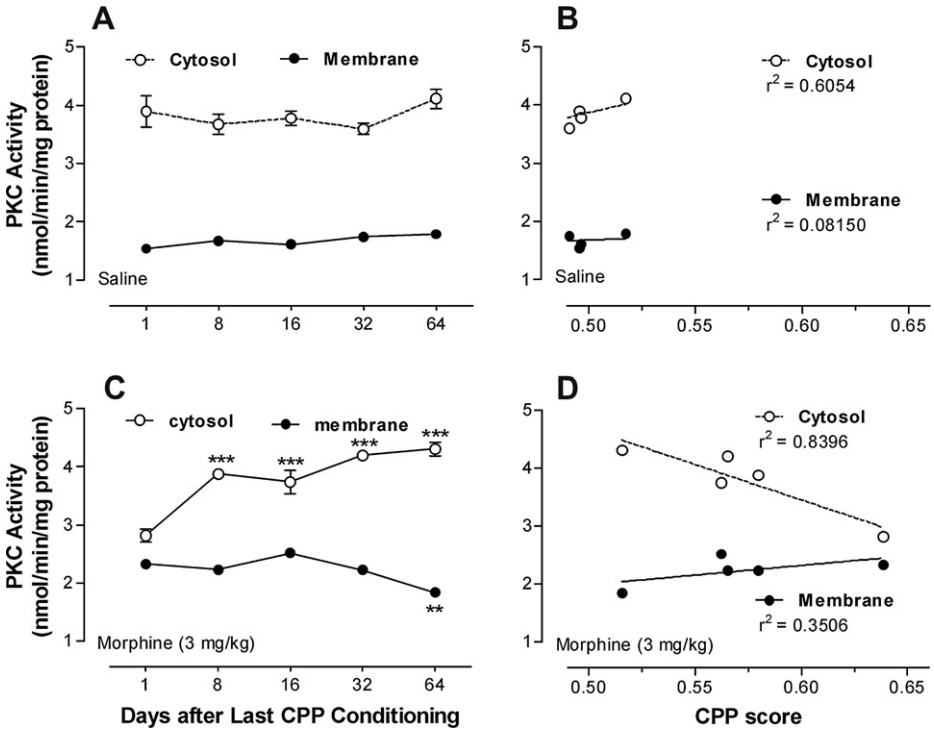

Fig. 2. Temporal-dependent change of the NAc PKC activity during CPP maintenance. A, C, PKC activity in NAc membrane and cytosol fractions during the maintenance of CPP conditioned by saline and morphine (3 mg/kg), respectively. Data were expressed as mean ± S.E.M and analyzed using two-way repeated measures ANOVA followed by Bonferroni *post-hoc* test. **, ***, $p < 0.01$, 0.001, compared with day 1 ($n = 4$ in each group). B, D, Correlation of PKC activity in the NAc cytosol and membrane fractions with the CPP score during the maintenance of CPP conditioned by saline and morphine (3 mg/kg), respectively. Data were expressed as the means of PKC activity and CPP scores, and analyzed by linear regression.

liquid nitrogen and stored at −80 °C until analysis. The tissue was homogenized in 250 μl ice-cold buffer containing 20 mM Tris–HCl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulfonyluoride, 25 mg/ml of leupeptin, 0.1 mg/ml of aprotinin and 0.32 M sucrose using a Potter-Elvehjem tissue grinder with Teflon pestle. The homogenate was then centrifuged at 1000 × g for 10 min and the supernatant was centrifuged at 26,000 × g for 1 h at 4 °C. The supernatant was used as the cytosol fraction. The pellet was then homogenized in homogenizing buffer containing 0.2% (w/v) Triton X-100. The homogenate was kept at 4 °C for 1 h with

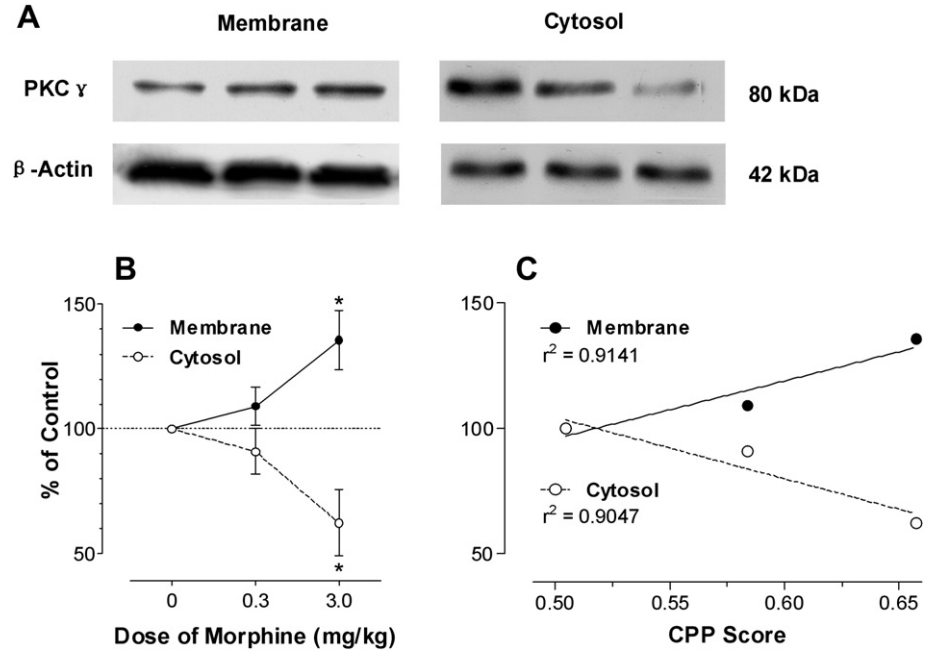

Fig. 3. Effects of morphine dose on the protein levels of PKCγ in CPP expression. A, Representative bands from Western blots. B, PKCγ activity in the NAc membrane and cytosol fractions from rats conditioned by morphine (0, 0.3, 3.0 mg/kg). Data were expressed as mean ± S.E.M of the normalized PKCγ content and analyzed using two-way repeated measures ANOVA followed by Bonferroni *post-hoc* test. *, $p < 0.05$, compared with saline (i.e. 0 mg/kg morphine) group ($n = 4$ in each group). C, Correlation of PKCγ content in the NAc cytosol and membrane fractions with the CPP score from CPP expression tests. Data were expressed as the means of PKC activity and CPP scores, and analyzed by linear regression.

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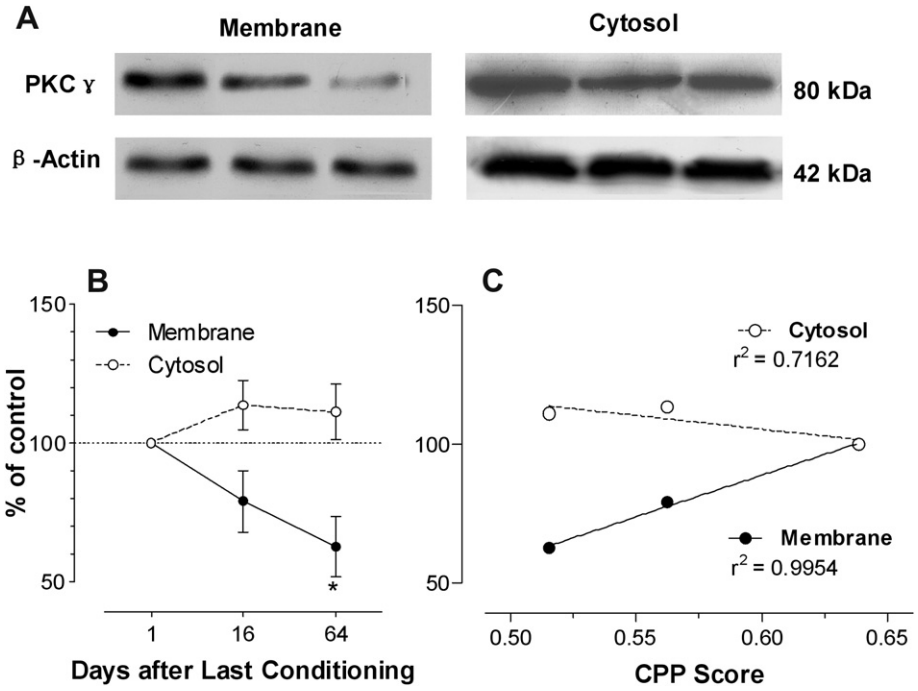

Fig. 4. Temporal-dependent changes of PKCγ protein levels during CPP maintenance. A, Representative bands from Western blots. B, PKCγ activity in the NAc membrane and cytosol fractions from rats during the CPP retention tests. Data were expressed as mean ± S.E.M of the normalized PKCγ content and analyzed using two-way repeated measures ANOVA followed by Bonferroni *post-hoc* test. *, $p < 0.05$, compared with day 1 ($n = 4$ in each group). C, Correlation of PKCγ content in the NAc cytosol and membrane fractions with the CPP score from CPP expression tests. Data were expressed as the means of PKC activity and CPP scores, and analyzed by linear regression.

occasional stirring and then centrifuged at 26,000 × g for 1 h at 4 °C. The resulting supernatant was used as the membrane fraction. Protein concentrations were determined using a BCA assay (Pierce, Rockford, IL).

2.8. Determination of PKC activity in membrane and cytosol fractions of rat brain

PKC activity in subcellular tissue fractions was measured following the procedure described by Dwivedi and Pandey (Dwivedi and Pandey, 1999). PKC activity was determined using the Amersham enzyme assay system, in which a PKC-specific targeting peptide as well as all the necessary cofactors was provided. Assay tubes (with a final incubation volume of 75 ml) contained 25 μl of a component mixture [3 mM Ca $(C_2H_3O_2)_2$, 75 μg/ml L-a-phosphatidyl-L-serine, 6 μg/ml PMA, 225 μM substrate peptide, and 7.5 mM dithiothreitol in 50 mM Tris–HCl containing 0.05% sodium azide, pH 7.5] and 25 μl of membrane or cytosol fraction. The reaction was initiated by addition of 25 μl of Mg-ATP buffer (10 μCi/ml [γ-^{32}P] ATP, 1.2 mM ATP, 72 mM $MgCl_2$, and 30 mM HEPES, pH 7.4) to each tube. The subcellular samples were incubated for 15 min at 37 °C, and the reaction was terminated by addition of 100 μl of the "stop" reagent (300 mM orthophosphoric acid containing carmosine red) to each tube. An aliquot of the solution from each tube (35 μl) was blotted onto individual peptide-binding papers. Papers were washed with 75 mM phosphoric acid twice for 5 min. Papers were dried, and the retained radioactivity was counted by a liquid scintillation counter. The result was expressed as nanomoles per minute per milligram of protein (Dwivedi and Pandey, 1999).

2.9. Immunoblotting of PKCγ

Equivalent amounts of membrane or cytosol fractions (50 μg) for each sample were resolved in 8% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes after electrophoresis. The membranes were incubated in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBST) containing 5% nonfat milk for 1 h at room temperature with agitation. Then the membranes were incubated with primary antibody diluted in TBST containing 5% nonfat dried milk (PKCγ, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. On the next day, the membranes were washed thrice for 5 min in TBST, followed by 1 h of incubation at room temperature with horseradish peroxidase-conjugated rabbit anti-goat IgG (Zhongshan Biotechnology, Beijing, China) diluted 1:10,000 in TBST containing 5% nonfat dried milk. After this incubation, the membranes were washed twice for 5 min and then twice for 10 min in TBST. The antigen–antibody peroxidase complex was finally detected by enhanced chemiluminescence (Zhongshan Biotechnology, Beijing, China) and visualized by exposure to Kodak film (Eastman Kodak, Kodak, NJ). The bands on the autoradiogram were quantified with the TotalLab 2.01 Analysis System (Phoretix, UK), and the optical density of each band of the PKCγ subunit was corrected by the optical density of the corresponding β-actin band. The values are presented as a percentage of the control.

2.10. Drugs

Morphine hydrochloride was purchased from the First Pharmaceutical Factory of Shenyang, China. γV5-3 (amino acids 659–664 [CRLVLAS] conjugated to Tat transmembrane carrier peptide (amino acid 47–57) via a cysteine–cysteine bond at its N-terminus) (Chen et al., 2001) and Tat ([CYGRKKRRQRRR]) (Wu et al., 2010), as well as biotin-labeled Tat-γV5-3 were synthesized by Chinese Peptide Company (Hangzhou, China). All drugs were dissolved in normal saline.

2.11. Statistical analysis

CPP score represents the index of place preference for each rat, calculated by dividing the time spent in the drug-paired compartment by the time spent in both conditioning compartments (Shi et al., 2004). Data were analyzed by commercially available software Graph Pad Prism 4.0. Results were presented as mean ± SEM. Results from Figs. 1–5 were analyzed with two-way repeated measures analysis of variance (ANOVA) followed by Bonferroni *post-hoc* tests, results from Tables 1 and 2 were analyzed with one-way ANOVA followed by Newman–Keuls *post-hoc* tests. The accepted level of statistical significance was $p < 0.05$.

3. Results

3.1. PKC activity was up-regulated in membrane fractions of the NAc in morphine-conditioned rats whereas that in cytosol was reduced

Different doses of morphine (0, 01, 0.03, 0.1, 0.3, 1.0, 3.0 and 10 mg/kg) were administered by intraperitoneal (i.p.) injection in the four sessions of conditioning as described by Ma et al. (2009). CPP test was performed in the followed day. Significant place preference was observed in the groups treated with morphine at doses from 0.3 to 10 mg/kg (CPP scores), with maximum response obtained at 3.0 mg/kg of morphine (Table 2). Using [^{32}P] labeling substrate method, we determined the activity of PKC in the NAc of

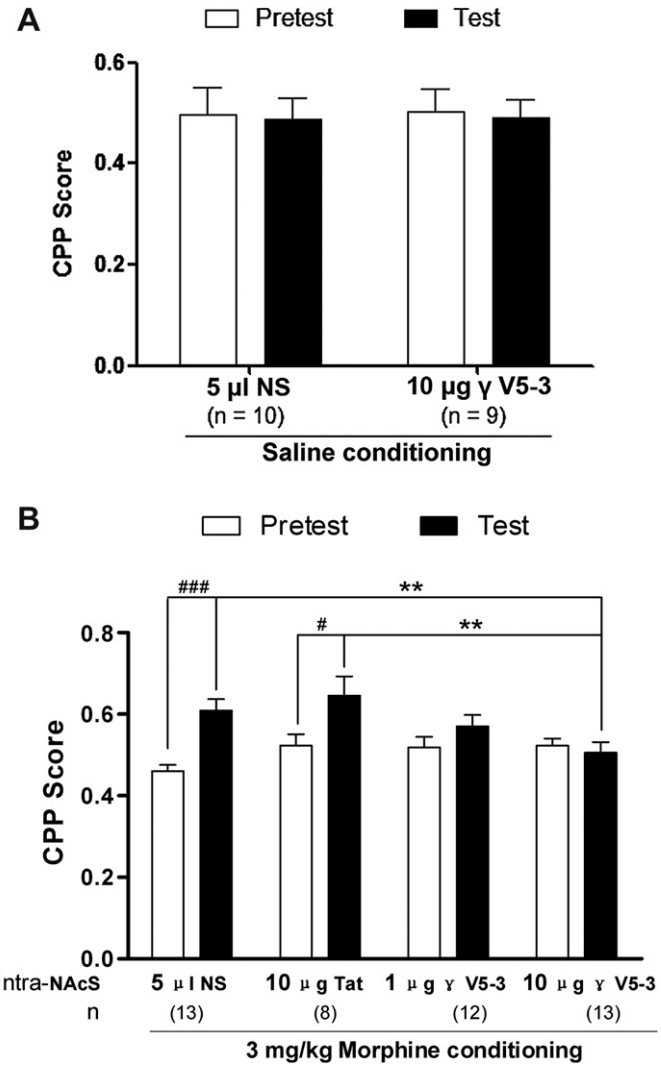

Fig. 5. Effects of intra-NAcS injection of γV5-3 on morphine-induced place preference. A. Intra-NAcS microinjection of 10 µg γV5-3 didn't induce any preference or aversion, comparing to that treated with saline. B. Effects of saline, Tat, 1 µg and 10 µg γV5-3 on morphine-induced place preference. #, ###, $p < 0.05$, 0.001, pretest *vs*. test. *, **, $p < 0.05$, 0.01, respectively, compared among groups with microinjections of different solutions. Data were expressed as mean ± S.E.M of CPP scores, and analyzed using two-way repeated measures ANOVA followed by Bonferroni *post-hoc* test.

rats conditioned with morphine at doses of 0, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10.0 mg/kg (Fig. 1A). Two-way repeated measures ANOVA showed significant effects of the dose of morphine ($F_{7, 24} = 2.55$, $p = 0.0413$), the membrane *vs*. cytosol fractions ($F_{1, 24} = 307.31$, $p < 0.0001$) and the interaction of these two factors ($F_{7, 24} = 26.06$, $p < 0.0001$). Bonferroni *post-hoc* test showed significant increases of PKC activity in the membrane protein (at least $p < 0.01$, between the rats conditioned by morphine of ≥0.1 mg/kg *vs*. saline) and significant decreases in the cytosol protein (at least $p < 0.01$, between the rats conditioned by morphine at ≥0.03 mg/kg *vs*. saline). Moreover, PKC activities in protein extracts from the membrane fraction presented strong positive correlations with CPP expression score ($r^2 = 0.8086$, $p = 0.0024$, Fig. 1B). Our data suggested that more PKC was transferred from cytosol to membrane (as an activated state) as the dose of morphine increased and, more importantly, PKC activity was positively correlated to the time spent in the morphine-paired chambers.

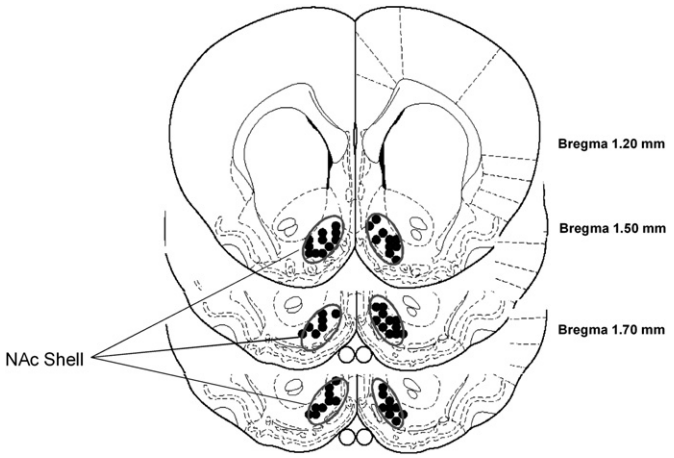

Fig. 6. Distribution of microinjection sites in the nucleus accumbens shell (gray circle), plotted on drawings of coronal sections from the atlas of Paxinos and Watson (1997).

3.2. The PKC activity was decreased in membrane fractions but increased in cytosol of the NAc during the retention of morphine CPP

Lower panel in Table 2 shows the behavioral scores during CPP retention induced by 3 mg/kg morphine. Bonferroni post-test after significant two-way ANOVA indicated that the CPP induced by 3 mg/kg morphine lasted until 64 days after the last conditioning. Two-way repeated measures ANOVA (fractions of membrane *vs*. cytosol, $F_{1, 15} = 665.47$, $p < 0.0001$; days after last conditioning, $F_{4, 15} = 15.87$, $p < 0.0001$; interaction of these two factors, $F_{4, 15} = 30.89$, $p < 0.001$), followed by Bonferroni *post-hoc* test showed the activity of PKC in the NAc membrane of morphine-conditioned rats decreased gradually during the abstinence from morphine ($p < 0.01$, day 64 *vs*. day 1), while the PKC activity in the NAc cytosol displayed an opposite change, i.e. increasing since 8 days after the last CPP conditioning ($p < 0.001$, day 8, 16, 32, 64 *vs*. day 1) (Fig. 2C). No significant differences were shown for the activity of PKC in the NAc of normal saline-conditioned rats (among days, $F_{4, 15} = 1.88$, $p = 0.1664$; interaction $F_{4, 15} = 1.27$, $p = 0.3241$), although there were differences between fractions of membrane *vs*. cytosol ($F_{1, 15} = 637.99$, $p < 0.0001$) (Fig. 2A). Moreover, PKC activities in protein extracts from the cytosol fraction presented strong positive correlations with CPP retention score ($r^2 = 0.8396$, $p = 0.0287$, Fig. 2D), suggesting that the translocation of PKC from cytosol to membrane decreased in parallel with the decrease of place preference to the morphine-paired chamber.

3.3. The translocation of PKCγ from cytosol to membrane in the NAc was increased when morphine CPP was established, but decreased during the retention of morphine CPP

We also determined the protein levels of PKCγ in the NAc of conditioned rats after the CPP expression test (representative bands of Western blots in Fig. 3A). Two-way repeated measures ANOVA showed a significant interaction between morphine doses and fractions ($F_{2, 9} = 15.6$, $p = 0.0012$) (Fig. 3B), although no significant effect was shown in the conditioning dose of morphine ($F_{2, 9} = 0.01$, $p = 0.9928$). Bonferroni *post-hoc* test showed a significant increase of PKCγ in the membrane fraction and a decrease in the cytosol fraction ($p < 0.05$, compared with saline-conditioned group). Moreover, protein level of PKCγ in extracts from the membrane and cytosol fractions presented strong positive and negative correlations, respectively, with CPP expression score ($r^2 > 0.9$, Fig. 3C), suggesting that the translocation of PKCγ from cytosol to

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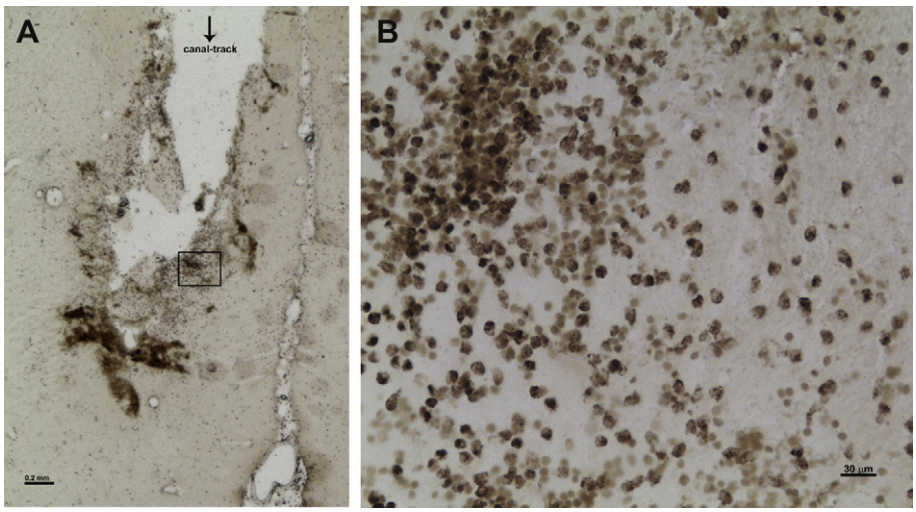

Fig. 7. Visualization of DAB reaction of Biotin-labeled Tat-γV5-3 in the NAc shell. A. Cannula track in the NAc shell. B. Enlargement of the boxed area in A.

membrane increased in parallel with the increase of place preference to the morphine-paired chamber after conditioning with high dose of morphine.

During the retention of morphine-conditioned CPP, the levels of PKCγ in membrane and cytosol fractions were also examined by Western blots (representative bands shown in Fig. 4A). Two-way repeated measures ANOVA showed a significant interaction between the two factors of day and fraction ($F_{2,\ 9} = 5.68$, $p = 0.0254$), although no significant effect was shown among the days after the last conditioning ($F_{2,\ 9} = 1.09$, $p = 0.3761$, Fig. 4B). Bonferroni *post-hoc* test showed a significant decrease of PKCγ in the membrane fraction 64 days after the last conditioning ($p < 0.05$, compared with 1 day after last conditioning). Moreover, protein level of PKCγ in extracts from the membrane fraction presented a strong positive correlation with CPP score in CPP retention ($r^2 = 0.9954$, $p = 0.0430$, Fig. 4C), suggesting a significant decrease of PKCγ activity in parallel with the decrease of place preference to the morphine-paired chamber.

Table 1
Locomotor activity in CPP tests. A. The locomotor activity in tests of CPP (for Figs. 1–4) expression (upper panel) and maintenance (lower panel) was assessed as described in the Materials and methods section. B. The locomotor activity in tests of CPP expression (for Fig. 5). Data are expressed as mean ± S.E.M. No significant differences of locomotor activity were detected by one-way ANOVA in both CPP expression and maintenance.

A. Locomotor activity in CPP tests of Figs. 1–4

CPP test		Locomotor activity	*n*
CPP expression			
Dose of morphine (mg/kg, i.p.)	0	410.0 ± 23.31	12
	0.01	451.6 ± 25.13	10
	0.03	442.6 ± 27.90	10
	0.1	460.9 ± 15.77	10
	0.3	441.8 ± 38.94	10
	1	430.9 ± 27.77	9
	3	422.6 ± 25.69	9
	10	434.9 ± 39.19	10
CPP maintenance			
Days after last CPP conditioning	1	451.8 ± 28.1	9
	8	414.8 ± 24.98	10
	16	417.0 ± 27.27	11
	32	428.3 ± 26.80	11
	64	437.0 ± 27.27	12

B. Locomotor activity in CPP tests of Fig. 5

CPP test		Locomotor activity	*n*
Group			
i.c.v.	i.p.		
Saline	Saline	387.6 ± 50.55	11
10 μg γV5-3	Saline	357.9 ± 43.21	9
Saline	3 mg/kg morphine	406.4 ± 34.82	14
5 μg Tat	3 mg/kg morphine	386.8 ± 53.34	8
1 μg γV5-3	3 mg/kg morphine	366.7 ± 51.08	12
10 μg γV5-3	3 mg/kg morphine	367.1 ± 43.18	13

3.4. Intra-NAcS infusion of PKCγ inhibitor γV5-3 blocked the acquisition of morphine CPP

Since there was an increase of PKCγ translocation after the acquisition of morphine-induced CPP, we next used the PKCγ translocation inhibitor, γV5-3 to investigate if translocation of PKCγ was necessary in the establishment of morphine-induced CPP. In order to confirm if γV5-3 could induce any preference or aversion by itself, intra-NAcS microinjection of saline or 10 μg γV5-3 30 min before saline conditioning was performed in saline control rats. Two-way repeated measures ANOVA showed no differences of the CPP scores between pre- *vs.* post-conditionings ($F_{1,\ 17} = 0.16$, $p = 0.6950$) and intra-NAcS microinjection between saline *vs.* γV5-3 ($F_{1,\ 17} = 0.01$, $p = 0.9418$) as well as no interaction effect of these two factors ($F_{1,\ 17} = 0.00$, $p = 0.9593$) (Fig. 5A), suggesting no influence of γV5-3 on place preference tests.

Fig. 5B showed the effect of intra-NAcS injection of 5 μl saline, 10 μg Tat, 1 μg γV5-3 and 10 μg γV5-3 on morphine-induced place preference. Two-way repeated measures ANOVA displayed that there was an obvious change in preference score between pre- and post-conditionings ($F_{1,\ 41} = 14.4$, $p = 0.0005$), though the four treatments didn't showed a significant effect ($F_{3,\ 41} = 2.33$, $p = 0.0888$), there's an interaction effect of morphine conditioning × intra-NAcS treatment ($F_{3,\ 41} = 3.81$, $p = 0.0169$). The followed Bonferroni *post-hoc* tests showed 3 mg/kg morphine-induced CPP was only observed in rats treated by microinjections of saline or Tat before each morphine conditioning ($p < 0.001$, 0.05, pretest *vs.* test, respectively), but not by microinjections of γV5-3 (1, 10 μg). Furthermore, significant differences of test scores were demonstrated between groups treated by saline *vs.* 10 μg γV5-3 and Tat *vs.* 10 μg γV5-3 ($p < 0.01$ respectively, two-way repeated measures ANOVA followed by Bonferroni *post-hoc* tests). These data indicated that inhibition of PKCγ translocation by γV5-3

Table 2
Summary of CPP scores. Data are expressed as mean ± S.E.M. One-way ANOVA and two-way ANOVA were used in the upper and lower panel, respectively. *, **, ***, $p < 0.05$, 0.01, 0.001, compared with saline (i.e. 0 mg/kg morphine) group; ##, $p < 0.01$ compared with 1 day after last conditioning. For the CPP expression: one-way ANOVA: $F(7, 72) = 9.169$, $p < 0.0001$; For the CPP retention: two-way ANOVA: Treatment (morphine or saline conditioning): $F(1, 104) = 37.56$, $p < 0.0001$; Time: $F(4, 104) = 1.75$, $p < 0.1456$; Interaction: $F(4, 104) = 3.32$, $p < 0.0133$.

CPP expression								
Morphine dose (mg/kg)	0 (i.e. saline)	0.01	0.03	0.1	0.3	1	3	10
CPP score	0.50 ± 0.01	0.50 ± 0.03	0.53 ± 0.03	0.56 ± 0.02	0.58 ± 0.01*	0.61 ± 0.01**	0.66 ± 0.01***	0.64 ± 0.03***
CPP retention								
Days after last CPP conditioning				1	8	16	32	64
Conditioned by		Saline		0.5 ± 0.01	0.47 ± 0.03	0.50 ± 0.02	0.51 ± 0.03	0.51 ± 0.01
		Morphine (3 mg/kg)		0.64 ± 0.02***	0.58 ± 0.02**	0.56 ± 0.02*	0.57 ± 0.01	0.52 ± 0.01##

attenuated morphine-induced place preference, and this effect was not produced by the transmembrane carrier Tat, which was used to help the inhibitor to penetrate the cell membrane (Wu et al., 2010).

4. Discussion

There has been a great amount of research on the mechanisms of morphine abuse, but it is still hard to clarify the downstream signaling pathways induced after morphine binds to μ-opioid receptors. A great number of molecules are involved in this complex process, including protein kinase C (Bailey and Connor, 2005; Martini and Whistler, 2007; Mayer et al., 1995; Narita et al., 2008).

The PKCs are a family of phospholipid-dependent serine/threonine protein kinases that are highly distributed in the brain. PKC plays a major role in cellular regulatory and signaling processes (Shu and Selmanoff, 1988; Zurgil and Zisapel, 1985). Once fully processed and phosphorylated, PKC can respond to second messengers, engage and phosphorylate downstream targets (Barnett et al., 2007; Leithe et al., 2003). There is accumulating evidence that PKC may contribute to morphine abuse (Benavides et al., 2005; Shukla et al., 2006; Ueda, 2004).

Narita et al. (2001) reported that an intracerebroventricular injection of calphostin C, a specific PKC inhibitor, produced a dose-dependent reduction in the morphine-induced place preference (Narita et al., 2001), indicating the PKC in the limbic forebrain may be critical for the development and/or maintenance of the reinforcing effects induced by morphine in mice. In the present study, we focused on the PKC activity of the NAc in acquisition and maintenance of morphine-induced CPP. We found that at different doses of morphine-induced CPP, the translocation of PKC from cytosol to membrane (which indicates PKC's activation) was increased in a morphine dose-dependent manner, while decreased gradually during the retention of morphine CPP (Figs. 1 and 2), suggesting that PKC activity in the NAc was involved in the acquisition and maintenance of rats' response to the drug associated environmental cues.

There are at least 10 isoforms of PKC that are divided into 3 groups related through their primary structure (Barnett et al., 2007; Newton, 2003). PKCγ is a specific member of the conventional PKCs, the most unique characteristics of this isoform is that it is only expressed in brain and spinal cord (Nishizuka, 1988).

It is well documented that the brain specific PKCγ is particularly involved in spatial learning and memory processes (Bowers et al., 1995; Nithianantharajah and Murphy, 2009; Van der Zee et al., 1997; Alvarez-Jaimes et al., 2004), and also in drug reward memory (Lai et al., 2008; Miyatake et al., 2005). Narita et al. (2001) reported that spatial experience and training resulted in activation of PKCγ. Also, the age-related impairments of spatial memory, as well as deficits in the flexible use of previously acquired information, may result from dysregulation of PKCγ (Colombo and Gallagher, 2002). Blockade of NAc-PKCγ translation via the NAc injection of antisense oligodeoxynucleotide caused impairments in the early phase of learning and retention of spatial information, which demonstrated that NAc-PKCγ plays a role during the early acquisition and consolidation of spatial learning (Alvarez-Jaimes et al., 2004).

There is also growing body of evidence showing that PKCγ is important in drug reward memory. It has been reported that the protein level of PKCγ was significantly up-regulated in membrane fractions of the limbic forebrain obtained from morphine-conditioned mice compared to those from saline-conditioned mice. However, the protein levels of PKC α, βI, βII, and ε were not affected in the same preparation. Furthermore, PKCγ knockout mice showed a deficit of morphine-induced CPP (Narita et al., 2001). These findings suggested that PKCγ in the limbic forebrain may be critical for the development of reinforcing effects induced by morphine in mice. In our research, we detected the content of PKCγ in NAc of morphine-conditioned rats. The level of PKCγ in the membrane was increased after the morphine CPP acquisition and decreased during its retention whereas that in the cytosol showed an opposite trend, which means there might be a positive relationship between the translocation of PKCγ from cytosol to membrane with the degree of preference to morphine-associated environmental cues.

Furthermore, we observed the effect of intra-NAcS infusion of PKCγ translocation inhibitor γV5-3 on the acquisition of morphine-induced CPP. The morphine CPP was blocked with administration of 1 or 10 μg γV5-3 into the NAcS before conditioning, and γV5-3 could not result in rats' preference or aversion itself, whereas the Tat transmembrane carrier and vehicle (normal saline) did not affect the morphine CPP. All these findings implied that the translocation of PKCγ from cytosol to membrane in the NAc is critical for the expression and maintenance of the morphine-related reinforcing effects.

The NAc is well-known as a pivotal region involved in rewarding effect induced by drug abuse. In our previous work, we demonstrated the level of NR2B-containing NMDA receptors in the NAc were elevated in morphine CPP rats, while inhibition of NR2B-containing NMDA receptors in the NAc blocked morphine CPP. However, the change of NR2B subunit levels in rats with morphine injection following the same schedule as in the CPP experiment but without the behavioral component of place preference was not significantly different from that in control rats with alternative saline injection only, suggesting that the changes in the concentration of the NR2B subunit were due to the combination of the activation of the reward circuit by morphine and the CPP paradigm but not by pharmacological actions of morphine alone (Ma et al., 2006, 2007). It has been showed that PKC can affect NR2B/NR1 NMDA currents by direct phosphorylation of the NR2B tail at residues S1303 and S1323 (Liao et al., 2001). Chen and Huang (1991) demonstrated that a selective μ-opioid receptor agonist DAMGO

Please cite this article in press as: Ping, X., et al., Essential role of protein kinase C in morphine-induced rewarding memory, Neuropharmacology (2011), doi:10.1016/j.neuropharm.2011.10.001

caused a sustained increase in glutamate-activated currents that were mediated by NMDARs, and this increase was PKC dependent (Chen and Huang, 1991), so it is reasonable to speculate that NR2B may be one of the downstream substrates of PKC when it is activated via morphine, and the change of PKC activity may be result from the morphine-associated learning but not the only pharmacological effect of morphine.

It has been reported that inhibition of PKC activity decreases morphine-induced c-Fos expression in the striatum (Harlan et al., 2004), and the c-Fos expression in the striatum is also dependent on AMPA receptors (Arai et al., 1994) and mGluR1 (Moroni et al., 1997). Interestingly, both AMPA receptors and mGluR1 mediated, morphine-induced c-Fos expression is PKC activity-mediated (Garcia et al., 2003). These could be a way by which PKC may take part in morphine-related reinforcing effects, which suggests a prominent role of receptors coupled to PKC activation in the actions of morphine in the striatum. That might be one of the mechanisms by which PKC contributed to morphine dependence.

5. Conclusion

The present studies indicate that the translocation and activation of PKC may be a critical step in the development and maintenance of morphine rewarding memory. Modulation of PKC, especially PKCγ translocation and activation in the NAc may prove useful for the management of morphine dependence.

Acknowledgments

Our work was supported by a grant (30970933) from the National Natural Science Foundation and the National Basic Research Program (2009CB522003) of China.

References

Alvarez-Jaimes, L., Betancourt, E., Centeno-Gonzalez, M., Feliciano-Rivera, M.Z., Rodriguez, D., Pena de, O.S., Maldonado-Vlaar, C.S., 2004. Spatial learning in rats is impaired by microinfusions of protein kinase C-gamma antisense oligodeoxynucleotide within the nucleus accumbens. Neurobiol. Learn. Mem. 81, 120–136.

Arai, A., Kessler, M., Xiao, P., Ambros-Ingerson, J., Rogers, G., Lynch, G., 1994. A centrally active drug that modulates AMPA receptor gated currents. Brain Res. 638, 343–346.

Bailey, C.P., Connor, M., 2005. Opioids: cellular mechanisms of tolerance and physical dependence. Curr. Opin. Pharmacol. 5, 60–68.

Barnett, M.E., Madgwick, D.K., Takemoto, D.J., 2007. Protein kinase C as a stress sensor. Cell Signal. 19, 1820–1829.

Benavides, M., Laorden, M.L., Marin, M.T., Milanes, M.V., 2005. Role of PKC-alpha, gamma isoforms in regulation of c-Fos and TH expression after naloxone-induced morphine withdrawal in the hypothalamic PVN and medulla oblongata catecholaminergic cell groups. J. Neurochem. 95, 1249–1258.

Bowers, B.J., Christensen, S.C., Pauley, J.R., Paylor, R., Yuva, L., Dunbar, S.E., Wehner, J.M., 1995. Protein and molecular characterization of hippocampal protein kinase C in C57BL/6 and DBA/2 mice. J. Neurochem. 64, 2737–2746.

Chen, L., Huang, L.Y., 1991. Sustained potentiation of NMDA receptor-mediated glutamate responses through activation of protein kinase C by a mu opioid. Neuron 7, 319–326.

Chen, L., Wright, L.R., Chen, C.H., Oliver, S.F., Wender, P.A., Mochly-Rosen, D., 2001. Molecular transporters for peptides: delivery of a cardioprotective epsilonPKC agonist peptide into cells and intact ischemic heart using a transport system, R(7). Chem. Biol. 8, 1123–1129.

Colombo, P.J., Gallagher, M., 2002. Individual differences in spatial memory among aged rats are related to hippocampal PKCgamma immunoreactivity. Hippocampus 12, 285–289.

Dwivedi, Y., Pandey, G.N., 1999. Effects of treatment with haloperidol, chlorpromazine, and clozapine on protein kinase C (PKC) and phosphoinositide-specific phospholipase C (PI-PLC) activity and on mRNA and protein expression of PKC and PLC isozymes in rat brain. J. Pharmacol. Exp. Ther. 291, 688–704.

Fisher, S.K., Heacock, A.M., Agranoff, B.W., 1992. Inositol lipids and signal transduction in the nervous system: an update. J. Neurochem. 58, 18–38.

Garcia, M.M., Anderson, A.T., Edwards, R., Harlan, R.E., 2003. Morphine induction of c-fos expression in the rat forebrain through glutamatergic mechanisms: role of non-n-methyl-D-aspartate receptors. Neuroscience 119, 787–794.

Harlan, R.E., Kailas, S.R., Tagoe, C.E., Garcia, M.M., 2004. Morphine actions in the rat forebrain: role of protein kinase C. Brain Res. Bull. 62, 285–295.

Lai, Y.T., Fan, H.Y., Cherng, C.G., Chiang, C.Y., Kao, G.S., Yu, L., 2008. Activation of amygdaloid PKC pathway is necessary for conditioned cues-provoked cocaine memory performance. Neurobiol. Learn. Mem. 90, 164–170.

Leithe, E., Cruciani, V., Sanner, T., Mikalsen, S.O., Rivedal, E., 2003. Recovery of gap junctional intercellular communication after phorbol ester treatment requires proteasomal degradation of protein kinase C. Carcinogenesis 24, 1239–1245.

Liao, G.Y., Wagner, D.A., Hsu, M.H., Leonard, J.P., 2001. Evidence for direct protein kinase-C mediated modulation of N-methyl-D-aspartate receptor current. Mol. Pharmacol. 59, 960–964.

Ma, Y.Y., Chu, N.N., Guo, C.Y., Han, J.S., Cui, C.L., 2007. NR2B-containing NMDA receptor is required for morphine-but not stress-induced reinstatement. Exp. Neurol. 203, 309–319.

Ma, Y.Y., Guo, C.Y., Yu, P., Lee, D.Y., Han, J.S., Cui, C.L., 2006. The role of NR2B containing NMDA receptor in place preference conditioned with morphine and natural reinforcers in rats. Exp. Neurol. 200, 343–355.

Ma, Y.Y., Meng, L., Guo, C.Y., Han, J.S., Lee, D.Y., Cui, C.L., 2009. Dose- and time-dependent, context-induced elevation of dopamine and its metabolites in the nucleus accumbens of morphine-induced CPP rats. Behav. Brain Res. 204, 192–199.

Martini, L., Whistler, J.L., 2007. The role of mu opioid receptor desensitization and endocytosis in morphine tolerance and dependence. Curr. Opin. Neurobiol. 17, 556–564.

Mayer, D.J., Mao, J., Price, D.D., 1995. The association of neuropathic pain, morphine tolerance and dependence, and the translocation of protein kinase C. NIDA Res. Monogr. 147, 269–298.

Miyatake, M., Narita, M., Shibasaki, M., Nakamura, A., Suzuki, T., 2005. Glutamatergic neurotransmission and protein kinase C play a role in neuron-glia communication during the development of methamphetamine-induced psychological dependence. Eur. J. Neurosci. 22, 1476–1488.

Moroni, F., Lombardi, G., Thomsen, C., Leonardi, P., Attucci, S., Peruginelli, F., Torregrossa, S.A., Pellegrini-Giampietro, D.E., Luneia, R., Pellicciari, R., 1997. Pharmacological characterization of 1-aminoindan-1,5-dicarboxylic acid, a potent mGluR1 antagonist. J. Pharmacol. Exp. Ther. 281, 721–729.

Narita, M., Aoki, T., Ozaki, S., Yajima, Y., Suzuki, T., 2001. Involvement of protein kinase Cgamma isoform in morphine-induced reinforcing effects. Neuroscience 103, 309–314.

Narita, M., Suzuki, M., Kuzumaki, N., Miyatake, M., Suzuki, T., 2008. Implication of activated astrocytes in the development of drug dependence: differences between methamphetamine and morphine. Ann. N. Y. Acad. Sci. 1141, 96–104.

Newton, A.C., 2003. Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. Biochem. J. 370, 361–371.

Nishizuka, Y., 1995. Protein kinase C and lipid signaling for sustained cellular responses. FASEB J. 9, 484–496.

Nishizuka, Y., 1988. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334, 661–665.

Nithianantharajah, J., Murphy, M., 2009. Experience on the Barnes spatial maze influences PKCgamma levels in the hippocampus. Int. J. Neurosci. 119, 1014–1030.

Paxinos, G., Watson, C., 1997. The Rat Brain in Stereotaxic Coordinates, third ed. Academic Press, Inc, San Diego, CA.

Rykx, A., De, K.L., Mikhalap, S., Vantus, T., Seufferlein, T., Vandenheede, J.R., Van, L.J., 2003. Protein kinase D: a family affair. FEBS Lett. 546, 81–86.

Shi, X.D., Wang, G.B., Ma, Y.Y., Ren, W., Luo, F., Cui, C.L., Han, J.S., 2004. Repeated peripheral electrical stimulations suppress both morphine-induced CPP and reinstatement of extinguished CPP in rats: accelerated expression of PPE and PPD mRNA in NAc implicated. Brain Res. Mol. Brain Res. 130, 124–133.

Shu, C., Selmanoff, M., 1988. Phorbol esters potentiate rapid dopamine release from median eminence and striatal synaptosomes. Endocrinology 122, 2699–2709.

Shukla, P.K., Tang, L., Wang, Z.J., 2006. Phosphorylation of neurogranin, protein kinase C, and Ca2+/calmodulin dependent protein kinase II in opioid tolerance and dependence. Neurosci. Lett. 404, 266–269.

Ueda, H., 2004. Locus-specific involvement of anti-opioid systems in morphine tolerance and dependence. Ann. N. Y. Acad. Sci. 1025, 376–382.

Van der Zee, E.A., Kronforst-Collins, M.A., Maizels, E.T., Hunzicker-Dunn, M., Disterhoft, J.F., 1997. gamma Isoform-selective changes in PKC immunoreactivity after trace eyeblink conditioning in the rabbit hippocampus. Hippocampus 7, 271–285.

Wu, Y., Ren, C., Gao, Y., Hou, B., Chen, T., Zhang, C., 2010. A novel method for promoting heterologous protein expression in *Escherichia coli* by fusion with the HIV-1 TAT core domain. Amino Acids 39, 811–820.

Zurgil, N., Zisapel, N., 1985. Phorbol ester and calcium act synergistically to enhance neurotransmitter release by brain neurons in culture. FEBS Lett. 185, 257–261.

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