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·Brief Communication·

Disruption of dopamine D1 receptor phosphorylation at serine 421 attenuates cocaine-induced behaviors in mice

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ABSTRACT

Dopamine D1 receptors (D1Rs) play a key role in cocaine addiction, and multiple protein kinases such as GRKs, PKA, and PKC are involved in their phosphorylation. Recently, we reported that protein kinase D1 phosphorylates the D1R at S421 and promotes its membrane localization. Moreover, this phosphorylation of S421 is required for cocaine-induced behaviors in rats. In the present study, we generated transgenic mice over-expressing S421A-D1R in the forebrain. These transgenic mice showed reduced phospho-D1R (S421) and its membrane localization, and reduced downstream ERK1/2 activation in the striatum. Importantly, acute and chronic cocaine-induced locomotor hyperactivity and conditioned place preference were significantly attenuated in these mice. These findings provide *in vivo* evidence for the critical role of S421 phosphorylation of the D1R in its membrane localization and in cocaine-induced behaviors. Thus, S421 on the D1R represents a potential pharmacotherapeutic target for cocaine addiction and other drug-abuse disorders.

Keywords: protein kinase D1; dopamine D1 receptor; phosphorylation; cocaine; addiction; conditioned place preference; locomotor activity

INTRODUCTION

Cocaine is one of the most widely abused drugs, and long-term abuse leads to addiction and serious health problems. To date, no pharmacologic intervention for cocaine addiction has been successfully developed[1]. Understanding the biological pathways and identifying new therapeutic targets may provide new insights into the treatment of cocaine addiction.

Cocaine induces a strong increase in extracellular dopamine (DA) levels by direct inhibition of DA transporters[2,3]. Increased extracellular DA in the dopaminergic mesocorticolimbic and nigrostriatal systems plays a critical role in the effects of cocaine and addiction[1]. DA acts through DA receptors, which can be grouped into two subclasses based on their structural, pharmacological, and physiological properties[4]. The D1-like subclass is composed of D1 and D5 receptors that couple to the G_s/adenylate cyclase/cyclic adenosine 3,5,-monophosphate (cAMP) signaling pathway. The D2-like subclass is composed of D2, D3, and D4 receptors that couple to the G_i protein, which leads to the inhibition of cAMP accumulation. Both D1 and D2 receptors, the most abundant subtypes *in vivo*, play key roles in reward and drug addiction. However, activation of the D1 receptor (D1R) is essential for the induction of many of the cellular and behavioral effects of cocaine, as demonstrated in D1R-knockout mice and in transgenic mice in which D1R- or D2R-expressing

medium spiny neurons are labeled by different fluorescent proteins[5,6].

Phosphorylation is one of the major post-translational modifications of the D1R. To date, three classes of protein kinases have been identified to be involved in D1R phosphorylation: G-protein-coupled receptor kinases (GRKs), cAMP-dependent protein kinase (PKA), and protein kinase C (PKC)[4]. The major effect of D1R phosphorylation is receptor desensitization, the process of diminishing receptor responsiveness under continuous agonist stimulation. GRK2-like (GRK2 and GRK3) and GRK4-like (GRK4 and GRK5) kinases have been reported to phosphorylate the D1R[7,8]. Second-messenger-activated protein kinases, such as PKA and PKC, are implicated in the heterologous desensitization of the D1R, which occurs when the activation of other G-protein-coupled receptors causes the desensitization of D1Rs in the same cell. Taken together, phosphorylation of D1Rs mediated by these protein kinases leads to receptor internalization or G-protein uncoupling, which are manifestations of functional receptor down-regulation.

Our recent studies revealed a new protein kinase, protein kinase D1 (PKD1), which is involved in D1R phosphorylation[9]. In contrast to the down-regulation of D1R function by GRK-, PKA-, or PKC-mediated phosphorylation, PKD1-mediated phosphorylation of the D1R at S421 promotes the membrane localization of D1Rs and contributes to the behavioral effects of cocaine. In this study, we provide *in vivo* evidence for the critical role of S421 phosphorylation of D1Rs in cocaine-induced behaviors using transgenic mice over-expressing mutant S421A-D1R in the forebrain (Tg-S421A).

MATERIALS AND METHODS

Antibodies and Drugs

Anti-phospho-serine 421-D1R antibody (pS421-D1R) was custom-made by GL Biochem (Shanghai, China); anti-D1R (sc-14001) and anti-extracellular signal-regulated kinase (ERK)1/2 (sc-93) were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phospho-ERK1/2 (4370S) was from Cell Signaling Technology (Beverly, MA); anti-GFP (11814460001) was from Roche (Indianapolis, IN); anti-β-actin (A5316) was from Sigma-Aldrich (St Louis, MO); and anti-transferrin receptor (TFR) (136800) was from Invitrogen (Carlsbad, CA).

Cocaine hydrochloride (Qinghai Pharmaceutical Co., Ltd, Qinghai, China) was dissolved in 0.9% physiological saline to a final concentration of 10 mg/mL and was injected into mice intraperitoneally at 20 mg/kg. Dopamine hydrochloride (Sigma-Aldrich) was dissolved in distilled water to a final concentration of 100 mmol/L and stored at −25°C.

Cell Culture and Transfection

HEK 293A cells were cultured in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum (HyClone, Logan, UT) at 37°C in 5% CO_2. Transfection was performed with Lipofectamine 2000 (Invitrogen). Cells were used 24 h post-transfection. YFP-D1R and YFP-S421A-D1R plasmids were described previously[9].

PKC Activity Assay

A non-radioactive PKC assay kit (V5330) from Promega (Madison, WI) was used. After treatment, cells were rinsed with cold PBS, and PKC activity was assayed immediately according to the manufacturer's protocol.

Generation of Transgenic Mice

S421A-D1R cDNA was sub-cloned into a 279-plasmid with a forebrain-specific promoter calcium/calmodulin-dependent protein kinase (CaMK)IIα, T2A, hrGFP, and SV40 poly A. The CaMKIIα promoter has been widely used for genetic manipulation by region-specific gene expression. Transgenic FVB mice were generated by pronuclear microinjection of linearized S421A-D1R plasmid into fertilized eggs. Transgenic mice were selected using genomic PCR with GFP-specific primers (forward: TTC ACC AAG TAC CCC GAG GA; reverse: TAG AAC TTG CCG CTG TTC A), and GAPDH was used as a control (forward: ATG ACA TCA AGA AGG TGG TG; reverse: CAT ACC AGG AAA TGA GCT TG). All experimental procedures were approved by the Animal Care and Use Committee of Peking University and all efforts were made to minimize discomfort of the animals.

DNA Extraction

Under inhalational anesthesia with isoflurane (Shandong Keyuan Pharmaceutical Co., Ltd, Shandong, China), the tip of the mouse tail (0.5 cm) was cut and digested with

60 μL DNA lysis buffer (100 mmol/L Tris, pH 8.0, 0.05 mmol/L EDTA, 0.1 mol/L NaCl, 0.5% SDS, and 2.5 mg/mL proteinase K) for 6 h at 55°C. Then, DNA was isolated with 200 μL Tris-EDTA buffer. The tubes were centrifuged at 12 000 r/min for 5 min, and the supernatant containing the DNA was subjected to polymerase chain reaction (PCR) and the PCR products were examined by 2% ethidium bromide agarose gel electrophoresis to determine the genotype (wild-type or transgenic).

Reverse Transcription PCR (RT-PCR)

Total RNA was isolated from the tissues with TRIzol reagent (Invitrogen) following the manufacturer's instructions. For each sample, 2 μg of total RNA was reverse-transcribed into cDNA with Oligo (dT) 15 primer and M-MLV reverse transcriptase (Promega, Madison, WI) for 1 h at 42°C. The products were amplified with Taq polymerase using a PCR program consisting of 95°C for 180 s as an initial denaturation step, followed by 35 cycles of 95°C for 50 s, 60°C for 30 s, and 72°C for 90 s. GAPDH served as the internal control. The PCR products were separated on 2% agarose gels containing ethidium bromide (Dingguo Changsheng Biotechnology, Beijing, China). The bands were visualized and photographed using a gel documentation system (Bio-Rad Laboratories, Hercules, CA).

Western Blot Analysis

After anesthesia (10% chloral hydrate, 0.3 g/kg, i.p.), the bilateral striata were removed from mice, quickly frozen in liquid nitrogen, and stored at –70°C until further processing. The tissues were homogenized in cold lysis buffer (containing 50 mmol/L Tris (pH 7.5), 250 mmol/L NaCl, 10 mmol/L EDTA, 0.5% NP-40, 1 μg/mL Leupeptin, 1 mmol/L PMSF, 4 mmol/L NaF) and total protein was extracted as described previously[10]. The immunoreactive bands were scanned and analyzed by densitometry with Quantity One Software (Bio-Rad Laboratories).

Immunofluorescence Staining

After anesthesia (10% chloral hydrate, 0.3 g/kg, i.p.), mice were perfused transcardially with 20 mL saline at 37°C, followed by 50 mL ice-cold 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). The brain was removed, postfixed in the same fixative overnight, and then transferred into 30% sucrose until it sank to the bottom of the container. Frozen tissue sections were cut coronally on a Leica cryostat (CM 1900, Heidelberg, Germany) at 15 μm.

For immunostaining, sections were blocked for 30 min at room temperature with 3% bovine serum albumin/0.3% Triton X-100, incubated with monoclonal anti-GFP antibody (1:1 000, Roche, Indianapolis, IN) overnight at 4°C, washed in PBS, and incubated with fluorescein isothiocyanate-labeled goat anti-mouse antibody for 1–2 h at room temperature. Finally, sections were stained with Hoechst 33258 (1 μg/mL) for 5 min, washed in PBS, and examined under a confocal microscope (FV1000, Olympus, Tokyo, Japan).

Isolation of Membrane Fraction

Tissues were homogenized in ice-cold lysis buffer (10 mmol/L HEPES, pH 7.4, 2 mmol/L EDTA, 320 mmol/L sucrose, and 2% (*v*/*v*) protease inhibitor). The homogenate was centrifuged at 1 000 g for 10 min to pellet the nuclei and cellular debris (P1). The supernatant (S1) was collected and further centrifuged for 45 min at 200 000 g. The crude membrane pellet (P2) was washed in 1 mL of membrane buffer (25 mmol/L HEPES, pH 7.4, 2 mmol/L EDTA, and protease inhibitors), and the membrane fraction was centrifuged again at 200 000 g for 30 min. Then, the P2 pellet was re-suspended in membrane buffer and homogenized (8–10 strokes) on ice. To solubilize the membranes, Triton X-100 was added to a final concentration of 1%, and the extract was incubated overnight at 4°C. The extract was subsequently centrifuged at 200 000 g for 45 min, and the pellet (P3) comprised the detergent-resistant membrane (DRM) fraction. The DRM pellet was washed with membrane buffer, recovered by centrifugation at 200 000 g for 30 min, and re-suspended in membrane buffer containing 1% Triton X-100 for further analysis.

Locomotor Activity

Locomotor activity was measured in a 40 × 40 cm^2 Plexiglas chamber and the mouse was monitored under infrared light. Each mouse was monitored for 30 min to measure the basal locomotor activity. In cocaine-induced locomotor hyperactivity, the mouse was placed in the chamber immediately after cocaine injection and monitored for at least 60 min. Data were collected with Anilab software

(Anilab, Ningbo, China), and the distance traveled (cm) every 5 min was calculated.

For statistical analysis, the total distance traveled during 30-min observation before (−30 to 0 min) and after (0 to 30 min) cocaine administration was calculated and compared between the two groups.

Place Preference Apparatus

Conditioning was conducted in black rectangular PVC boxes (350 × 150 × 150 mm^3) containing three chambers separated by guillotine doors. The two large, black conditioning chambers A and C (each 150 × 150 × 150 mm^3) were separated by a small gray center choice chamber (150 × 50 × 150 mm^3). Chamber A had 4 light-emitting diodes (LEDs) forming a square on the wall and a stainless-steel mesh floor. Chamber C had 3 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 mouse spent in each chamber by means of infrared beam crossings.

Cocaine-Induced Conditioned Place Preference

Cocaine-induced conditioned place preference (CPP) was performed using an unbiased, counterbalanced protocol. The CPP score was defined as the time (in seconds) spent in the cocaine-paired chamber minus the time spent in the saline-paired chamber.

Pre-test (day 0): Baseline preference was assessed by placing each mouse in the center chamber (chamber B) of the CPP apparatus and allowing free access to all chambers for 15 min. The time spent in each chamber was recorded. Most of the mice spent approximately the same time in each side-chamber. Mice that stayed 180-s longer in one chamber than the other were excluded. For mice that met this criterion, the preferred compartment was paired with saline treatment, while the non-preferred side was paired with cocaine treatment. In addition, cocaine-paired chamber assignment was counterbalanced, whereby half of the mice in each group experienced cocaine pairing in one side chamber while the other half did so in the opposite chamber.

Conditioning (days 1–8): Mice 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. In detail, mice received cocaine on days 1, 3, 5, and 7, and were immediately confined to one side-chamber for 45 min. On days 2, 4, 6, and 8, mice received saline and were immediately confined to the opposite chamber for 45 min. Then the mice were returned to their home cages.

CPP test (day 9): 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.

Open Field Test

The apparatus consisted of a large area made of wood and surrounded by 50-cm high walls. The floor was 25 cm × 25 cm. The illumination in the test room was provided by a 40 W lamp in the center, 60 cm above the floor. Each mouse was gently placed in the center of the open field, and its behavior was videotaped for 5 min. The time each mouse spent in the center was recorded with the Smart Video Tracking System (v2.5.21, Panlab, Spain).

Elevated Plus Maze

The elevated plus maze was 80 cm above the floor and was composed of two open and two closed (opposite) arms arranged in a cross. During a test, a mouse was placed in the central square facing an open arm. The behavior of the mouse was recorded for 5 min *via* a video recorder mounted above the maze, and the Smart Video Tracking System was used to calculate the amount of time spent in each arm. The apparatus was wiped clean between tests.

Statistical Analyses

Data are expressed as mean ± SEM. The statistical analyses were performed using Prism 5.0 software. Differences between groups were compared using either Student's *t*-test or a two-way repeated measures ANOVA, followed by Bonferroni *post hoc* tests. Statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Generation of S421A-D1R Transgenic Mice

First, we generated transgenic mice over-expressing mutant S421A-D1R in the forebrain (Tg-S421A mice) *via* the CaMKIIα promoter (Fig. 1A). The transgenic mice

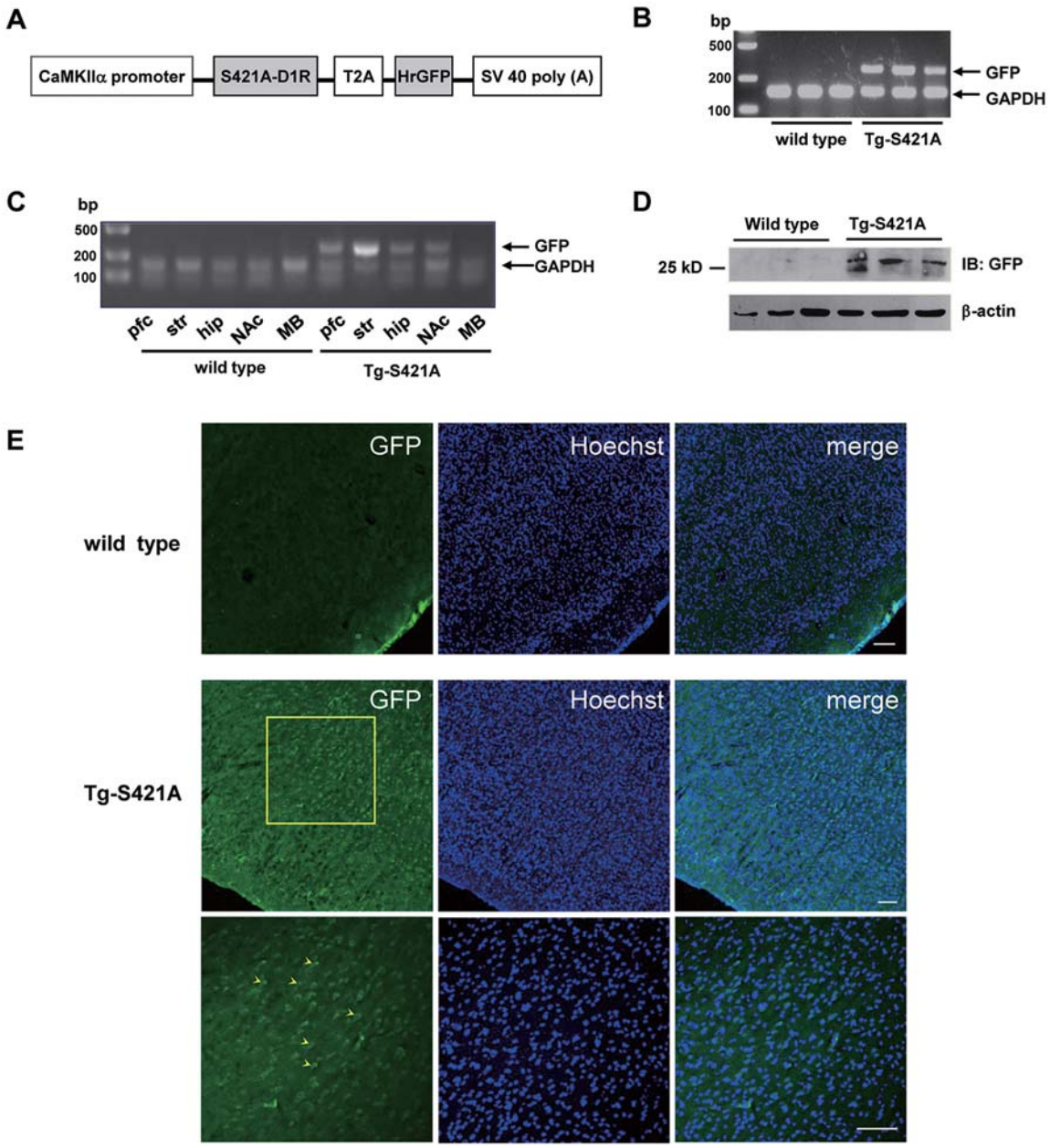

Fig. 1. Generation of S421A D1R-transgenic (Tg-S421A) mice. A: Schematic of the transgene constructs. B: PCR genotyping assay for the transgenic mice. C: RT-PCR of transgene GFP mRNA expression. pfc, prefrontal cortex; str, striatum; hip, hippocampus; NAc, nucleus accumbens; MB, midbrain. D: Western blots of transgene GFP expression in striatal extracts. E: Immunofluorescence staining of GFP expression in the forebrain cortex from WT and Tg-S421A mice. Enlarged images of the boxed area are shown below. Arrowheads indicate the typical GFP-positive neurons. Scale bars, 100 μm.

were comparable to the wild-type (WT) mice in body weight, survival rate, and home-cage behaviors. The mice were genotyped by PCR of the inserted GFP gene (305 bp, Fig. 1B). Furthermore, RT-PCR was used to detect GFP expression in the prefrontal cortex, striatum, hippocampus, and nucleus accumbens (Fig. 1C). There was no detectable GFP expression in the midbrain. To further confirm GFP protein expression in the striatum

of transgenic mice, we performed Western blot analysis and a GFP band of ~27 kD was detected in the striatal extracts from Tg-S421A mice but not in WT mice (Fig. 1D). In addition, immunofluorescence staining confirmed GFP expression in the forebrain cortex of Tg-S421A mice, and the large pyramidal neurons were easily identified by fluorescence (Fig. 1E). Altogether, these findings revealed GFP overexpression in the forebrain of Tg-S421A mice.

Reduced Membrane Localization and Downstream ERK Signaling of D1Rs in Tg-S421A Mice

The goal of developing the Tg-S421A mice was to disrupt phosphorylation of the D1R at S421 *in vivo*, so we performed Western blot analysis to test whether this phosphorylation was reduced in the forebrain of transgenic mice. The level of phospho-D1R (S421) in striatal extracts from Tg-S421A mice was markedly lower than in WT mice (P <0.05, Fig. 2A). The reduction of pS421-D1R in the transgenic mice may have been for the following two reasons. First, there is only a single site difference between WT-D1R and S421A-D1R. It is likely that S421A retains the ability to occupy the kinase catalytic domain of PKD1. However, the phosphorylation process is disrupted by this S421A mutation. Thus, less PKD1 protein is available for the phosphorylation of WT-D1R. Second, expression of exogenous D1R might competitively inhibit the synthesis of WT-D1R, leading to a reduction of WT-D1R production. So, the substrate of PKD1 is reduced and the level of pS421-D1R is decreased.

Meanwhile, contrary to the down-regulation of phospho-D1R, the total D1R expression was increased in the striatal extracts from Tg-S421A mice (P <0.05, Fig. 2A). The highest levels of D1R are found in the nigrostriatal, mesolimbic, and mesocortical areas, such as the striatum, nucleus accumbens, substantia nigra, amygdala, and frontal cortex[4]. Thus, the heterotopic expression of S421A

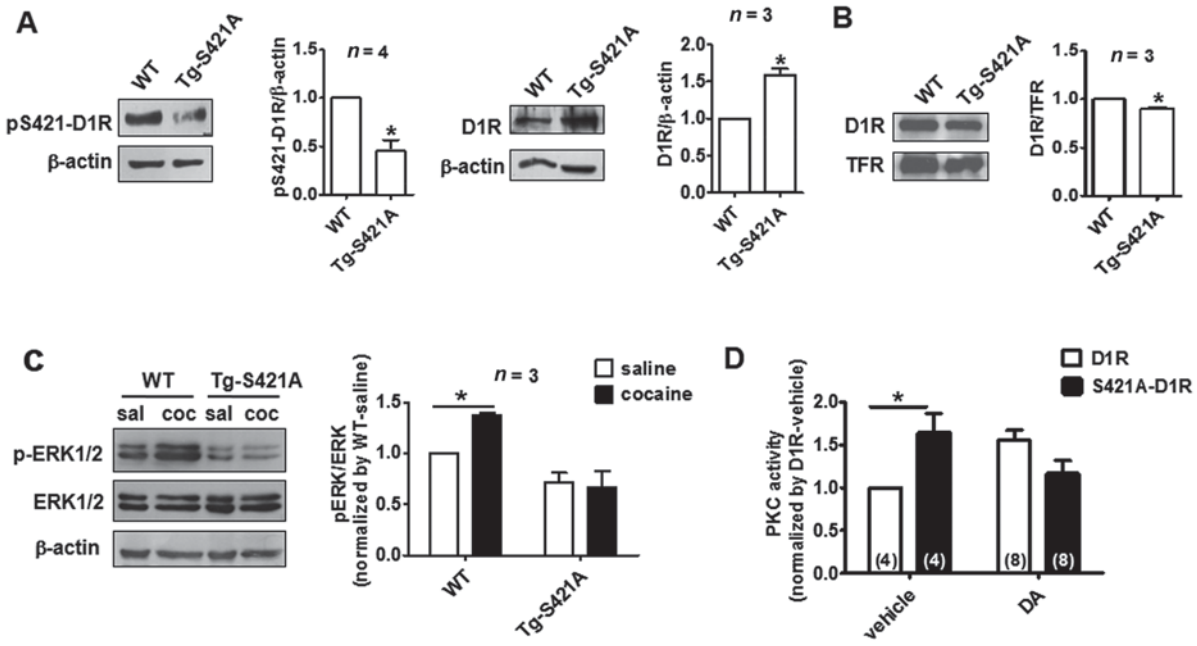

Fig. 2. Reduced phospho-D1R (S421) and reduced membrane localization of D1R in the striatum of Tg-S421A mice. A: Western blot analysis of phospho-D1R (S421) (left panels) and total D1R expression (right panels) in striatal extracts from WT and Tg-S421A mice. *P <0.05, Student's paired t-test. B: Membrane localization of D1R in the striatum of WT and Tg-S421A mice. *P <0.05, Student's paired t-test. C: Western blot analysis of cocaine-induced ERK1/2 activation in striatal extracts from WT and Tg-S421A mice. *P <0.05, two-way ANOVA followed by Bonferroni *post hoc* test. Sal, saline; coc, cocaine. D: Assay of PKC activity induced by DA treatment (10 μmol/L, 10 min) in D1R- or S421A-D1R-transfected HEK 293 cells. *P <0.05, two-way ANOVA followed by Bonferroni *post hoc* test.

D1R in excitatory neurons driven by the CaMKIIα promoter might lead to increased total D1R levels in the forebrain of Tg-S421A mice. In addition, this finding also provided evidence for the transgene S421A-D1R expression in the striatum of transgenic mice.

Our recent studies indicated that phosphorylation of the D1R at S421 promotes receptor membrane localization[9], so we compared the levels of D1R localized in the membrane fractions in the striatum of WT and Tg-S421A mice. The membrane localization of D1R was reduced in Tg-S421A mice compared with WT mice (P <0.05, Fig. 2B), consistent with our previous findings. However, the underlying mechanism is unknown. It is possible that receptor trafficking between membrane and intracellular compartments is disrupted by S421A mutation. If this is the case, the D1R might increase mainly in the intracellular fraction.

To directly assess the effect of downregulation of pS421-D1R on receptor signaling, we examined the activation of ERK1/2 in striatal extracts 20 min after an injection of cocaine (20 mg/kg, i.p.) in WT and Tg-S421A mice. As expected, an acute cocaine injection induced the activation of ERK1/2 in the striatum in WT mice (P <0.05, Fig. 2C), but this was not found in Tg-S421A mice. In addition, we assessed DA-induced PKC activation in D1R- and S421A-D1R-transfected HEK 293 cells. DA treatment (10 μmol/L, 10 min) induced a 1.65 ± 0.33-fold increase of PKC activity in D1R-transfected cells (P <0.05, Fig. 2D). However, no significant changes of PKC activity were observed in S421A-D1R-transfected cells, suggesting that disruption of S421 phosphorylation of D1R also affects D1R-G_q-PLC signaling and subsequent PKC activation. Taken together, these data suggest that the downstream signaling of D1R is affected by the disruption of S421 phosphorylation.

Attenuation of Cocaine-Induced Locomotor Hyperactivity in Tg-S421A Mice

Cocaine is a prototypical psychomotor stimulant that increases locomotor activity[11]. In the following studies, we compared the acute and chronic cocaine-induced locomotor activity of WT and Tg-S421A mice. Prior to cocaine injection (-30 to 0 min), the locomotor activity did not differ between WT and Tg-S421A mice (Fig. 3A). Quantitative analysis also showed no significant difference between the two groups

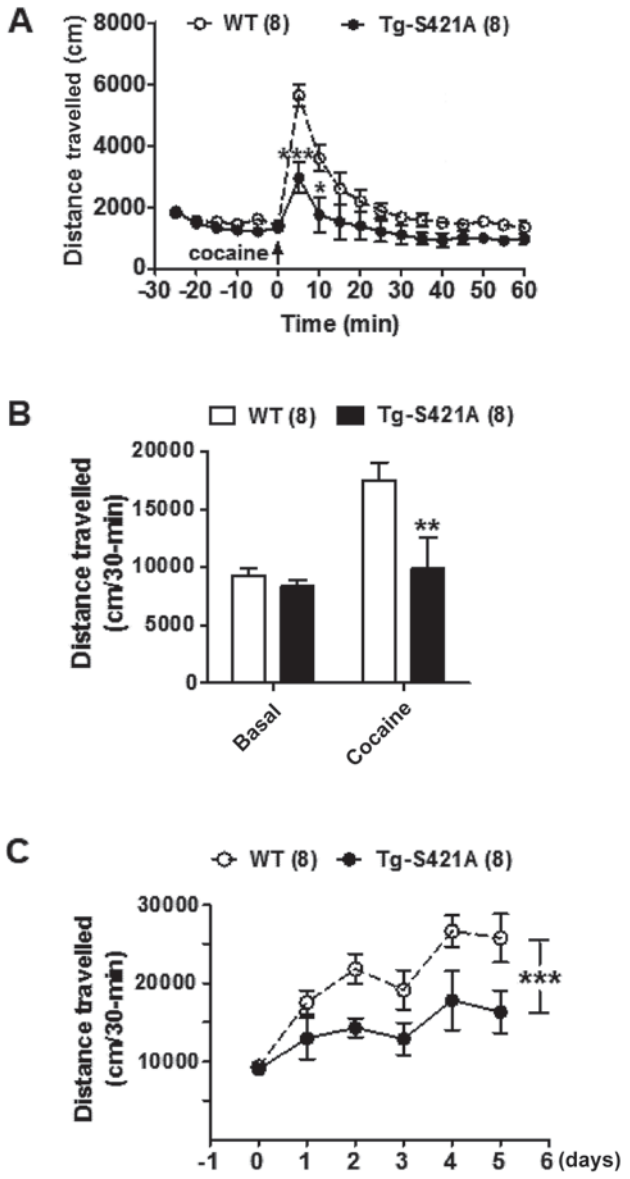

Fig. 3. Attenuation of cocaine-induced locomotor hyperactivity in Tg-S421A mice. A: Time course of locomotor activity in WT and Tg-S421A mice before (-30–0 min) and after cocaine injection (20 mg/kg, single i.p. injection; 0–60 min). *P <0.05, ***P <0.001, Student's unpaired t-test. B: Total distances traveled during -30–0 min before and 0–30 min after cocaine administration in WT and Tg-S421A mice. **P <0.01, two-way ANOVA followed by Bonferroni *post hoc* test. C: Chronic cocaine-induced locomotor activity by repeated, daily injection of cocaine (20 mg/kg) for 5 consecutive days in WT and Tg-S421A mice. Total distance traveled after cocaine administration (0–30 min) was calculated for each group. ***P <0.001, two-way ANOVA.

(Fig. 3B). These results suggest that S421A mutation of D1R does not affect locomotor activity under physiological conditions. As expected, acute cocaine injection induced hyperactivity in both groups. However, at 5 and 10 min after cocaine injection, the locomotor activity of transgenic mice was markedly lower than that of WT mice (P <0.001 and P <0.05, respectively; Fig. 3A). Quantitative analysis indicated that acute cocaine-induced hyperactivity is significantly attenuated in Tg-S421A mice (P <0.01, Fig. 3B).

As repeated cocaine exposure is more common than single-use in practice, we measured the changes in locomotor activity during chronic cocaine administration in Tg-S421A mice by injecting cocaine (20 mg/kg, i.p.) once daily for 5 consecutive days. We compared the locomotor activity during the first 30 min after cocaine administration (WT *versus* Tg-S421A mice). It is known that repeated administration of cocaine progressively enhances the induced locomotor activity[12]. In the present study, a gradual increase in locomotor activity was observed over the 5 consecutive days in both groups. However, the increase was reduced in the Tg-S421A mice compared to the WT mice (P <0.001, Fig. 3C). Taken together, our results indicate that inhibiting S421 phosphorylation of the D1R significantly attenuates acute and chronic cocaine-induced locomotor hyperactivity.

Attenuation of Cocaine CPP in Tg-S421A Mice

CPP is a widely-used, standard procedure for assessing the rewarding effects of drugs in rodent models[13,14]. We therefore determined whether cocaine-induced CPP was altered in Tg-S421A mice. As expected, cocaine promoted the establishment of CPP in WT mice, as shown by the increment of time spent in the cocaine-associated compartment (Fig. 4A). However, Tg-S421A mice had reduced preference for the cocaine-paired context compared with the WT mice (P <0.001, Fig. 4A). As a control, conditioning with saline did not induce place preference in WT and Tg-S421A mice (Fig. 4A). These results indicate that inhibition of D1R phosphorylation at S421 attenuates the rewarding effect of cocaine.

Because the ERK pathway plays a critical role in cocaine addiction[15,16], we examined ERK activation after the cocaine-CPP test in WT and Tg-S421A mice. Western blot analysis showed that the p-ERK1/2 levels in striatal extracts from transgenic mice were lower than those in WT mice (P <0.05, Fig. 4B).

DA neurotransmission is implicated in the regulation of motor activity, motivation and reward, cognitive processes, and emotional responses[17,18]. Anxiety, one of the most common negative emotions, might have a negative effect on CPP. Thus, we evaluated the anxiety levels of Tg-S421A mice with the open-field test and the elevated plus maze test. The results showed no significant differences between the WT and Tg-S421A mice in the following parameters: number of times entering the open arms and time spent in the open arms during the elevated plus maze test (Fig. 4C), and time spent in the center during the open-field test (Fig. 4D). These data argue against the possibility of a negative emotional effect on the cocaine-rewarding behaviors in Tg-S421A mice.

The present experiments using Tg-S421A mice provide important *in vivo* evidence for the critical role of S421 phosphorylation of D1R in cocaine addiction. Protein kinase-mediated phosphorylation of the D1R has been extensively studied and shown that GRKs 2–5 are all capable of phosphorylating D1R in cell culture models[4]. However, the *in vivo* role of GRKs in D1R signaling in drug addiction remains to be determined[12,19,20]. For example, although GRK6 seems to be the most prominent GRK in both the dorsal and ventral striatum, observations in mice lacking GRK6 indicate that the D2R is its physiological target[21,22]. In addition to GRKs, the second-messenger activated protein kinases, PKA and PKC, can also phosphorylate the D1R. For example, PKA phosphorylates T268 in the third cytoplasmic loop and T380 in the carboxyl-terminus of the D1R, which regulates either the rate of desensitization or the intracellular trafficking of the receptor once internalized[23,24]. D1R can also be phosphorylated either constitutively or heterologously by PKC (including PKC α, βI, γ, δ, and ε) at S259 in the 3rd intracellular loop and S397, S398, S417, and S421 in the carboxyl terminus, which results in the inhibition of receptor-G-protein coupling and downstream signaling[25-27]. In these studies, the authors reported that PKC can phosphorylate S421 of the D1R. However, no further experiments were performed to elucidate the exact role of each of the five phosphorylation sites. Similar to the GRK studies, the above results were mostly obtained from cell culture systems. The physiological role of PKA- and PKC-mediated phosphorylation of D1Rs remains unknown.

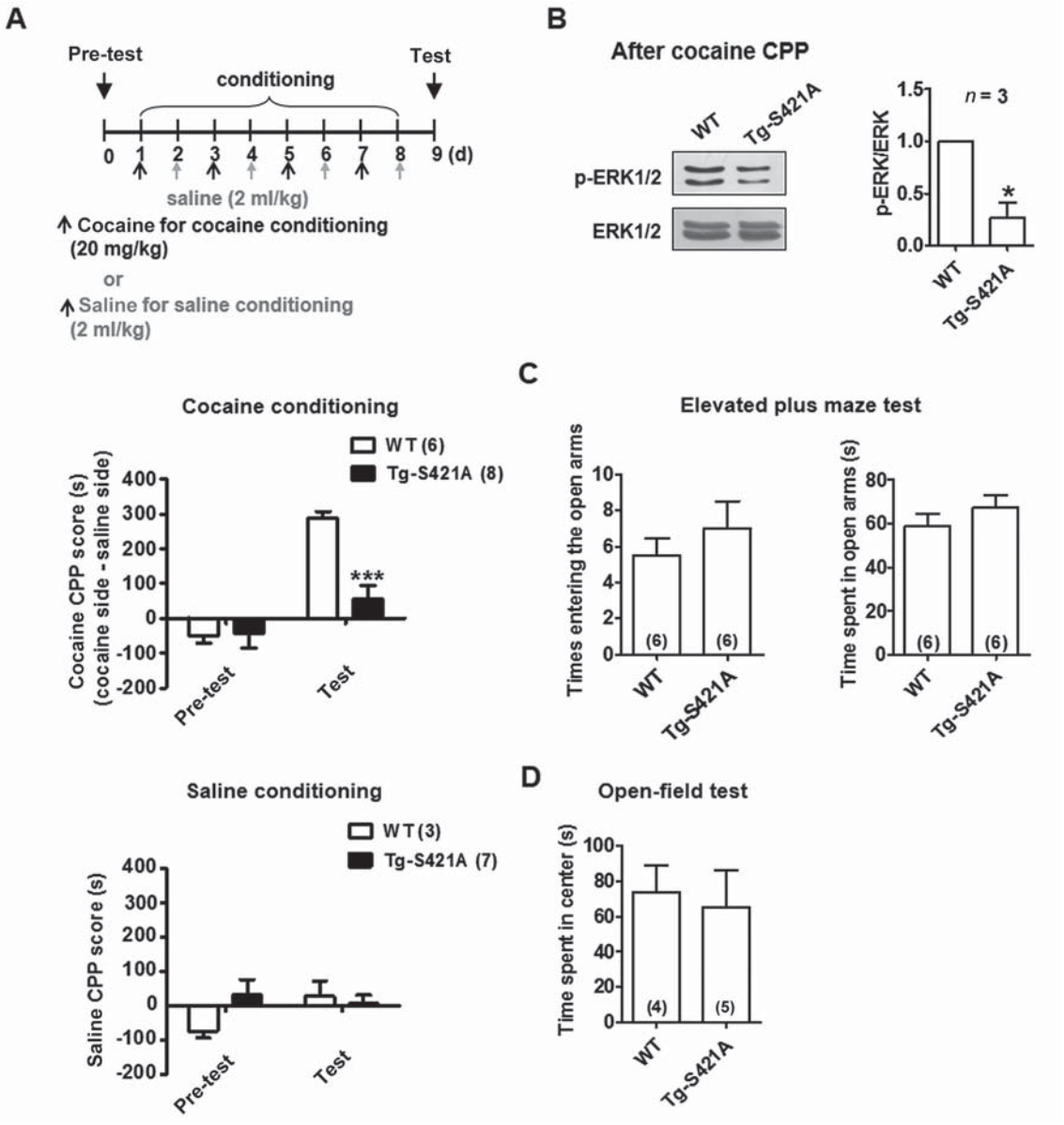

Fig. 4. Attenuation of cocaine-induced conditioned place preference (CPP) in Tg-S421A mice. A: Upper panel: Timeline of drug administration, CPP training, and testing. Middle panel: CPP scores of WT and Tg-S421A mice before cocaine CPP training (pre-test) and 1 day after training (test). ******P* **<0.001, two-way ANOVA followed by Bonferroni *post hoc* test. Lower panel: CPP scores of WT and Tg-S421A mice subject to saline conditioning. B: Western blot analysis of p-ERK1/2 levels in striatal extracts from WT and Tg-S421A mice immediately after the cocaine CPP test. ****P* **<0.05, Student's paired *t*-test. C and D: Anxiety-like behavior of the Tg-S421A transgenic mice in the elevated plus maze test (C) and open-field test (D). Student's unpaired *t*-test.

PKD1, a relatively new member of the CaMK family, plays an important role in neuronal development, neuroprotection, and learning[28]. Recently, we revealed the critical role of PKD1 in cocaine addiction through phosphorylating S421 of the D1R[9]. Lentivirus-mediated PKD1 knockdown effectively reduced the increase in locomotor activity after acute cocaine injection. Moreover, the essential role of S421 of the D1R in receptor membrane localization, downstream ERK signaling, and cocaine-induced behaviors in rats was revealed. The present study provides *in vivo* evidence for

the critical role of S421 of the D1R in receptor membrane localization and behavioral responses to cocaine in mice. Similarly, the critical role of CaMKII-mediated phosphorylation of S229, located in the third loop of the D3 receptor, in the regulation of receptor signaling and the motor response to cocaine, has been demonstrated by another research group[29,30]. Taken together, our findings implicate S421 of the D1R as a potential therapeutic target for the treatment of cocaine addiction and other drug-abuse disorders.

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