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Protein Kinase D1-Dependent Phosphorylation of Dopamine D1 Receptor Regulates Cocaine-Induced Behavioral Responses

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The dopamine (DA) D1 receptor (D1R) is critically involved in reward and drug addiction. Phosphorylation-mediated desensitization or internalization of D1R has been extensively investigated. However, the potential for upregulation of D1R function through phosphorylation remains to be determined. Here we report that acute cocaine exposure induces protein kinase D1 (PKD1) activation in the rat striatum, and knockdown of PKD1 in the rat dorsal striatum attenuates cocaine-induced locomotor hyperactivity. Moreover, PKD1-mediated phosphorylation of serine 421 (S421) of D1R promotes surface localization of D1R and enhances downstream extracellular signal-regulated kinase signaling in D1R-transfected HEK 293 cells. Importantly, injection of the peptide Tat-S421, an engineered Tat fusion-peptide targeting S421 (Tat-S421), into the rat dorsal striatum inhibits cocaine-induced locomotor hyperactivity and injection of Tat-S421 into the rat hippocampus or the shell of the nucleus accumbens (NAc) also inhibits cocaine-induced conditioned place preference (CPP). However, injection of Tat-S421 into the rat NAc shell does not establish CPP by itself and injection of Tat-S421 into the hippocampus does not influence spatial learning and memory. Thus, targeting S421 of D1R represents a promising strategy for the development of pharmacotherapeutic treatments for drug addiction and other disorders that result from DA imbalances.
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INTRODUCTION

Cocaine is one of the most abused psychostimulants used by humans. Chronic, long-term use of cocaine can result in addiction, which is a state of compulsive drug use. At present, cocaine addiction has become a serious issue worldwide. However, there are currently no effective medications available for the treatment of cocaine addiction (Shorter and Kosten, 2011). Understanding the neurobiological mechanisms responsible for cocaine addiction and identifying candidate targets for pharmacotherapeutic intervention is of great importance.

It is well known that the mesolimbic dopamine (DA) system has a critical role in drug addiction and the reward pathway. Cocaine induces a strong and rapid increase in extracellular DA in the mesolimbic reward circuit by inhibiting DA transporters (Chen *et al*, 2006; Giros *et al*, 1996; Schmitt and Reith, 2010). DA exerts its functions via binding to DA receptors, which can be grouped into two subclasses: D1-like receptors (D1R and D5R) and D2-like receptors (D2R, D3R, and D4R; Beaulieu and Gainetdinov, 2011). Both D1R and D2R, the most abundant subtypes of DA receptors *in vivo*, are critically involved in drug addiction and the reward pathway. However, activation of D1R is an absolute requirement for the induction of many of the cellular and behavioral effects of cocaine (Bateup *et al*, 2010; Bateup *et al*, 2008; Bertran-Gonzalez *et al*, 2008; Xu *et al*, 1994).

The protein kinase D (PKD) family is composed of three members, PKD1/PKCμ, PKD2, and PKD3/PKCν. To date, PKD1 is the best-characterized isoform of this family (Rozengurt *et al*, 2005; Steinberg, 2012). PKD1 has a vital role in various important biological processes, including cell proliferation and differentiation, vesicle trafficking and secretion, immune regulation and cardiac hypertrophy, and contraction (Rozengurt, 2011). In the nervous system, we first found that PKD1 has an important role in inflammatory heat hyperalgesia through an interaction with transient receptor potential vanilloid 1 (Wang *et al*, 2004; Zhu *et al*, 2008). Subsequently, studies by us and others have shown

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that PKD1 also participates in other nervous system processes, including neuronal development (Bisbal *et al*, 2008; Yin *et al*, 2008), neuroprotection (Stetler *et al*, 2012), and learning (Fu *et al*, 2009). Although the function of PKD1 has been extensively studied in a variety of cellular models, its physiological role, especially in the nervous system, is largely unknown.

Herein, we report the following: (1) PKD1 modulates cocaine-induced behaviors via phosphorylating D1R at serine 421 (S421); (2) PKD1 potentiates the surface localization of D1R and downstream extracellular signal-regulated kinase (ERK) signaling in D1R-transfected cell line; (3) the cell-permeable Tat fusion-peptide targeting S421 (Tat-S421), targeting phosphorylation of S421 of D1R, exhibits an inhibitory effect on cocaine-induced behaviors. Given the pressing need for effective disease-modifying treatments for addiction, the identification of the molecular basis of cocaine addiction and the anti-addiction effects of Tat-S421 are highly valuable findings.

MATERIALS AND METHODS

Antibodies

Anti-phospho-D1R (p-S421) antibody was custom-made by GL Biochem (Shanghai, China). Anti-PKD1 antibody (sc-639), anti-D1R antibody (sc-14001), and anti-ERK1/2 antibody (sc-93) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-PKD1 (p-S744/748) antibody (2054S) and anti-phospho-ERK1/2 antibody (4370S) were purchased from Cell Signaling Technology (Danvers, MA). Anti-β-actin antibody (A5316) was purchased from Sigma (St Louis, MO). Anti-transferrin receptor antibody (13–6800) was purchased from Invitrogen (Carlsbad, CA). Anti-GFP antibody was purchased from Roche Diagnostics (Indianapolis, IN).

Plasmid Construction and Mutation

The EYFP-N1-D1R construct was a gift from Professor Xuechu Zhen at Soochow University. The construct to fuse glutathione *S*-transferase (GST) with the carboxyl terminus of the D1R (GST-D1R-CT) was generated using the *Eco*RI/*Xho*I sites and subcloned into pGEX-5x-1 (GE Healthcare, Little Chalfont, UK). The construct myc-his-PKD1 was generated using the *Xho*I/*Eco*RI sites and subcloned into pcDNA 3.1/myc-His C (Invitrogen). Mutants of GST-D1R-CT and myc-his-DNPKD1 (D727A) were created with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All constructs were confirmed by DNA sequencing (BGI, Beijing, China).

Drug and Peptides

Cocaine HCl (Qinghai Pharmaceutical, Qinghai, China) was dissolved in 0.9% physiological saline to a final concentration of 10 mg/ml. Tat-D1R-CT-S421 (RKKRRQRRR-PLEKLSPALS-VILD) and Tat-D1R-CT-S421A (RKKRRQRRR-PLEKLSPALA-VILD) were synthesized and purified by GL Biochem (Shanghai, China). The mass and purity of the peptides were verified by HPLC. Peptides were dissolved in ddH_2O to a concentration of 10 μg/μl and 1 μl peptide was injected bilaterally into the specific brain regions, and the peptide was administered in 4 min (0.25 μl/min) followed by an additional 1 min to allow diffusion before the injection needle was taken out.

shRNA Lentivirus

GFP-PKD1-shRNA lentivirus was constructed and amplified by Genechem (Shanghai, China). The sequence 5′-GGTTCT GGACAGTTCGGAA-3′ was connected to the U6-vshRNA-UBI-GFP. Lentivirus expressing shRNA targeting a non-specific sequence were used as controls. For i*n vivo* knockdown of PKD1, 1×10^8 lentiviral particles were injected bilaterally into the dorsal striatum. Five days later, further experiments were performed.

Cell Culture and Transfection

HEK 293A cells were maintained in DMEM plus 10% fetal bovine serum (Hyclone, Logan, UT) at 37 °C in 5% CO_2. Transfections of cells were performed with Lipofectamine 2000 (Invitrogen). Cells were used 24–36 h post transfection.

Cell Surface Biotinylation Assay and Western Blot Analysis

We performed the cell surface biotinylation assay and western blotting as previously described (Xing *et al*, 2012). Immunoreactive bands were scanned and analyzed quantitatively by densitometry with Quantity One Software (Bio-Rad, Hercules, CA).

In Vitro Kinase Assay

The *in vitro* kinase assay was conducted as previously described (Yang *et al*, 2007). Briefly, 20 ng of active PKD1 (Upstate Biotechnology, Lake Placid, NY) was resuspended in assay buffer to a volume of 25 μl and a final concentration of 0.8 ng/μl. To initiate the reaction, 10 μl of the phosphorylation mixture (assay buffer containing 5 μCi of [γ-^{32}P]ATP and one of the following GST fusion proteins: GST, GST-D1R-CT, or GST-D1R-CT S421A) was added. The mixture was incubated at 30 °C for 30 min, and the reaction was terminated by the addition of SDS-PAGE sample buffer. After boiling for 5 min, the samples were subjected to SDS-PAGE. The gels were stained with Coomassie blue, dried, and exposed to X-ray film for autoradiography.

Animals

Male Sprague–Dawley rats (weighing 250–270 g on arrival) were housed in groups of five in a temperature- (23 ± 2 °C) and humidity- (50 ± 5%) controlled environment. The animals were maintained on a 12 h light/dark cycle with *ad libitum* access to food and water. All experiments were performed according to the guidelines of the Animal Care and Use Committee of Peking University.

Surgery and Stereotactic Injection

Rats were anesthetized with 10% chloral hydrate (m/v, i.p.). Stainless steel guide cannulas (0.67 mm outer diameter)

were bilaterally implanted 1 mm above the dorsal striatum, nucleus accumbens (NAc), or hippocampus. The cannulas (for the NAc core or shell) were angled toward the midline at 16° to avoid penetrating the lateral ventricles. The NAc core coordinates were anterior/posterior + 1.5 mm, medial/lateral ± 3.8 mm, and dorsal/ventral − 6.2 mm; the coordinates for the NAc shell were anterior/posterior: + 1.8 mm, medial/lateral ± 3.2 mm, and dorsal/ventral − 6.6 mm; the coordinates for the dorsal striatum were anterior/posterior + 1.0 mm, medial/lateral ± 2.5 mm, and dorsal/ventral − 5.5 mm; and the coordinates for the CA1 region of the hippocampus were anterior/posterior + 3.5 mm, medial/lateral ± 2.0 mm, and dorsal/ventral: 2.0 mm. The cannulas were fixed to screws in the skull with dental cement. Internal cannulas were replaced with dummy cannulas to keep the cannulas in place and prevent infection. The rats were given at least 5 days to recover before the experiments.

Place Preference Apparatus

Conditioning was conducted in black-colored rectangular PVC boxes ($795 \times 230 \times 250\,mm^3$) containing three chambers separated by guillotine doors. The two large, black conditioning chambers (A and C, $280 \times 220 \times 225\,mm^3$) were separated by a small gray center choice chamber (B, $135 \times 220 \times 225\,mm^3$). Chamber A had four light-emitting diodes (LEDs) forming a square on the wall and a stainless-steel mesh floor. Chamber C had four LEDs forming a triangle on the wall and a stainless-steel rod floor. Chamber B had a flat stainless steel floor. A computer interface was used to record the time that the rat spent in each chamber by means of infrared beam crossings.

Cocaine-Induced Conditioned Place Preference

Baseline preference was assessed by placing the rats in the center compartment of the conditioned place preference (CPP) apparatus and allowing *ad libitum* access to all compartments for 15 min. On subsequent conditioning days, the rats were trained for 8 consecutive days with alternating injections of cocaine (20 mg/kg, i.p.) or saline (2 ml/kg, i.p.) in the designated compartments. After each injection, the rats were confined to the corresponding conditioning chambers for 45 min and then returned to their home cages. Tests for the expression of CPP in a drug-free state (15 min duration) were performed on the first day after the training sessions. The procedure during testing was the same as during the initial baseline preference assessment. The CPP score was defined as the time (in seconds) spent in the reward-paired chamber minus the time spent in the saline-paired chamber during CPP testing.

Locomotor Activity

Locomotion was measured in 40×40 cm plexiglass chambers, and the rat was recognized by infrared radiation. Data were collected with Anilab software for locomotor activity (Anilab, Ningbo, China) that calculated the total distance traveled (cm) every 5 min by measuring the position of the rat.

Morris Water Maze Performance

A circular black pool (150 cm in diameter, 60 cm in height) was filled to a depth of 32 cm with water at a temperature of 22 ± 1 °C. The pool was divided into four quarters. An invisible black platform (12 cm in diameter) made of Plexiglass was located 1 cm below the water in the center of the selected quadrant. The animals were trained four times in different quadrants on the first day, and then for the last four days at approximately the same time each day. The sequence of quadrants was different on each subsequent day. The time limit for each animal was 90 s and the intertrial interval was 30 s, which was spent on the platform to remember the location. The rats rested for 20 min between two consecutive training sessions. A video camera mounted directly above the water maze was linked to a computer and recorded the time to reach the hidden platform (escape latency), the length of the swim path (distance traveled), and percentage of time spent in the target quadrant for each rat. The day after the last learning trial, each rat was given a single 60 s probe trial without a platform.

Statistical Analysis

The data are expressed as the mean ± SEM. Statistical analysis was performed using Prism 5.0 software. Differences between groups were compared using Student's *t*-test, one-way ANOVA analysis followed by Newman-Keuls post-hoc test or two-way ANOVA analysis followed by Bonferroni's post-hoc test. Statistical significance was set at $p < 0.05$.

RESULTS

PKD1 Contributes to Cocaine-Induced Locomotor Hyperactivity by Phosphorylating D1R at S421

We found increased p-PKD1 (S744/748) in rat striatal extracts after an acute cocaine injection ($t_3 = 3.51$, $p < 0.05$, Figure 1a). To further examine the role of PKD1 in cocaine-induced behaviors, we injected PKD1-shRNA lentivirus into the rat dorsal striatum (caudate and putamen nucleus) to knockdown PKD1 expression. As shown in Figure 1b and c, basal locomotor activity was unaffected by PKD1 knockdown. Cocaine injection induced locomotor hyperactivity in non-silencing shRNA lentivirus-injected rats. However, cocaine-induced locomotor hyperactivity was significantly attenuated in the PKD1-shRNA lentivirus-injected group ($F_{1,8} = 35.23$, $p < 0.001$, Figure 1c), suggesting that PKD1 activation contributes to cocaine-induced locomotor hyperactivity. Effective knockdown of PKD1 was subsequently verified by western blot analysis ($t_4 = 3.29$, $p < 0.05$, Figure 1d).

As D1R is a critical mediator for DA signaling in drug addiction, we tested whether D1R exerts functions as a downstream target of PKD1. Using bioinformatics analysis, we identified two potential PKD1 phosphorylation sites (I/L/VXK/RXXS/T) in the intracellular domains of D1R, S229 in the third intracellular loop and S421 in the carboxyl terminus (Supplementary Figure 1b and c). As S421 was conserved among different species, we concentrated on this

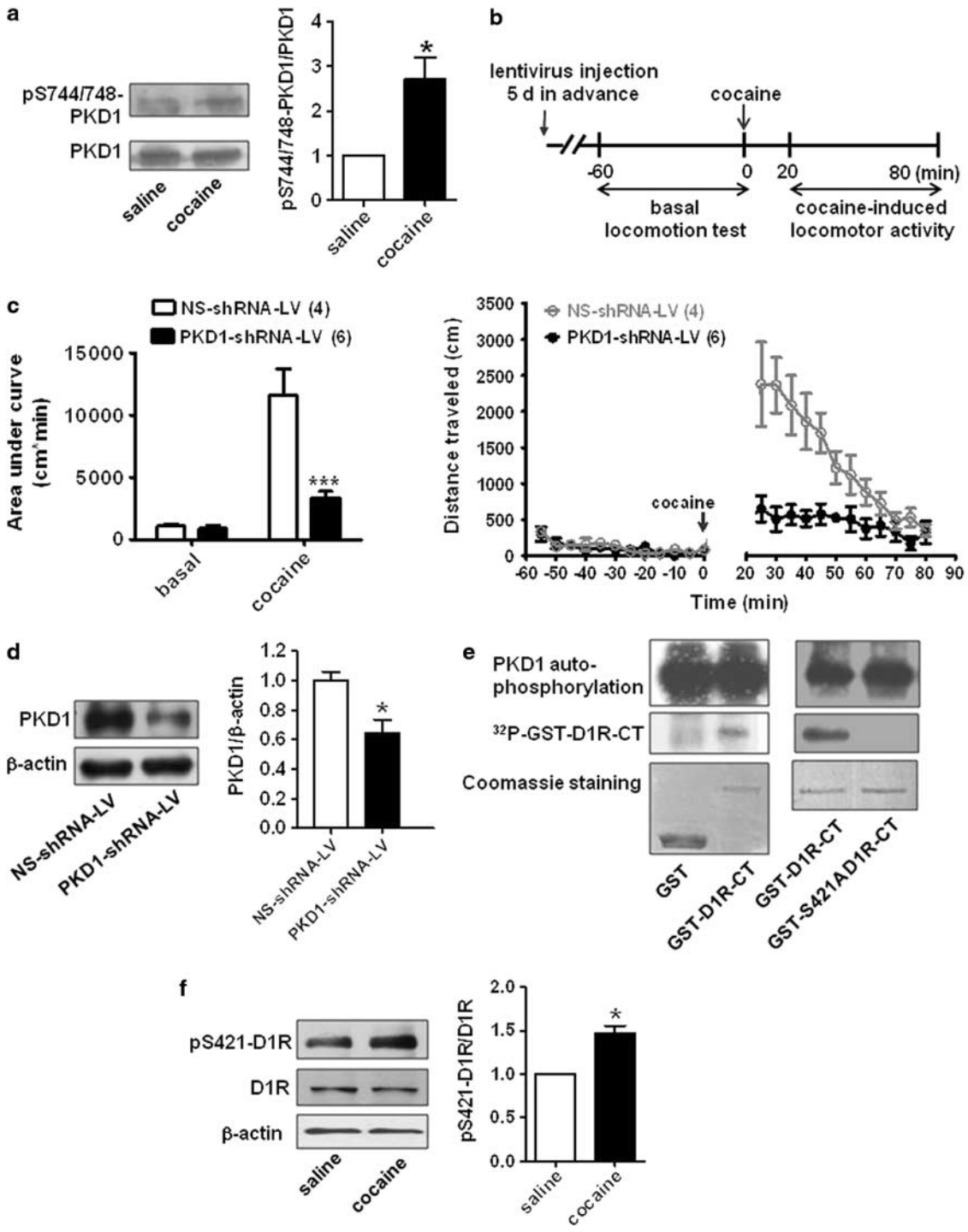

Figure 1 Activation of protein kinase D1 (PKD1) following acute cocaine exposure and phosphorylation of serine 421 (S421) in the dopamine D1 receptor (D1R) carboxyl terminus by PKD1. (a) Increased p-PKD1 (S744/748) in striatal extracts 20 min after an acute cocaine injection (20 mg/kg, intraperitoneally (i.p.)) in rats. $^{*}p<0.05$, $n=4$, *t*-test. (b) Top: timeline of non-silencing short hairpin RNA (shRNA) or PKD1-shRNA lentivirus injection into the bilateral dorsal striatum, cocaine administration, and locomotion test. Bottom: time course of horizontal locomotor activity in non-silencing shRNA lentivirus (NS-shRNA-LV)-injected or PKD1-shRNA lentivirus (PKD1-shRNA-LV)-injected rats. (c) Inhibition of cocaine-induced locomotor hyperactivity by PKD1 knockdown in the dorsal striatum. Total locomotor activity was quantified as the area under the curve during a 30-min observation period under basal conditions (− 30–0 min) and 20–50 min after cocaine administration. $^{***}p<0.001$, two-way analysis of variance analysis followed by Bonferroni *post-hoc* test. (d) Verification of PKD1 knockdown by PKD1-shRNA lentivirus injection. After the behavioral tests, striatal tissues were removed and total protein was extracted followed by SDS-polyacrylamide gel electrophoresis and immunoblotting. $^{*}p<0.05$, $n=3$, *t*-test. (e) *In vitro* kinase assay showing phosphorylation of the D1R carboxyl terminus (D1R-CT) by purified PKD1 and abolishment of the phosphorylation by S421A mutation (S421A D1R-CT). The bands detected by Coomassie blue represent loading for each lane. (f) Increased p-D1R (S421) in striatal extracts 20 min after an acute cocaine injection in rats. $^{*}p<0.05$, $n=3$, *t*-test.

site in the following studies. An *in vitro* kinase assay demonstrated direct phosphorylation of the D1R carboxyl terminus by PKD1, and mutation of S421 to alanine (S421A) completely abolished the phosphorylation (Figure 1e). Furthermore, acute cocaine administration increased p-D1R (S421) in striatal extracts ($t_3 = 5.57$, $p < 0.05$, Figure 1f). These data indicate that PKD1 directly phosphorylates D1R *in vitro* and *in vivo*.

Reduction of Surface D1R by Inhibiting PKD1 Activity or by Disrupting S421 Phosphorylation

Next, we examined the role of PKD1 in D1R functional regulation. First, western blot analysis showed that total levels of D1R were unaffected by cotransfection of PKD1 or DN-PKD1 in D1R-transfected HEK 293 cells (Figure 2a, lysates). As the surface levels of D1R were finely tuned via

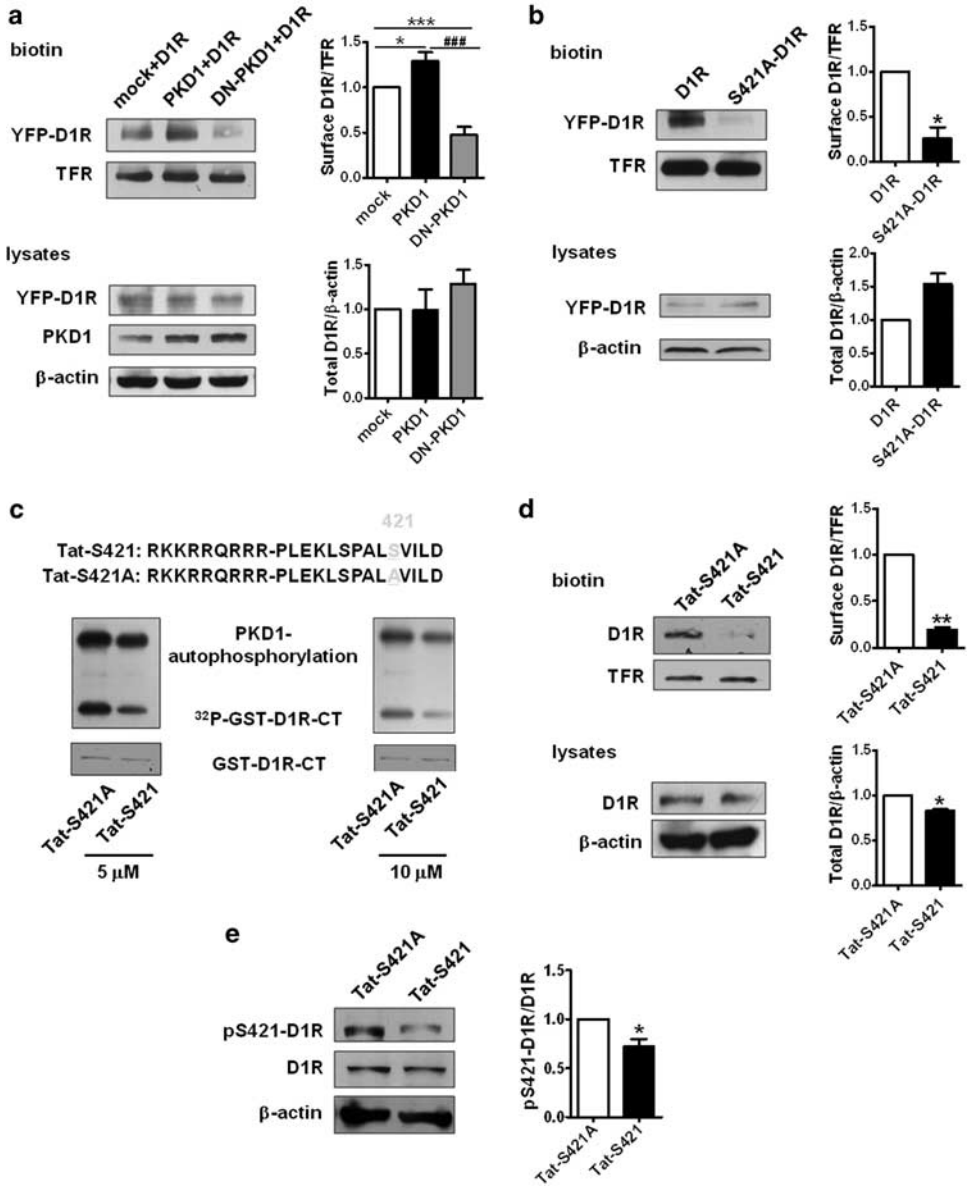

Figure 2 Regulation of the surface localization of dopamine D1 receptor (D1R) by protein kinase D1 (PKD1) activity. (a) Upregulation of surface D1R (biotin) by overexpression of PKD1 and downregulation of surface D1R by cotransfection of dominant-negative PKD1 (DN-PKD1). No effect of PKD1 activity on total levels of D1R (lysates) was observed. HEK 293 cells were cotransfected with YFP-D1R and myc-his-PKD1, or the kinase dead mutant myc-his-D727A PKD1. Biotinylation assays were performed 24 h after transfection to detect surface levels of D1R. $n = 5$ for biotin, $n = 3$ for lysates. $^{*}p < 0.05$, $^{***}p < 0.001$ *vs* mock; $^{\#\#\#}p < 0.001$ *vs* PKD1, one-way analysis of variance followed by Newman–Keuls *post-hoc* tests. (b) Reduced surface levels of D1R (biotin) by the S421A mutation. Total levels of D1R (lysates) were comparable between D1R-transfected and S421A D1R-transfected groups. HEK 293 cells were cotransfected with myc-his-PKD1 and YFP-D1R, or YFP-S421A-D1R. Biotinylation assay was performed 24 h after transfection to detect the surface levels of D1R. $^{*}p < 0.05$, $n = 3$, *t*-test. (c) Top: sequence of Tat-fusion peptides constructed according to the PKD1 phosphorylation site, S421 in the D1R carboxyl terminus, and the S421A mutant peptide. Bottom: inhibition of the phosphorylation of the D1R carboxyl terminus (D1R-CT) by Tat fusion-peptide targeting S421 (Tat-S421) treatment; 5 or 10 μM Tat-S421 or Tat-S421A control peptide was added to the kinase assay buffer and incubated for 30 min followed by SDS-polyacrylamide gel electrophoresis electrophoresis and autoradiography. (d) Reduced surface (biotin) and total (lysates) D1R in SK-N-MC cells following Tat-S421 treatment (5 μM, 2 h). $^{*}p < 0.05$, $^{**}p < 0.01$, $n = 3$, *t*-test. (e) Decreased p-D1R (S421) in striatal extracts from Tat-S421-injected (3.6 nmol/side, bilateral dorsal striatum injection) rats compared with Tat-S421A-injected rats. $^{*}p < 0.05$, $n = 4$, *t*-test.

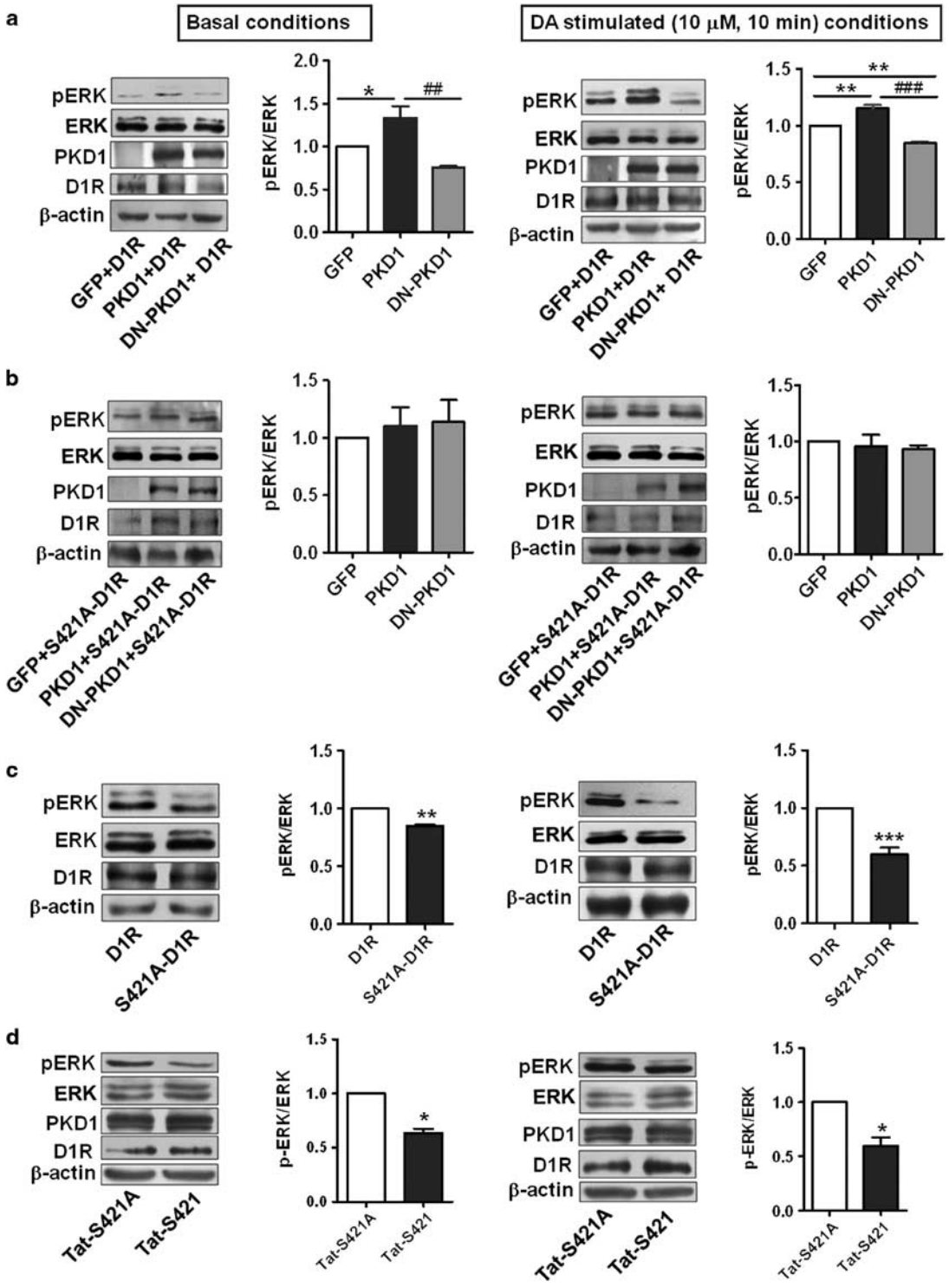

Figure 3 Inhibition of extracellular signal-regulated kinase (ERK) activation under both basal and dopamine (DA)-stimulated conditions by inhibiting protein kinase D1 (PKD1) activity or by disrupting serine 421 (S421) phosphorylation of DA D1 receptor (D1R). (a) Effects of PKD1 activity on ERK activation under both basal and DA-stimulated conditions. Cotransfection of GFP-PKD1 promoted ERK activation, whereas cotransfection of DN-PKD1 decreased it. The experiments were performed on HEK 293 cells cotransfected with GFP, GFP-PKD1, or GFP-DN-PKD1, and YFP-D1R plasmids. $n=3$. $^{*}p<0.05$, *vs* GFP, $^{\#\#}p<0.01$ *vs* PKD1. One-way analysis of variance (ANOVA) followed by Newman–Keuls *post-hoc* tests. (b) The single site mutation of D1R at S421 abolished the effects of PKD1 activity on ERK activation under both basal and DA-stimulated conditions. The experiments were performed on HEK 293 cells co-transfected with GFP, GFP-PKD1, or GFP-DN-PKD1, and YFP-S421A D1 plasmids. $n=3$. $^{**}p<0.01$ *vs* GFP, $^{\#\#\#}p<0.001$ *vs* PKD1. One-way ANOVA analysis followed by Newman–Keuls *post-hoc* tests. (c) The single site mutation of D1R at S421 inhibited ERK activation under both basal and DA-stimulated conditions. The experiments were performed on HEK 293 cells co-transfected with YFP-D1 or YFP-S421A D1, and GFP-PKD1 plasmids. $n=7$. $^{**}p<0.01$, $^{***}p<0.001$, *t*-test. (d) Inhibition of basal and DA-induced ERK activation by Tat fusion-peptide targeting S421 (Tat-S421) peptide (5 μM, 2 h) treatment in HEK 293 cells co-transfected with myc-his-PKD1 and YFP-D1R plasmids. $n=3$. $^{*}p<0.05$, *t*-test.

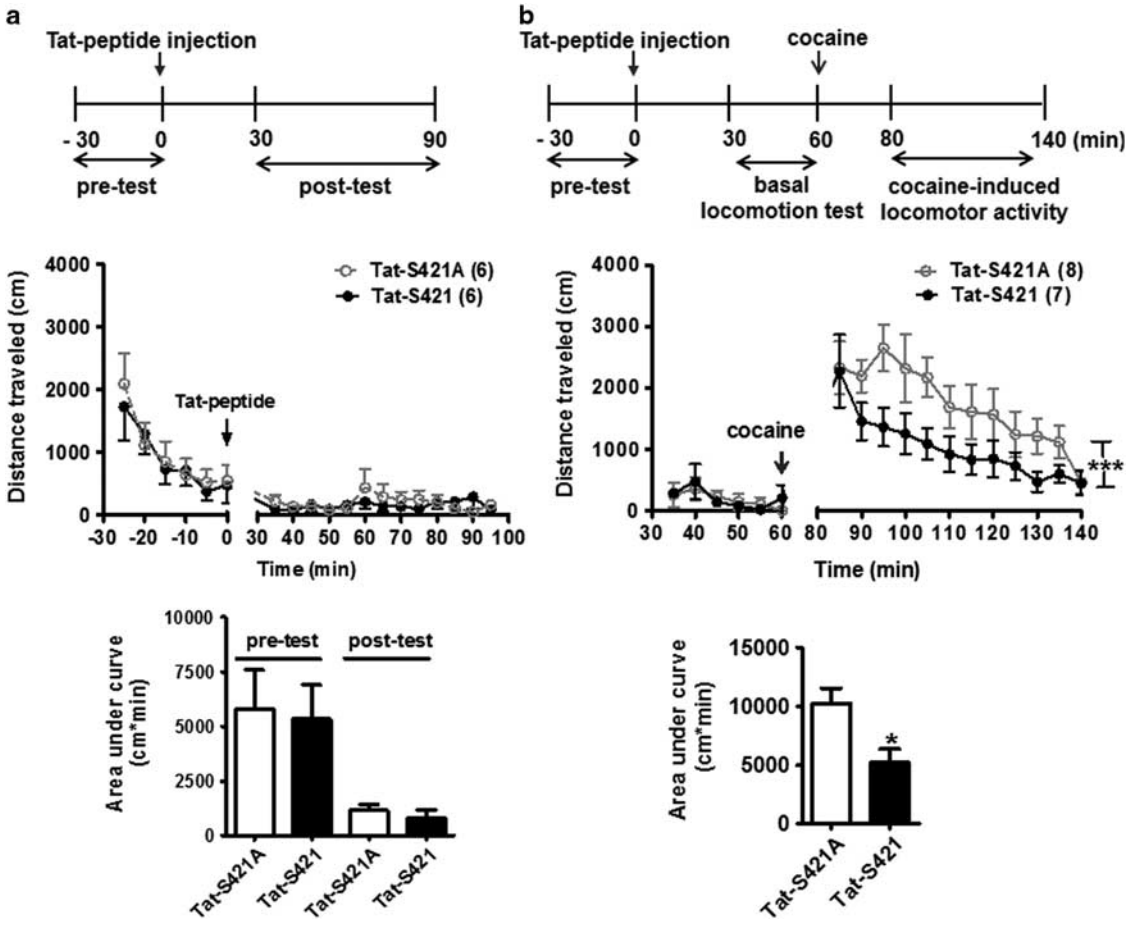

Figure 4 Attenuation of the cocaine-induced locomotor hyperactivity following the injection of the Tat fusion-peptide targeting S421 (Tat-S421) peptide. (a) Top: timeline of the bilateral Tat-peptide injection into the dorsal striatum and locomotion test. Middle: time course of horizontal locomotor activity in the Tat-S421-injected and Tat-S421A-injected rats. Bottom: no significant difference in total locomotor activity between the Tat-S421-injected and Tat-S421A-injected rats was detected. Total locomotor activity was quantified as the area under the curve during a 30-min observation time before the Tat-peptide injection (− 30–0 min) and 30–60 min after the Tat-peptide injection. (b) Top: timeline of the bilateral Tat-peptide injection into the dorsal striatum, cocaine administration, and locomotion test. Middle: time course of horizontal locomotor activity in the Tat-S421-injected and Tat-S421A-injected rats before and after cocaine injection. Bottom: inhibition of cocaine-induced locomotor hyperactivity following intrastriatal injection of Tat-S421 (3.6 nmol/side). Total locomotor activity was quantified as the area under the curve during a 30-min observation time (20–50 min after cocaine administration). $^{*}p<0.05$, *t*-test.

agonist-induced internalization and recycling, we performed surface biotinylation assay to detect whether surface levels of D1R can be regulated by PKD1 activity. Figure 2a illustrates that cotransfection of PKD1 increased surface D1R ($F_{2,12}=28.59$, $p<0.05$), whereas cotransfection of DN-PKD1 decreased it ($F_{2,12}=28.59$, $p<0.0001$, Figure 2a), suggesting that PKD1 can regulate the surface localization of D1R.

The role of S421 phosphorylation in the surface localization of D1R was further examined. S421A mutation significantly reduced surface D1R ($t_2=5.8$, $p<0.05$), whereas no significant difference of the total levels of D1R and S421A-D1R was detected (Figure 2b). Tat-mediated intracellular delivery of functional peptides is an effective intervention method that has been proved by us and others (Futaki, 2005; Ji *et al*, 2006; Zhang *et al*, 2010). Thus, we constructed a Tat-S421 peptide (Figure 2c, top) to competitively inhibit the phosphorylation of D1R on S421. An *in vitro* kinase assay demonstrated that Tat-S421 effectively inhibited S421 phosphorylation of the D1R carboxyl terminus (Figure 2c, bottom). Next, the effect of the Tat-S421 peptide on the surface localization of D1R was examined in SK-N-MC cells, which have been shown to possess endogenous D1R (Chen *et al*, 2003; Sidhu and Fishman, 1990; Zhen *et al*, 1998). As shown in Figure 2d, the surface levels of D1R were significantly reduced by Tat-S421 treatment, as were total D1R levels (for biotin, $t_2=29.44$, $p<0.01$; for lysates, $t_2=8.17$, $p<0.05$, Figure 2d). Importantly, bilateral injection of Tat-S421 (3.6 nmol/side) into the rat dorsal striatum significantly reduced p-D1R (S421) in striatal extracts ($t_3=3.6$, $p<0.05$, Figure 2e). Collectively, these data indicate that phosphorylation of D1R at S421 is required for its surface localization.

Impairment of D1R Signaling by Disrupting S421 Phosphorylation

The direct consequence of reduced surface D1R is inhibition of its downstream signaling cascade. Thus, we

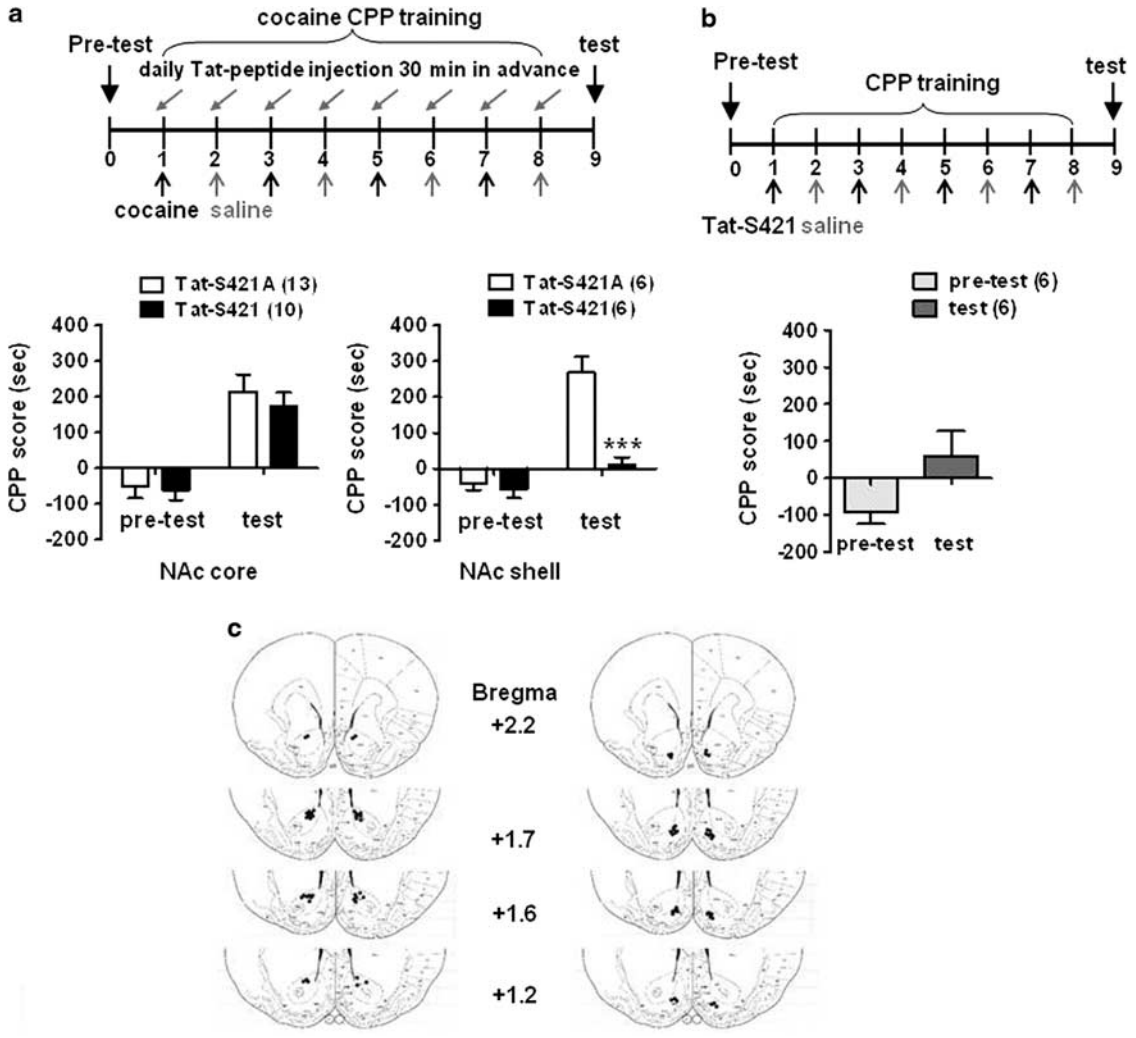

Figure 5 Attenuation of cocaine-induced conditioned place preference (CPP) by the injection of Tat fusion-peptide targeting S421 (Tat-S421) into the nucleus accumbens (NAc). (a) Top: timeline of Tat-peptide administration, cocaine-CPP training, and test. Bottom: attenuation of cocaine-induced CPP by bilateral injection of the Tat-S421 peptide (3.6 nmol/side) into the shell (right) but not into the core (left) of the NAc. ***$p<0.001$, two-way analysis of variance followed by Bonferroni *post-hoc* test. (b) Top: timeline of Tat-peptide administration, CPP training, and test. Bottom: Tat-peptide injection into the NAc shell did not induce the establishment of CPP. (c) Locations of intra-accumbal injection sites from the rats summarized in a and b.

examined whether the activation of ERK1/2, a critical downstream mediator of the D1R signaling cascade (Fricks-Gleason and Marshall, 2011; Lu *et al*, 2006), was suppressed by inhibiting PKD1 activity or by disrupting S421 phosphorylation of D1R. Studies in D1R-transfected HEK 293 cells indicated that cotransfection with PKD1 increased ERK1/2 phosphorylation under both basal ($F_{2,\ 6}=13.42$, $p<0.05$, *vs* GFP, Figure 3a) and DA-stimulated conditions ($F_{2,\ 6}=67.41$, $p<0.01$, *vs* GFP, Figure 3a). Conversely, cotransfection with DN-PKD1 reduced DA-induced ERK1/2 activation ($F_{2,\ 6}=67.41$, $p<0.01$, *vs* GFP, Figure 3a). However, transfection of the S421A mutant D1R abolished the effect of PKD1 activity on ERK1/2 phosphorylation (Figure 3b), suggesting that S421 is required for the regulatory effect of PKD1 on D1R signaling. Moreover, the S421A D1R-transfected cells showed reduced ERK1/2 activation under both basal conditions ($t_3=10.16$, $p<0.01$, Figure 3c) and DA-stimulated conditions ($t_6=6.87$, $p<0.001$, Figure 3c). In addition, Tat-S421 application in D1R-transfected HEK 293 cells also inhibited basal and DA-stimulated ERK1/2 activation (for basal conditions: $t_2=9.0$, $p<0.05$; for DA-stimulated conditions: $t_2=5.2$, $p<0.05$, Figure 3d). Thus, Tat-S421 may be used to reduce the efficacy of D1R signaling.

Attenuation of Cocaine-Induced Locomotor Hyperactivity by the Tat-S421 Peptide

Next, the effect of Tat-S421 on cocaine-induced locomotor hyperactivity was observed via bilateral injection into the dorsal striatum. No significant effect of the Tat-S421 peptide on basal locomotor activity was observed (Figure 4a). However, cocaine-induced locomotor hyperactivity was significantly attenuated in Tat-S421 peptide-injected rats compared with the Tat-S421A peptide-injected group ($t_{12}=2.8$, $p<0.05$, Figure 4b, bottom). The total locomotor

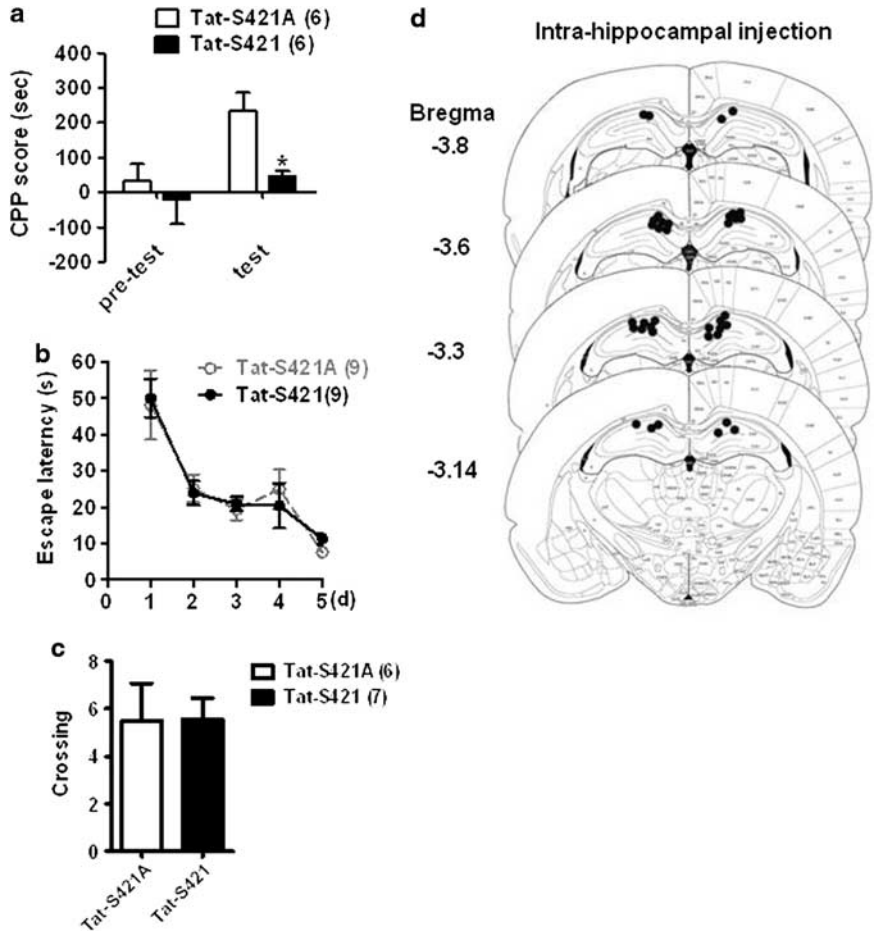

Figure 6 Attenuation of cocaine-induced conditioned place preference (CPP) by the injection of Tat fusion-peptide targeting S421 (Tat-S421) into the hippocampus. (a) Attenuation of cocaine-induced CPP by the bilateral injection of Tat-S421 peptide (3.6 nmol/side) into the hippocampus. *$p<0.05$, two-way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* test. (b, c) No effect of intrahippocampal injection of Tat-fusion peptides on spatial learning was detected. Escape latency time, two-way ANOVA analysis followed by Bonferroni *post-hoc* test, (b) and numbers of annulus (would-be platform) crossings, *t*-test, (c) were not significantly different between the Tat-S421-injected and Tat-S421A-injected rats. (d) Locations of intrahippocampal injection sites in the rats.

activity after cocaine injection in the control group was ~10 000 (Figure 4b, bottom), which is similar to the results shown in Figure 1c, suggesting the stability of cocaine-induced locomotor activity in the present studies. Together, these data indicate that Tat-S421 could be used to attenuate cocaine-induced locomotor hyperactivity.

Attenuation of Cocaine-Induced Reward Behaviors by the Tat-S421 Peptide

In the following studies, we tested whether *in vivo* application of Tat-S421 could attenuate cocaine-induced CPP in rats. As the NAc is known to be an important center in the reward circuit (Exley *et al*, 2012; Seif *et al*, 2011; Suto and Wise, 2011), we injected Tat-fusion peptides into the core or shell of the NAc to observe their effects on cocaine-induced CPP. Figure 5a illustrates that bilateral injection of Tat-S421 into the NAc shell had a significant inhibitory effect on the establishment of cocaine-induced CPP ($F_{1,10}=22.36$, $p<0.001$, Figure 5a). However, no effect of Tat-S421 injection into the NAc core was observed (Figure 5a). This finding was consistent with previous studies, which showed that addictive drugs preferentially increased DA in the NAc shell rather than the core. The NAc shell is the primary DA terminal area affected by acute exposure to addictive drugs (Anderson *et al*, 2003; Aragona *et al*, 2008; Di Chiara and Bassareo, 2007).

As a control, injection of Tat-S421 into the NAc shell did not induce CPP by itself (Figure 5b). Verification of the injection sites is shown in Figure 5c. Collectively, these data indicate that disrupting S421 phosphorylation of D1R by infusing the Tat-S421 peptide into the NAc shell during the acquisition period attenuates cocaine-induced place preference.

Similarly, intrahippocampal injection of the Tat-S421 peptide using a similar paradigm as in Figure 5a greatly inhibited the establishment of cocaine-induced CPP ($F_{1,10}=5.68$, $p<0.05$, Figure 6a). In addition, the possible effects of the Tat-S421 peptide on spatial learning and memory were examined. Figure 6b and c illustrate that no significant differences in latency and the number of annulus (would-be platform) crossings were detected between the two groups, which excluded the possibility of decreased ability of the rats to learn the association between environmental cues and the rewarding effects of cocaine following Tat-S421

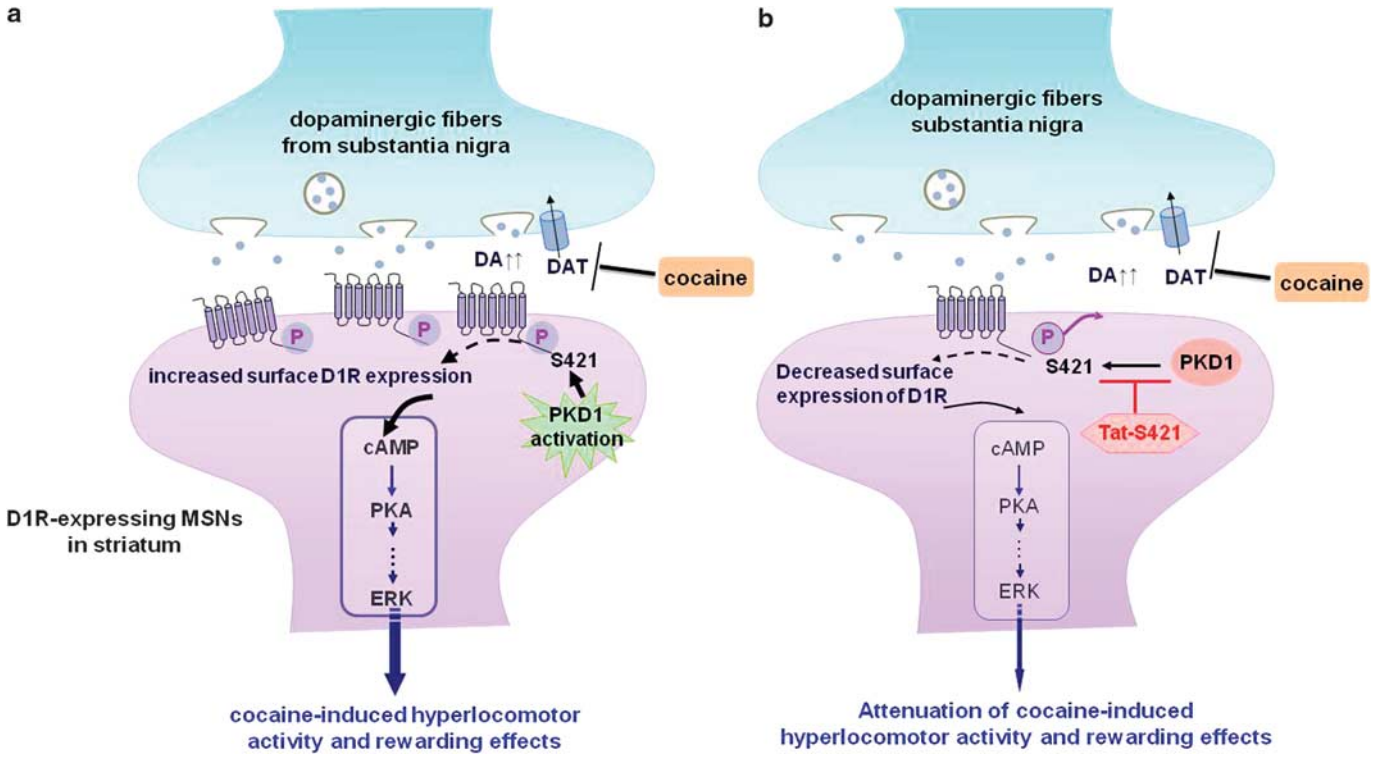

Figure 7 Working model. (a) Cocaine administration induces protein kinase D1 (PKD1) activation in the striatum. Next, PKD1 phosphorylates serine 421 (S421) in the carboxyl terminus of dopamine D1 receptor (D1R), leading to increased surface levels of D1R, enhanced downstream extracellular signal-regulated kinase (ERK) signaling, and cocaine-induced locomotor hyperactivity and rewarding effects. (b) Disrupting S421 phosphorylation of D1R with the Tat-S421 peptide reduced surface D1R levels, suppressed downstream ERK signaling, and attenuated cocaine-induced behavioral responses. DAT, dopamine transporter; MSN, medium spiny neuron.

injection. Verification of the injection sites within the hippocampus is shown in Figure 6d. Taken together, the above data suggest that Tat-S421 might be used to attenuate cocaine-induced behavioral responses and is a potential therapeutic candidate for cocaine addiction.

DISCUSSION

Phosphorylation is an important post-translational modification of D1R. In total, 32 potential serine/threonine phosphorylation sites are located within the intracellular domains of D1R (Supplementary Figure 1a). The two main phosphorylation domains lie within the carboxyl terminus and the third intracellular loop of D1R, which contain 20 and 8 potential phosphorylation sites, respectively (Kim *et al*, 2004; Rankin and Sibley, 2010). In general, GRKs mediate agonist-induced phosphorylation of D1R, whereas PKA and PKC contribute to heterologous receptor phosphorylation (Gainetdinov *et al*, 2004). PKA can phosphorylate T268 in the third intracellular loop and T380 in the carboxyl terminus of D1R, which regulates either the rate of desensitization or intracellular trafficking of the receptor once internalized (Jiang and Sibley, 1999; Mason *et al*, 2002). Constitutive or heterologous PKC (including PKCα, βI, γ, δ, and ε) phosphorylates D1R and dampens DA activation of the receptor, thus attenuating D1R-mediated signaling (Gardner *et al*, 2001; Rankin and Sibley, 2010; Rex *et al*, 2008; Rex *et al*, 2010). Here we provided both *in vitro* and *in vivo* evidence for the involvement of PKD1 in D1R phosphorylation.

PKD1 activation in response to activation of Gα_q-coupled GPCR by agonists, such as norepinephrine, angiotensin II, and neurotensin, has been extensively investigated (Iwata *et al*, 2005; Yuan *et al*, 2003; Zugaza *et al*, 1997). D1R can couple to Gα_q proteins in addition to the classic Gα_s/AC/cAMP signaling pathway (Lee *et al*, 2004). Activated Gα_q promotes the stimulation of PLC isoforms and catalyzes the hydrolysis of phosphatidylinositol 4,5-biphosphate to produce Ins (1,4,5)P3 and DAG. DAG induces a dramatic conversion of PKD1 from an inactive to an active form. In addition, DAG activates novel PKCs (PKCδ, ε, η, and θ), which mediate transphosphorylation of PKD1 on S744 followed by autophosphorylation on S748 (Bisbal *et al*, 2008). Although the precise role of S748 phosphorylation in PKD1 activity is still under debate, S744 phosphorylation of PKD1 was positively correlated with PKD1 catalytic activity. In our studies, we found increased phospho-PKD1 (S744/S748) following acute cocaine administration, which sufficiently indicated the activation of PKD1. Direct evidence for the contribution of PKD1 to cocaine-induced behavioral effects comes from *in vivo* knockdown studies. Lentivirus-mediated PKD1 knockdown in the dorsal striatum significantly reduced cocaine-induced locomotor hyperactivity, indicating that PKD1 activity is essential for cocaine-induced locomotor hyperactivity.

In most cases, PKD1 phosphorylates a sequence characterized by L/V/I at position -5 and R/K at position-3

(Rozengurt, 2011). The S421 identified in the present study does not fit the classical consensus phosphorylation sites for PKD1 (Supplementary Figure 1c). This inconsistency may explain why D1R has not been identified as a PKD1 substrate in previous studies.

It was reported that the single substitution of T360 of D1R, a GRK2 phosphorylation site, abolished the rapid agonist-induced desensitization of D1R. However, the single substitution of T446 abolished agonist-induced receptor internalization without any significant effect on receptor desensitization (Lamey *et al*, 2002). These findings indicate that agonist-induced desensitization and internalization of D1R are regulated by separate and distinct residues within the carboxyl terminus of D1R. Here we identified S421 as a critical site for surface expression of D1R. The single site mutation of S421A reduced the surface levels of D1R and impaired DA-induced ERK1/2 activation. Thus, phosphorylation of D1R at S421 is required for surface localization of D1R and downstream ERK signaling.

Taken together, our studies reveal that PKD1 is involved in cocaine-induced behavioral responses (Figure 7). The underlying mechanisms might be related to its promotion of surface localization of D1R through phosphorylating S421 in the carboxyl terminus of D1R. To our knowledge, this is the first report showing the critical role of S421 in surface D1R expression, downstream ERK signaling, and behavioral responses to cocaine. Importantly, we constructed a cell-permeable peptide, the Tat-S421 peptide, which inhibited cocaine-induced locomotor hyperactivity and cocaine CPP. Moreover, no significant side effects of Tat-S421 application in the brain regions were observed. Thus, S421 of D1R may represent a promising candidate for pharmacotherapeutic intervention of DA signaling for the treatment of drug addiction.

FUNDING AND DISCLOSURE

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)