

ORIGINAL ARTICLE

Identification of Piccolo as a regulator of behavioral plasticity and dopamine transporter internalization

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Dopamine transporter (DAT) internalization is a mechanism underlying the decreased dopamine reuptake caused by addictive drugs like methamphetamine (METH). We found that Piccolo, a presynaptic scaffolding protein, was overexpressed in the nucleus accumbens (NAc) of the mice repeatedly administered with METH. Piccolo downexpression by antisense technique augmented METH-induced behavioral sensitization, conditioned reward and synaptic dopamine accumulation in NAc. Expression of Piccolo C₂A domain attenuated METH-induced inhibition of dopamine uptake in PC12 cells expressing human DAT. Consistent with this, it slowed down the accelerated DAT internalization induced by METH, thus maintaining the presentation of plasmalemmal DAT. In immunostaining and structural modeling Piccolo C₂A domain displays an unusual feature of sequestering membrane phosphatidylinositol 4,5-bisphosphate, which may underlie its role in modulating DAT internalization. Together, our results indicate that Piccolo upregulation induced by METH represents a homeostatic response in the NAc to excessive dopaminergic transmission. Piccolo C₂A domain may act as a cytoskeletal regulator for plasmalemmal DAT internalization, which may underlie its contributions in behavioral plasticity.

Molecular Psychiatry advance online publication, 15 January 2008; doi:10.1038/sj.mp.4002132

Keywords: Piccolo; dopamine transporter; methamphetamine; behavioral plasticity; C₂A domain

Introduction

Dopamine transporter (DAT), a member of the Na⁺/Cl⁻-dependent transporters in the dopaminergic neurons, is critical for terminating dopamine (DA) neurotransmission and contributes to the abuse potential of psychostimulants. The stimulating and reinforcing effects of drugs result from enhanced synaptic DA accumulation in specific brain areas like nucleus accumbens (NAc). Cocaine and methamphetamine (METH; or its analogue amphetamine) elevate extracellular DA by inhibiting DA reuptake through DAT and, in the case of METH, also by promoting reverse transport of nonvesicular DA, reducing plasma membrane DAT through internalization, and displacing DA from synaptic vesicle (SV) to the cytoplasm.^{1,2}

Membrane trafficking of DAT is closely associated with DA homeostasis and synaptic plasticity, and increasing evidences have showed that METH-like drugs are able to modulate this dynamic process.³ The internalization of plasmalemmal DAT is a clathrin-mediated process,^{4,5} and internalized DAT can be sorted to endosomal compartments where they may be recycled to cell surface and/or lysosome for degradation.⁶ Inhibition of endocytic machinery assembly can attenuate amphetamine- or phorbol ester-mediated DAT internalization,⁷ whereas expression of endosomal proteins like Rab5 in endosomal vesicles promotes amphetamine-induced intracellular DAT accumulation.⁸ These findings strongly suggest that manipulation of endocytic components could be an important manner for regulating DAT internalization.

Piccolo, a component of the presynaptic cytoskeletal matrix, is assembled ultrastructurally as an electron-dense region of filaments at the active zone (AZ). It is proposed to play a scaffolding role in regulating AZ assembly,⁹ actin cytoskeleton and SV trafficking.^{10,11} Piccolo contains multiple subdomains including PDZ domain and Ca²⁺/phospholipid binding (C₂A and C₂B) domains, each of which exhibits

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Received 17 June 2007; revised 14 September 2007; accepted 21 September 2007

distinctive features.^{10,12} PDZ domain may interact with other presynaptic molecules involving molecule anchoring and assembly at AZ.¹³ C₂A domain shows an unusual ability to sense intracellular changes of Ca²⁺ levels and then trigger the association with membrane phospholipids (PIs) via electrostatic interaction.¹⁴ Notably, it interacts with phosphatidylinositol 4,5-bisphosphate (PIP₂),¹⁵ a critical molecule for actin dynamics and endocytosis. It is well established that PIP₂ coordinates membrane fusion with actin filament to promote membrane movement, and recruits accessory adaptors for clathrin-coated pits.¹⁶ Therefore, modulation of plasmalemmal PIP₂ may affect PIP₂-dependent biological processes like membrane trafficking and endocytosis.

In this study we find that Piccolo serves as a negative presynaptic modulator for behavioral hypersensitivity and blunts excessive dopaminergic synaptic plasticity by regulating plasmalemmal DAT internalization. Moreover, Piccolo C₂A domain may contribute to such distinct effects by targeting membrane PIP₂.

Materials and methods

Material

A pCMV-hDAT expression plasmid was kindly provided by Dr Marc Caron (Duke University Medical Center). The expression plasmids of pCMV-HA-Piccolo-PDZ (amino acid 3900–4244), pCMV-Myc-Piccolo-C₂A (amino acid 4704–5610) and pGEX4T-GST-p13192 (amino acid 4364–4755; named p13192) were constructed as previously described.¹⁷ The following antibodies were used: hDAT and tyrosine hydroxylase (TH; Chemicon International Inc., Billerica, MA); hemagglutinin epitope (HA) and c-Myc (Cell Signaling, Billerica, MA); GST (Amersham Biosciences, Uppsala, Sweden); Piccolo and Rim 2 (Synaptic Systems, Albany, OR); PIP₂ (Assay Designs, Ann Arbor, MI, USA); syntaxin 1A (Santa Cruz Biotechnology, Santa Cruz, CA); synaptophysin (Sigma-Aldrich, St Louis, MO). The following reagents were used: botulinum neurotoxin (Bont)/C1 and Bont/B (Wako Pure Chemical Industries Ltd, Osaka, Japan); sulfo-NHS-biotin and immobilized streptavidin (Pierce, Rockford, IL).

RT-PCR and real-time RT-PCR

Isolation of total RNA from the NAc of mice was performed using RNeasy Mini Kit (QIAGEN, Hilden, Germany). The mRNA productions from nine target cDNA sequences of Piccolo were assayed by reverse transcription (RT)-PCR, followed by electrophoresis. The forward and reverse primers for the nine sequences were shown in Supplementary Table 1. Piccolo mRNA levels in brain NAc were validated by quantitative real-time RT-PCR using an iCycler System (Bio-Rad, Hercules, CA). Briefly, isolation of total RNA was performed using RNeasy Mini Kit (QIAGEN). For reverse transcription, 1 µg RNA was converted into a cDNA by a standard 20 µl reverse

transcriptase reaction using oligo (dT) primers (Invitrogen, Hercules, CA) and Superscript II RT (Bio-Rad Laboratories, Hercules, CA, USA). Total cDNA (1 µl) was amplified in a 25 µl reaction mixture using 0.1 µM each of forward and reverse primers and Platinum Quantitative PCR SuperMix-UDG (Invitrogen). The primer and dye probes were designed by Nippon Gene Co. Ltd (Tokyo, Japan) using Primer Express software. The forward primer was 5'-GGATAGCGACAAGGTTTTCC-3' (base pair 4180–4200) with reverse being 5'-TTCAACCGAATCATAGGATGCTC-3' (base pair 4257–4279), and the dye probe was 5'-CACAAAGAGAATCCTGAGCTGGTCGATGA-3' (base pair 4192–4220). Ribosomal mRNA was used and determined as control for RNA integrity with TaqMan ribosomal RNA control reagents.

Antisense

An antisense oligodeoxynucleotide (AS; 5'-CTCTGCCAAACTTC-3') and a scramble oligodeoxynucleotide (SC; 5'-AACGTAGTCACGTAG-3') were synthesized by Nippon Gene Co. Ltd. C57BL/6 mice were infused intracerebroventricularly with AS or SC (1 µl h⁻¹, 10 nmol ml⁻¹), made in regular artificial cerebrospinal fluid (CSF) or CSF alone, using an implanted Alzet minipump (AP -0.5 mm, ML +1.0 mm from bregma, DV -2.0 mm from the skull).

Locomotor activity and CPP Test

Locomotor activity was measured using an infrared detector (Neuroscience, Tokyo, Japan) as our previous report.¹⁸ The mice were injected with METH (1 mg kg⁻¹, s.c.) daily for 5 days (day 1–5), followed by locomotor activity measurement at days 1, 3 and 5. Conditioned place-preference (CPP) test was carried out according to the methods as described before but with modification in conditioning.¹⁹ Briefly, a mouse was allowed to move freely between transparent and black boxes for 20 min once per day for 3 days (from day 2 to day 0) in the preconditioning. In the mornings from days 1 to 3, the mouse was treated with METH (1 mg kg⁻¹, s.c.) and put in nonpreferred box for 20 min. After an interval of 12 h the mouse was treated with saline and put in the side opposite to the METH-conditioning box for 20 min. On day 4, the post-conditioning test was performed without drug treatment, and place-conditioning behavior was expressed as post-value minus pre-value.

Microdialysis

C57BL/6 mice were anesthetized before a guide cannula was implanted in the NAc (AP +1.7 mm, ML -0.8 mm from bregma, DV -4.0 mm from the skull).¹⁹ Meanwhile, a mini osmotic pump filled with AS, SC (10 nmol ml⁻¹) or CSF was implanted intracerebroventricularly as described above. Equal numbers of animals were assigned to METH and saline pretreatment groups. Dialysis probes were inserted to the guide cannula the night prior to the experiment. Microdialysis samples were collected every 10 min (2.0 µl min⁻¹). The DA output was presented as

relative to the baseline (the average concentration of four consecutive stable samples defined as 100%).

Western blotting and immunostaining

To determine expression of Piccolo, brain tissue or cell lysate was solubilized in homogenization buffer (150 mM NaCl, 1 mM EDTA, 10 mM Tris, 100 mM Na₂CO₃, pH 11.5) with a mixture of protease inhibitor. After shaking for 30 min and centrifugation at 4 °C, supernatants were subjected to SDS-PAGE (4% polyacrylamide) and transferred to polyvinylidene difluoride membranes. Mouse brains or cultured cells were fixed in 4% paraformaldehyde in PBS and permeabilized with 0.4% Triton X-100.

Cell culture, transfection and [³H]DA uptake

PC12 cells (Riken Bioresource Center Cell Bank, Tsukuba, Japan) were cultured on polyornithine-coated culture coverslips in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum (FBS).⁶ For stable expression of hDAT, PC12 cells were transfected with pCMV-hDAT using Lipofectamine 2000 (Invitrogen). A stably transfected pool was selected with 800 µg ml⁻¹ geneticin (Invitrogen). For transient expression, the cells were transfected with the plasmids expressing different domain of Piccolo. The primary cultured dopaminergic neurons were separated from ventral midbrains of rat embryos (day 14). [³H]DA uptake in hDAT-PC12 cells was performed as described before.²⁰ Briefly, cells were washed in Krebs-Ringers-HEPES (KRH) buffer twice before assay. Uptake was initiated by adding 1 µM 3, 4-(ring-2,5,6-³H)-DA (Perkin Elmer, Waltham, MA) containing 10⁻⁵ M pargyline and 10⁻⁵ M ascorbic acid. Uptake proceeded for 10 min at 23 °C and was terminated by three rapid washes in ice-cold KRH buffer. Accumulated [³H]DA was determined by liquid scintillation counting (Beckman LS6500). Nonspecific uptake was defined in the presence of 10 µM GBR12909 (Sigma).

Cell-surface biotinylation and internalization assays

Biotinylation internalization assays were performed as described previously.⁶

Structural models

Molecule models of Piccolo C₂A domain were generated using the amino-acid sequence data from Protein Data Bank (Gi:42543545). The C₂A domain models were energy minimized using Molecular Operating Environment (MOE) software (Chemical Computing Group, Montreal, Canada) to fix any mismatches between the various structural segments. All calculations used an MMFF94x force field and a cutoff distance of 9.5 Å for nonbinding interactions. ASEDock of the MOE program was used for phospholipids and/or Ca²⁺ ions docking stimulation. DSviewer Lite software (Accelry Inc., San Diego, USA) was used for modeling of the electrostatic surface.

Statistics

All data were expressed as means ± s.e.m. Statistical significance was determined by a one-way ANOVA, followed by the Bonferroni-Dunn test for multigroup comparisons. Differences were considered significant when *P* < 0.05.

Results

Overexpression of Piccolo in the NAc of METH-treated mice

The reasons for pursuing Piccolo for intensive investigation arose from our preliminary findings in PCR-select cDNA subtraction strategy (Clontech Laboratories, Palo Alto, CA, USA) for detecting the

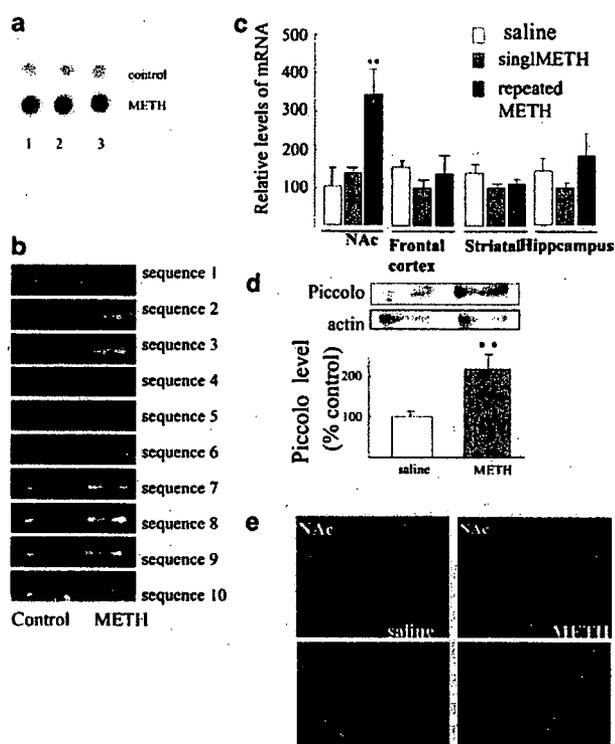


Figure 1 Piccolo expression in nucleus accumbens (NAc) was upregulated by repeated methamphetamine (METH) administration. (a) Piccolo overexpression in the NAc of a representative mouse after daily METH administration for 5 days was shown. (b) RT-PCR analysis revealed a significant increase in the productions of the target sequences of Piccolo induced by METH. (c) Piccolo mRNA production was elevated significantly in NAc, rather than in the frontal cortex, striatal and hippocampus, of the mice treated with repeated METH. Data are expressed as percent of mRNA level of NAc in saline-treated mice (*n* = 6). ***P* < 0.01, compared with saline or single dosing of METH (in NAc). (d) Western blotting analysis showed the elevation of Piccolo level in NAc responding to repeated METH. ***P* < 0.01, compared with saline. (e) Immunostaining showed the elevation of Piccolo immunoreactivity in the NAc of the mice administrated with repeated METH. Saline-treated mice (left column) and METH-treated mice (right column).

affected genes in the NAc by METH. The C57BL/6J mice were daily administrated with METH (2 mg kg⁻¹, s.c.) for 5 days, and Piccolo mRNA production in the NAc was found to increase by 240% in comparison to that of saline-treated mice (Figure 1a). Although little is known about the function of Piccolo in drug-induced behavioral sensitization, its subcellular localization, molecular functions and interacting partners led us to presume that Piccolo overexpression elicited by METH could be involved in DA signaling strength and presynaptic plasticity.

We performed a series of experiments to validate the results from PCR-select cDNA subtraction. After the mice were daily administrated with METH (1 mg kg⁻¹, s.c.) for 5 days, Piccolo mRNA levels in the NAc were measured semiquantitatively by RT-PCR. As Piccolo possesses several splicing domain structures, we amplified and analyzed 10 different target sequences. As shown in Figure 1b, repeated METH administration significantly elevated the mRNA productions of the target sequences of Piccolo in NAc. To confirm such alterations, the mRNA productions of Piccolo in different brain regions were measured quantitatively by real-time RT-PCR 2 h after single METH dosing (1 mg kg⁻¹, s.c.) or the final injection of daily METH administration (1 mg kg⁻¹, s.c.) for 5 days. As shown in Figure 1c, the levels of Piccolo mRNA in the frontal cortex, striatal or hippocampus were not affected by either single or repeated METH administration. Remarkably, Piccolo mRNA level in the NAc was increased following repeated METH administration ($F_{(2,15)} = 5.58$; $P < 0.05$), whereas it was not altered by single METH injection. We then examined Piccolo expression in the NAc using western blotting. Consistently, Piccolo protein level in NAc was elevated apparently after repeated METH administration ($t_{(1,8)} = 7.35$; $P < 0.01$; Figure 1d). Immunostaining also revealed a strengthened Piccolo immunoreactivity in NAc of the mice treated with repeated METH (Figure 1e). Taken together, our data suggest a selective increase of Piccolo expression in NAc of behaviorally sensitized mice induced by repeated METH dosing, rather than a global increase of the brain. Because NAc is a brain area closely associated with drug dependence, we presumed that Piccolo overexpression may be involved in dopaminergic plasticity in neural circuits, which is critical for reward.

Piccolo modulates behavioral plasticity and synaptic DA concentration in NAc

To correlate Piccolo expression with the behavioral and neurochemical phenotype to METH, we utilized an AS strategy, which has been widely used to manipulate gene expressions in the brain via intracerebroventricular infusion.²¹ The designed AS, which directs against nucleotides 2452–2466, has been demonstrated to downregulate successfully the expression of Piccolo in previous studies.¹⁷ Additionally, a SC was used as a control.

The mice were infused continuously with AS, SC or CSF using implanted osmotic minipumps for 3 days before daily saline or METH administration (1 mg kg⁻¹, s.c.) for 5 days. Such infusion was sustained till the end of each behavioral test. Locomotor activities of mice were measured at days 1, 3 and 5 immediately after drug injection (Figure 2a). There was no difference among Piccolo AS-, SC- or CSF-treated mice in baseline locomotor activity throughout a 30 min habituation period (data not shown) or in response to saline (Figure 2b). Repeated METH administration caused a progressive hyperlocomotion in mice, and interestingly, AS-pretreated mice developed a greater hyperlocomotor activity than those treated with SC or CSF after METH administration for 3 days ($F_{(2,15)} = 5.47$; $P < 0.05$; Figure 2c). Furthermore, such enhanced hyperlocomotor activity was sustained till day 5 despite that the difference was not significant compared with that of SC- or CSF-pretreated mice.

We then investigated the potential role of Piccolo in the rewarding effects by the CPP, a classical conditioning paradigm in which animals learn to prefer an environment associated with drug exposure. The mice were infused with AS, SC or CSF for 3 days before the training of CPP (Figure 2d). As shown in Figure 2e, the CSF-treated mice showed baseline preference for either side of the test chambers prior to METH administration, and developed the significant place conditioning after training with METH ($F_{(5,42)} = 9.12$; $P < 0.05$). Notably, the Piccolo AS-pretreated mice showed approximately a double degree of place conditioning compared to those treated with SC or CSF, indicating that the AS-treated mice developed an enhancement of rewarding effect to METH. The mice were killed immediately after the behavioral test to measure Piccolo protein levels in NAc. Piccolo expression in NAc responding to METH was dramatically increased, whereas AS effectively decreased its expression (Figure 2f). These results indicate that Piccolo downregulation was sufficient to confer METH-enhanced sensitization and rewarding effect, which is mediated predominantly by the dopaminergic system. No evidence of neurotoxicity in pathological histology was found outside of the mechanical disruption produced by implantation of the infusion cannula in our experimental conditions (data not shown).

We finally measured DA release in the NAc by a microdialysis technique. The mice were infused with Piccolo AS, SC or CSF for 3 days before daily METH administration (1 mg kg⁻¹, s.c.) for 3 days (Figure 2g). The basal levels of DA in NAc did not differ among CSF-, AS- or SC-treated mice (CSF, 0.58 ± 0.21 nM; AS, 0.49 ± 0.17 nM; SC, 0.60 ± 0.18 nM) before the final challenge of METH. As expected, DA levels in the NAc were markedly increased immediately after the final challenge of METH. Obviously, AS pretreatment promoted METH-induced DA release in the NAc compared with SC or CSF ($F_{(2,9)} = 5.874$; $P < 0.05$; Figure 2h). These data strongly supported

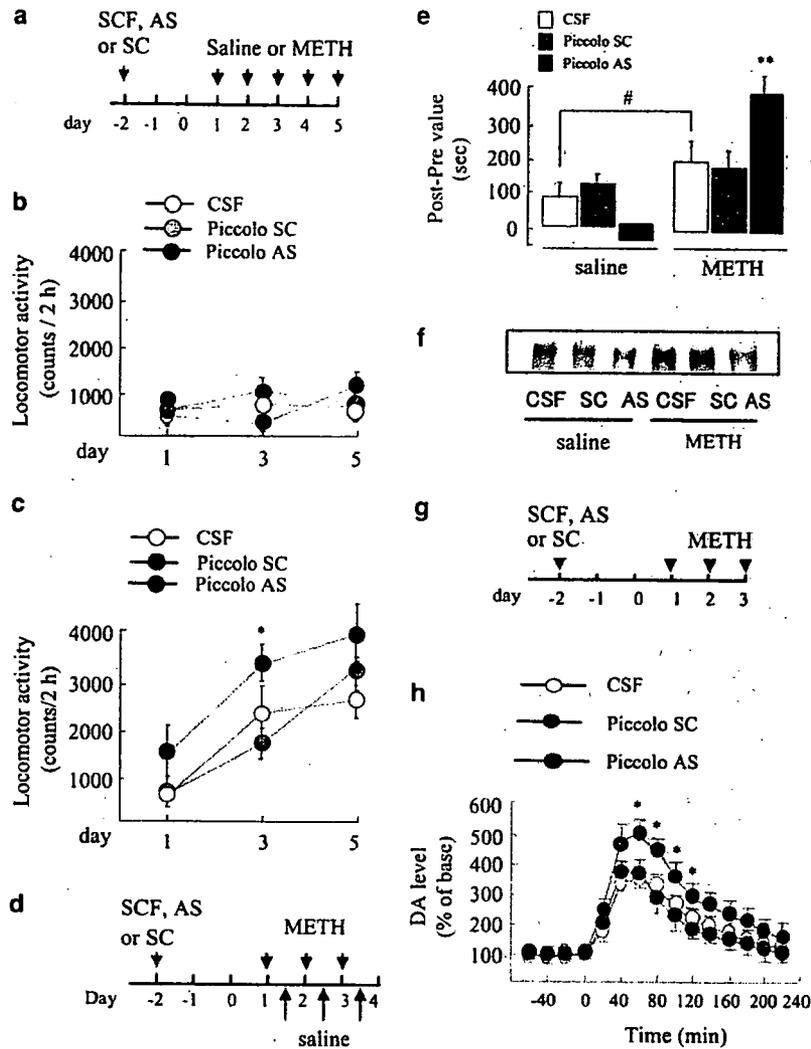


Figure 2 Downregulation of Piccolo expression with antisense oligodeoxynucleotide (AS) promoted methamphetamine (METH)-induced behavioral and synaptic plasticity. (a–c) Mice were infused intracerebroventricularly with Piccolo AS, scramble oligodeoxynucleotide (SC) or cerebrospinal fluid (CSF) for 3 days before daily saline (b) or METH (c) administration for 5 days. Locomotor activities were measured at days 1, 3 and 5 ($n=6$). $*P<0.05$, compared with SC or CSF. (d, e) Mice were infused with Piccolo AS, SC or CSF for 3 days before conditioned place-preference (CPP) training. On day 4, the post-conditioning test was performed ($n=8$). $**P<0.01$, compared with SC or CSF in METH-treated groups, $*P<0.05$, compared with CSF in saline-treated group. (f) The representative immunoblots from western blotting indicated that Piccolo expression in the nucleus accumbens (NAc) was inhibited by AS in the mice treated by repeated METH. (g, h) Mice were infused with AS, SC or CSF for 3 days, followed by daily METH administration for 3 days. Microdialysis was conducted after the final METH injection ($n=4$). $*P<0.05$, compared with SC or CSF at the same time point.

the findings in behavioral tests, suggesting that the enhanced accumulation of DA in NAc resulted from AS may contribute to the amplified responsiveness to METH; moreover, Piccolo may play a role in modulating synaptic DA concentration. Taken these results together, Piccolo overexpression in NAc may present a mechanism of opposing the behavioral responsiveness to METH.

Piccolo is colocalized in dopaminergic neurons

To study whether Piccolo is expressed in dopaminergic neurons, double immunostaining was performed in primary cultured dopaminergic neurons. The

immunoreactivities of Piccolo and TH revealed an extensive overlap along neuronal projections, indicating that Piccolo is present at dopaminergic synapse (Figure 3a). Notably, abundant Piccolo immunoreactivity was observed as clusters and puncta at the dopaminergic terminals (Figure 3b). Moreover, we also found that almost all of the DAT-immunopositive clusters were present at Piccolo-containing clusters situated along dendritic profiles (Figure 3c), implying the potential interplay of these two molecules. These results strongly support the conclusion that Piccolo is a shared component of the dopaminergic synapses.

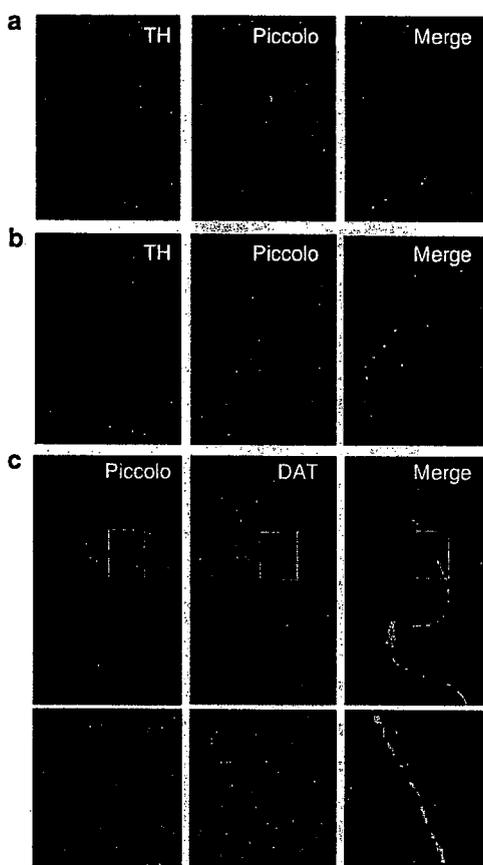


Figure 3 Expression of Piccolo in dopaminergic neurons. (a) Double immunostaining showed that Piccolo is expressed in tyrosine hydroxylase (TH)-positive neurons. (b) Abundant expression of Piccolo is present at the presynaptic component of cultured dopaminergic neurons. (c) Dopamine transporter (DAT) immunoreactivity along the dendritic profiles is paralleled with that of Piccolo.

Piccolo C₂A domain attenuates the inhibition of DA uptake induced by METH through modulating plasmalemmal DAT expression

Total DAT expression levels showed no changes when hDAT-PC12 cells were exposed to either METH (1 μ M) for various time periods or concentrations for 30 min (Figures 4a and b). However, the level of cell surface hDAT was reduced in time-dependent manner, and importantly, such reduction was paralleled with the extent of the inhibition of [³H]DA uptake ($F_{(4,15)} = 25.6$, $P < 0.001$; Figure 4c). Similar results were also obtained in dose-dependent studies, which showed a good correlation of the level of surface hDAT and [³H]DA uptake responding to various concentrations of METH ($F_{(4,15)} = 73.0$, $P < 0.001$; Figure 4d).

The schematic representations of C₂A domain, PDZ domain and a fragment between C₂A domain and PDZ domain are shown in Figure 4e. The C₂A domain, PDZ domain or the fragment were expressed in hDAT-PC12 cells to investigate the changes in [³H]DA uptake. We found that the cells transfected

with C₂A domain showed a slight, but not significant, increase in [³H]DA uptake in response to saline; moreover, transfection of PDZ domain or p13192 did not alter [³H]DA uptake, either (Figure 4f, left panel). We then pretreated the cells with 1 μ M METH for 30 min, followed by [³H]DA uptake assay. METH obviously inhibited [³H]DA uptake, and importantly, C₂A domain-transfected cells showed a higher level of [³H]DA uptake compared with empty pCMV (Stratagene, La Jolla, CA; $F_{(3,20)} = 18.68$, $P < 0.01$), indicating that the C₂A domain expression could attenuate METH-induced inhibition of DA uptake (Figure 4f, right panel).

Because an increase in DA uptake could be resulted from more DAT molecules expressed at the cell surface, we introduced these vectors into hDAT-PC12 cells, and analyzed plasmalemmal hDAT expression by cell-surface biotinylation. The expression levels of cell-surface hDAT did not increase significantly after transfection of C₂A domain, PDZ domain or p13192 in basal conditions (Figure 4g). When the cells were pretreated with 1 μ M METH for 30 min, C₂A domain transfection significantly attenuated the decrease in cell surface hDAT level compared to pCMV ($F_{(3,8)} = 14.61$, $P < 0.01$), whereas PDZ domain and p13192 showed no effects (Figure 4h). Such change was consistent with that of [³H]DA uptake shown in Figure 4f, indicating that Piccolo C₂A domain may attenuate the METH-induced inhibition of DA uptake and maintain DAT expression at cell surface.

Piccolo C₂A domain modulates DAT internalization by a mechanism of membrane association

Given that DAT can be internalized and/or recycled, we speculated that the decreased loss of membrane DAT induced by METH in C₂A domain-transfected cells could be resulted from attenuated DAT internalization. To test this hypothesis, DAT internalization was measured by reversible biotinylation in hDAT-PC12 cells. We found that C₂A domain expression could not affect the basal DAT internalization, as revealed by the similar amount of internalized DAT among all groups (Figure 5a). However, DAT internalization was significantly attenuated by C₂A domain expression when the cells were exposed to 1 μ M METH for 30 min ($F_{(3,8)} = 8.55$, $P < 0.01$; Figure 5b). Expression of both PDZ domain and p13192 failed to affect the basal or METH-induced DAT internalization. Double immunostaining for hDAT and c-Myc-tagged C₂A domain showed the similar findings that the cells transfected with C₂A domain still maintained a strong plasmalemmal hDAT immunoreactivity responding to METH, whereas a relatively large amount of internalized hDAT was observed in cytosolic compartments of the cells transfected with empty pCMV (Figure 5c). These results indicated that Piccolo C₂A domain attenuates METH-induced DAT internalization, which accounts for the decrease in the loss of DAT at cell surface.

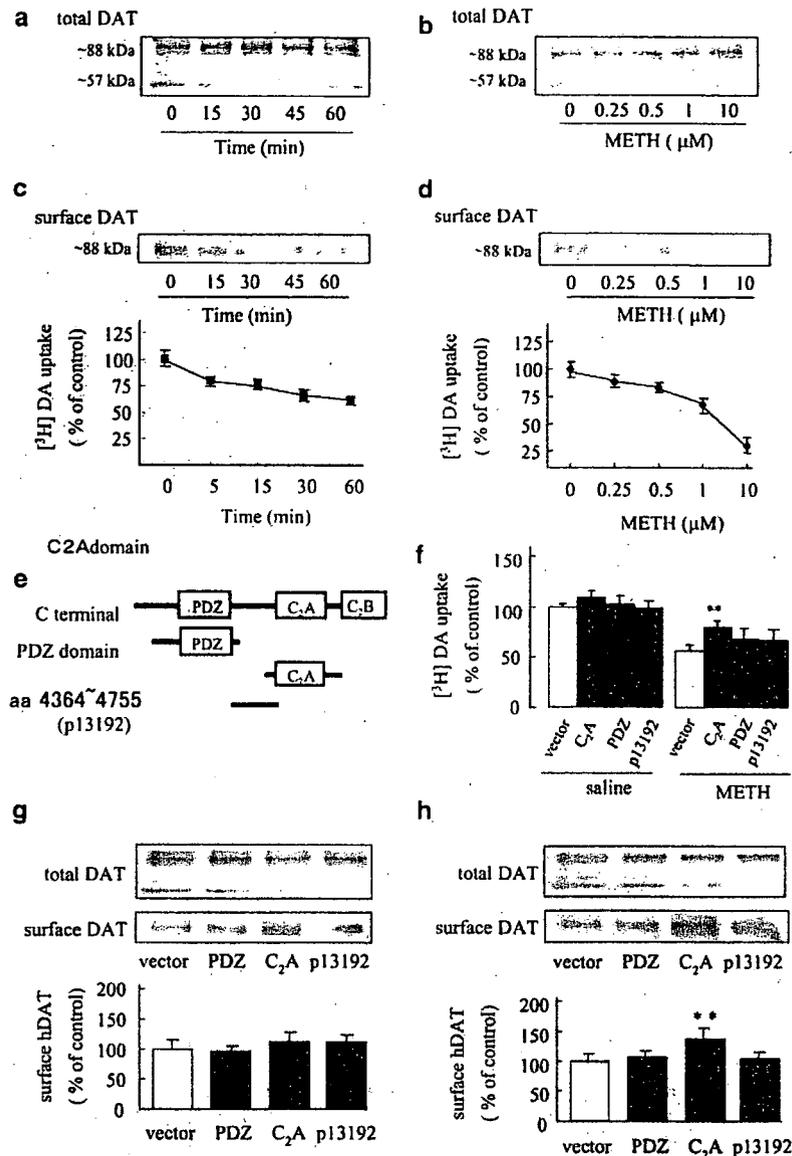


Figure 4 Piccolo C₂A domain increased dopamine (DA) uptake and dopamine transporter (DAT) surface expression. (a, b) Methamphetamine (METH) could not alter the total DAT expression levels in hDAT-PC12 cells in both time- (a) and dose-dependent studies (b). (c) METH (1 μ M) decreased plasmalemmal DAT expression (top) in time-dependent manner, which was paralleled with the decrease in [³H]DA uptake (bottom). ***P* < 0.01, compared with the basal level. (d) METH decreased DAT expression at the cell surface dose-dependently (top), which was consistent with the decrease in [³H]DA uptake (bottom). ***P* < 0.01 and **P* < 0.05, compared with the basal level. (e) Schematic representations of C₂A domain, PDZ domain and a fragment (amino acid 4364–4755). (f) Piccolo C₂A domain attenuated the METH-induced inhibition of [³H]DA uptake (right panel), but failed to change the basal DA uptake (left panel) (*n* = 6). ***P* < 0.01, compared with pCMV. (g, h) Piccolo C₂A domain could not influence DAT surface expression in hDAT-PC12 cells responding to saline (g). However, it attenuated METH-induced loss of surface DAT (h). ***P* < 0.01, compared with pCMV in METH-treated group.

To study the potential mechanism underlying the action of Piccolo C₂A domain on DAT internalization, we introduced C₂A domain into hDAT-PC12 cells and then analyzed membrane subcellular distributions of Piccolo, C₂A domain, hDAT as well as PIP₂. The cells were homogenized in regular RIPA buffer containing 1% Triton-X 100, and separated into a soluble supernatant and a particulate membrane fraction (120 000 g,

60-min pellet). The latter was solubilized again in RIPA buffer or RIPA buffer containing 0.1 M Na₂CO₃ (pH 11.5), which can extract a major part of detergent-resistant Piccolo protein from brain tissues.¹² As shown in Figure 5d, Piccolo, Piccolo C₂A domain and PIP₂ did not fractionate like a soluble cytosolic protein but was mainly found in membrane sediment extracted by Na₂CO₃, indicating that a substantial

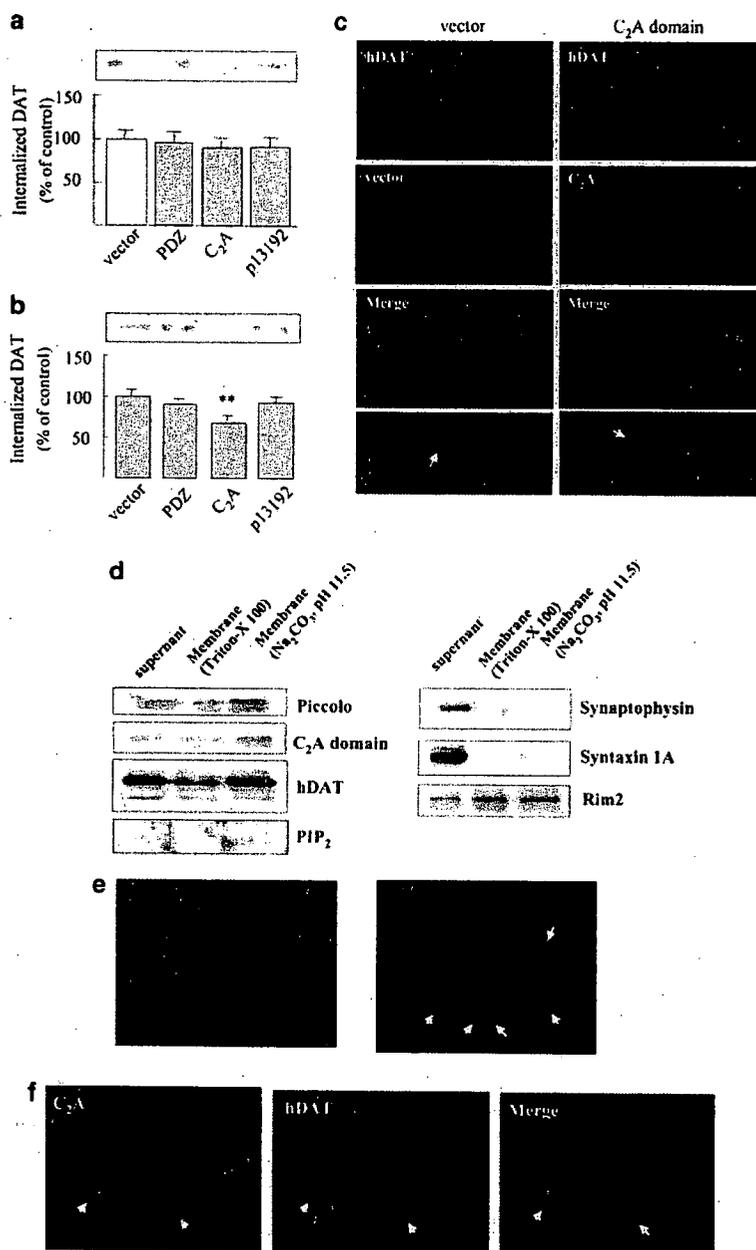


Figure 5 Piccolo modulates dopamine transporter (DAT) internalization by a mechanism of membrane association. (a, b) After transfection with various vectors the hDAT-PC12 cells were biotinylated and treated with either saline (a) or 1 μ M methamphetamine (METH) (b) for 30 min to initiate endocytosis. Top, representative blots of internalized hDAT. Bottom, quantitation of hDAT immunoreactivity. $P < 0.01$, compared with empty vector (pCMV) in METH-treated group. (c) The internalization of hDAT (red) was triggered by exposure of 1 μ M METH for 30 min. The cells transfected with empty vector (pCMV) show enriched internalized hDAT (left panel), whereas the cells transfected with c-Myc-tagged-C₂A domain (green) reveal the strong plasmalemmal hDAT immunoreactivity (right panel). Internalized hDAT is depicted. (d) Distributions of Piccolo, c-Myc-tagged C₂A domain, hDAT, PIP₂ and other presynaptic proteins in hDAT-PC12 cells. The cells and membrane fractions were extracted with RIPA buffer containing 0.1 M Na₂CO₃ (pH 11.5) or not. (e) The transfected Piccolo C₂A domain specially targets plasma membrane in hDAT-PC12 cells. (f) Piccolo C₂A domain shows a paralleled immunoreactivity pattern at plasmalemmal rafts with hDAT (arrowhead).

fraction of membrane-bound Piccolo, C₂A domain and PIP₂ are associated with the same plasmalemmal rafts. Interestingly, a significant amount of hDAT was also recovered in both soluble fraction and membrane

sediment extracted by Na₂CO₃, indicating that a relatively major part of membrane DAT is localized at the same subcellular fraction with Piccolo C₂A domain and PIP₂. The similar distributions of these

components in lipid raft fractions hint that C₂A domain-PIP₂ interaction may be involved in the distribution of plasmalemmal DAT. In contrast, syntaxin 1A and synaptophysin, the integral membrane proteins, were almost completely recovered in soluble cytosolic fraction, but not in a detergent-resistant fraction. Rim 2, a scaffolding protein with C₂ domain, is known to interact with Piccolo and to regulate presynaptic events. However, its similar subdistribution in the three fractions was different from that of Piccolo C₂A domain. To get an insight into the interplay among DAT, Piccolo C₂A domain and PIP₂, double immunostaining was performed. We found that Piccolo C₂A domain mainly anchored nonuniformly to the inner leaflet of plasma membrane (Figure 5e), which is consistent with its property of targeting membrane PIP₂. Notably, the distribution pattern of C₂A domain resembled that of hDAT, as revealed by the paralleled immunoreactivities at membrane microdomains (Figure 5f).

Internalization of plasmalemmal DAT is PIP₂-dependent

The concept of PIP₂ as a spatially localized regulator of membrane trafficking is clearly illustrated by its key role in clathrin-mediated endocytosis for transporter. If plasmalemmal DAT is triggered to internalize by METH, it should be accompanied by PIP₂ for recruiting endocytic adaptors through PIP₂-binding modules. To test this idea, hDAT and PIP₂ were double-stained in hDAT-PC12 cells after treatment of saline or 1 μM METH for 30 min. Surprisingly, the internalized DAT triggered by METH was found to colocalize with the PIP₂ in the cytosolic compartment (Figure 6, bottom panel), whereas the saline-treated cells only showed the constitutively internalized PIP₂ and DAT (Figure 6, top panel). These results further demonstrated that DAT internalization is also a clathrin-dependent process requiring the assembly of endocytic components like PIP₂.

Interaction of Piccolo C₂A domain and PIP₂

Although Piccolo C₂A domain binding to PIP₂ has been demonstrated using artificial membranes,¹⁵ there is no evidence indicating interaction of the two molecules in living models. We first investigated whether plasmalemmal clusters of Piccolo immunoreactivity coincide with sites of local PIP₂ accumulation using double immunostaining. The clusters of Piccolo immunoreactivities in dendrite profile colocalized precisely with those of PIP₂ in the primary cultured dopaminergic neurons (Figure 7a). Moreover, the localization of transfected C₂A domain in hDAT-PC12 cells was similar with that of PIP₂, which revealed a patchy staining pattern at plasma membrane (Figure 7b). Importantly, the clusters with strong immunoreactivity of C₂A domain also showed substantially larger and stronger labeling macroscopic of PIP₂ clusters, indicating that C₂A domain may sequester PIP₂, thus augmenting the formation of microscopically detectable plasmalemmal PIP₂ clusters.

To better understand the interaction of the two molecules, we generated a PIs binding model of Piccolo C₂A domain with Ca²⁺ docking. As show in Figure 7c, the three-dimensional structure indicated that the predicted PIs binding sites are Ca²⁺-binding loops at the top of C₂A domain, which shows the similar binding residues for phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP) and PIP₂. Notably, the crystal packing contacts for PIP₂ were the clusters of basic/aromatic residues including 4668–4670 (DNN), 4697–4698 (QK), 4738–4743 (DYDRFS) and 4746 (D). The potential importance of these residues is highlighted by the fact that they are completely conserved among rat, mouse, human and chicken Piccolo.²² Calculation of the electrostatic surface potential of C₂A domain showed that PIP₂ binding sites are positively charged (Figure 7d), further indicating that clustering PIs by C₂A domain depends on electrostatic interactions between the

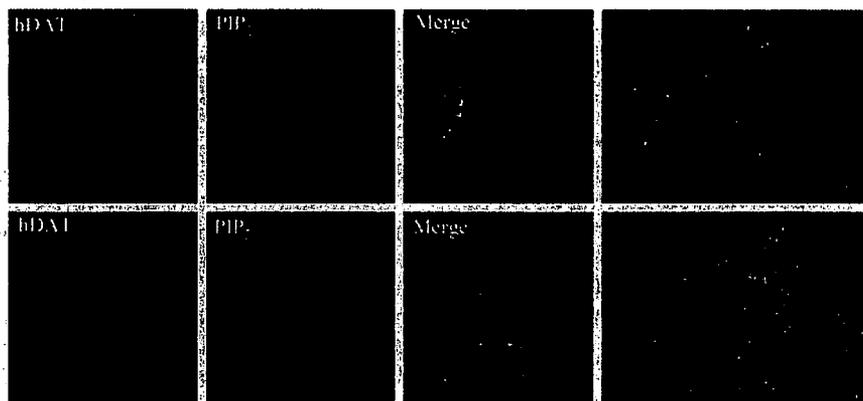


Figure 6 Piccolo C₂A domain attenuates dopamine transporter (DAT) internalization responding to methamphetamine (METH). Double-immunostaining of PIP₂ (red) and hDAT (green) in hDAT-PC12 cells. The internalization of hDAT was promoted by METH, which is accompanied by PIP₂ (bottom panel). The saline-treated cells show strong immunoreactivities of both hDAT and PIP₂ (top panel).

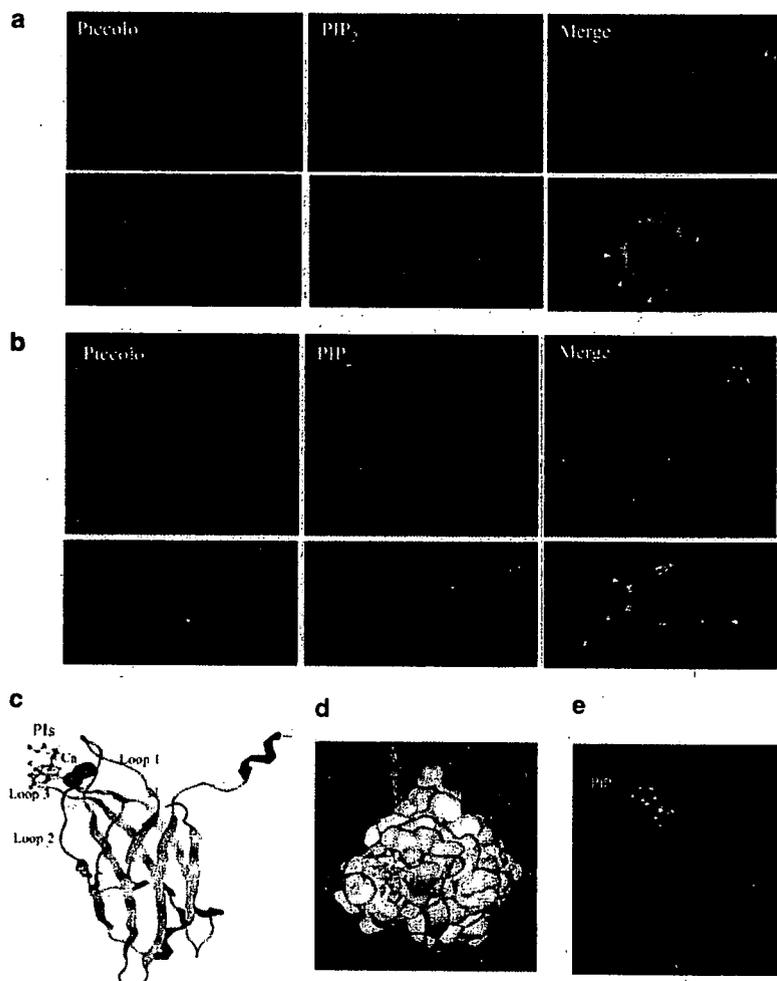


Figure 7 Interaction of Piccolo and PIP₂. (a) PIP₂ (red) colocalizes precisely with Piccolo (green) along the presynaptic terminal in primary cultured dopaminergic neurons (arrowed). (b) PIP₂ (red) accumulates at plasmalemmal rafts, where it colocalizes with Piccolo (green) in hDAT-PC12 cells. Arrowheads point to regions of intense staining of PIP₂ and Piccolo. (c) Model of Piccolo C₂A domain with three bound Ca²⁺ ions on top (green spheres). The top surface of C₂A domain shows the binding sites for the headgroups of PIs. (d) Surface plot showing the electrostatic potential of C₂A domain. Blue, positive; red, negative charge; white, neutral. PIP₂ is pointed. (e) Space-filling model of PIP₂ is shown on top in pink C₂A domain, which provides a cupped shape of polybasic region to accommodate PIP₂.

positively charged residues in proteins and the negatively charged headgroups of PIs. The lowest binding energies of Piccolo C₂A domain for PI, PIP and PIP₂ with Ca²⁺ docking were -59.491 , -93.229 and -102.642 Kcal, respectively, suggesting a specific interaction between PIP₂ and C₂A domain. Furthermore, the space-filling model showed that PIP₂ is tightly packed against the top surface of C₂A domain, which forms a favorable pocket to accommodate the moiety of PIP₂ (Figure 7e).

Piccolo regulated DAT function not through syntaxin 1A
As syntaxin 1A has been demonstrated to regulate the expressions and activities of serotonin transporter (SERT) and γ -aminobutyric acid (GABA) transporters,^{23,24} Piccolo might regulate DAT surface

expression through interaction with syntaxin 1A. We first investigated whether syntaxin 1A could bind to Piccolo, though syntaxin is identified to bind to Piccolo.²⁵ The lysates from hDAT-PC12 cells were immunoprecipitated with anti-syntaxin 1A, followed by hDAT immunoblotting. As shown in Supplementary Figure 1a, hDAT were present in the lysate. As expected, we also detected co-immunoprecipitation of hDAT and syntaxin 1A in following immunoprecipitation with anti-hDAT (Supplementary Figure 1b). These results showed an apparent association of these two molecules, which was supported by previous reports.²⁶ We then investigated whether syntaxin 1A could regulate DAT activity. The hDAT-PC12 cells were pretreated with Bont/C1, a toxin that specifically cleaves syntaxin 1A, followed by

[³H]DA uptake assay; moreover, Bont/B that specifically cleaves the vesicle *N*-ethylmaleimide-sensitive factor attachment receptor protein synaptobrevin was used as a control. As shown in Supplementary Figure 1c, Bont/C1 (0.5–5 nM) failed to alter the [³H]DA uptake in the cells treated with saline. Although Bont/C1 slightly elevated [³H]DA uptake in the cells exposed to METH compared with Bont/B, the difference was not significant. To exclude that such incapability of Bont/C1 in modulating DA uptake was a result of the low concentration or short exposure time, we treated the cells with Bont/C1 at 0.25 μM for 6 h. However, [³H]DA uptake was also not altered (data not shown). Additionally, exposure of METH at the concentration ranging from 0.5–20 μM for 30 min did not alter the expression level of syntaxin 1A in hDAT-PC12 cells (data not shown). Taken together, these data suggest that DAT and syntaxin 1A may mechanically, but not functionally, interact. Given the incapability of syntaxin 1A itself in modulating DAT, it unlikely mediates the role of Piccolo in regulating DAT expression at plasma membrane.

Discussion

The contribution of dopaminergic transmission to behavioral sensitization has been well recognized. Expression of certain proteins appears to be compensatory adaptation to the excessive DA signaling, which could be biologically adaptive mechanisms contributing to addiction. Nevertheless, some proteins likely function in a reverse manner. For example, we have previously found that the expression of tissue plasminogen activator plays a positive role in morphine-induced synaptic plasticity,¹⁹ whereas tumor necrosis factor-α expression in NAC inhibits METH-induced dependence.¹⁸ Piccolo expression was upregulated by repeated METH administration and partial knockdown of Piccolo expression by antisense technique led to elevated synaptic DA concentration in the NAC and two major behavioral manifestations in mice: heightened hyperlocomotor activity and rewarding effect. These findings strongly show that Piccolo overexpression elicited by METH may serve as a homeostatic mechanism that prevents behavioral sensitization by maintaining the expression and activity of the plasmalemmal DAT.

The human Piccolo gene contains more than 25 exons spanning over 350 kb of genomic DNA maps to 7q11.23-q21.3, a region of chromosome 7 implicated as a linkage site for autism and Williams Syndrome.²² Therefore, dysfunction of Piccolo may be involved in cognitive impairment and mental retardation.²⁷ The mechanism underlying Piccolo upregulation caused by METH remains to be elucidated. Nevertheless, inhibitory feedback to the excessive DA signaling would be a plausible candidate.

Piccolo has been reported to localize at the GABAergic and glycinergic presynaptic terminal,¹⁰ and our findings in immunostaining demonstrated

that it is also expressed at dopaminergic presynaptic terminal. DAT can be internalized from the plasma membrane at a relatively rapid rate, which provides a mechanism by which the turnover rate and density of the plasmalemmal DAT can be quickly and finely modulated.^{6,8} Signaling molecules, glycosylation and DAT substrates have been shown to regulate DAT membrane trafficking. Given those findings *in vivo* behaviors tests and the properties of Piccolo, we assumed that Piccolo may play a role in modulating DA flux and DAT distribution at dopaminergic terminals. To address this issue, we investigated DA uptake and membrane DAT expression in hDAT-PC12 cells expressing different functional domain of Piccolo. METH caused DA uptake inhibition in parallel with decreased DAT surface expression, which was well consistent with those works defining the dynamically internalized DAT in hDAT-PC12 cells triggered by amphetamine. These results further support the notion that redistribution of surface DAT caused by METH-like drugs may present an important mechanism underlying the consequently reduced DAT activity. Our data showed that Piccolo C₂A, but not PDZ domain, attenuated METH-induced DA uptake inhibition by retaining DAT expression at cell surface. Because DAT can be internalized and/or recycled, we speculated that the decreased loss of membrane DAT could be resulted from attenuated DAT internalization. Such hypothesis was demonstrated by reversible biotinylation, which revealed the decreased DAT internalization in C₂A domain-transfected cells responding to METH.

It is well established that PIP₂ functions in regulating cytoskeleton, channels and transporters, and membrane trafficking at presynaptic terminal.^{16,28,29} Especially, PIP₂ is essential at several stages of endocytosis for the sequential recruitment of adaptor and accessory proteins to endocytic sites.^{30,31} METH rapidly causes both DAT internalization and conformational rearrangement to an intracellularly oriented transporter from which DA is released. Such process is proposed to be a drastic membrane movement and requires PIP₂ to assemble various molecules to form endocytic compartment. Significantly, we found that PIP₂ exhibits a similar distribution pattern with DAT at membrane microdomains. Furthermore, internalized DAT triggered by METH is accompanied with PIP₂ in endocytic compartments. These results indicate that PIP₂ is an important regulator in the process of DAT internalization.

A couple of scaffolding proteins such as GAP43, CAP23 and Dap160 have shown their ability to sequester membrane PIP₂, thus potentially modulating the endocytic process.^{32,33} In this study we obtained several evidences further supporting the notion that Piccolo can electrostatically sequester PIP₂. Firstly, Piccolo C₂A domain may laterally bind membrane PIP₂, and augment PIP₂ clusters in hDAT-PC12 cells. In principle, the augmented clusters could represent the sequestration of phospholipids like PIP₂ at the plasma membrane.³⁴ Secondly, the crystal

packing contacts for PIP₂ were the clusters of basic/aromatic residues, which exhibit a universal capability of sequestering membrane PIP₂.³⁵ Thirdly, the space-filling model showed that Piccolo C₂A domain may pocket PIP₂ by a cupped shape of polybasic region, where the local positive potential electrostatically attracts the negatively charged PIP₂. Finally, C₂A domain shows stronger interacting potential with PIP₂ than PI or PIP. Our results are consistent with previous investigations indicating that PIs binding with Piccolo C₂A domain is largely driven by electrostatic interaction.¹⁵

Based on these findings, we speculated that Piccolo C₂A domain may regulate METH-triggered DAT internalization through sequestering PIP₂, and the findings in immunostaining strongly support this prediction. Piccolo C₂A domain mainly anchors nonuniformly to the inner leaflet, which is accompanied with the retention of DAT and PIP₂ at membrane microdomains; moreover, it clearly attenuated METH-triggered DAT and PIP₂ internalization in cytosol. These results show that Piccolo may sequester or 'control' locally PIP₂ by C₂A domain in membrane raft and suppress PIP₂-dependent endocytic process, thus leading to the attenuated DAT internalization.

How does the Piccolo C₂A domain-PIP₂ interaction fulfill a function in modulating DAT internalization and psychostimulant responsiveness? An explanation could be that the endocytic process for DAT internalization is inhibited directly through PIP₂ sequestration. Given the strong dependence of the endocytic machinery on PIP₂, more membrane PIP₂ is considerably mobilized for the accelerated DAT internalization triggered by METH. This situation would place the endocytic machinery of dopaminergic presynaptic terminal in a compromised position of insufficient availability of PIP₂, and thus slowing down the DAT internalization. Similarly, a dominant-negative mutant of dynamin I, a component of endocytic machinery, inhibits both PKC- and amphetamine-dependent DAT internalization;^{7,36} interruption of adaptor proteins present in clathrin-coated pits like epsin interferes with DAT endocytosis.³⁷ Another explanation could be that Piccolo C₂A domain may retain DAT at cell surface by promoting membrane stability. METH causes both DAT internalization and conformational rearrangement to an intracellularly oriented transporter from which DA is released. In this process PIP₂ acts as a positive regulator in modulating actin filament assembly and membrane movement by creating membrane microdomains and binding proteins with lipid-specific interaction.^{38,39} Therefore, overexpressed Piccolo elicited by METH may enhance the association with membrane PIP₂ or other PIs through C₂A domain and disturb PIP₂-dependent actin assembly, thereby strengthening membrane stability and weakening DAT internalization. In this case, Piccolo may function as a general stabilizer for plasma membrane and DAT. It is worth noting that protein interacting with C kinase 1 (PICK1), a skeletal

component, may also stabilize and maintain DAT at plasma membrane.⁴⁰

Piccolo likely binds to syntaxin 1A through its C₂A domain, because synaptotagmin C₂A domain which shares a great structural similarity with Piccolo C₂A domain interacts with syntaxin 1A.^{15,40} Syntaxin 1A directly regulates the expressions and activities of SERT and GABA transporter.^{23,24} Interestingly, a recent work has identified that syntaxin 1A also binds to DAT.²⁶ However, Piccolo C₂A domain appears not to regulate METH-induced DAT internalization through syntaxin 1A, because DA uptake is not affected when syntaxin 1A is inhibited.

Our findings reveal that Piccolo is capable of regulating METH-induced DAT internalization, leading to the change of DA signaling and synaptic strength. The precise mechanism underlying the role of C₂A domain-PIP₂ interplay in DAT internalization remains to be determined. No matter which mechanism could be more reasonable, sequestration of PIP₂ in lateral domains through C₂A domain appears to be important for Piccolo to regulate DAT internalization. Therefore, a greater understanding of the molecular regulators for PIP₂, which governs DAT trafficking, would shed light on the modulation of DAT surface presentation. Further investigation measuring membrane fluorescence resonance energy transfer and PIP₂ turnover/mobilization will help interpret the contribution of the proposed mechanisms.

The present investigation illustrates a paradigm that Piccolo, a presynaptic scaffolding protein, targets membrane PIP₂ by its C₂A domain, contributing to the regulation of DAT internalization. Piccolo upregulation may represent a homeostatic response of dopaminergic neurons in the NAc to excessive dopaminergic transmission, dampening hypersensitivity and rewarding effect.

Acknowledgments

We are thankful to Dr Seino Susumu and Dr Shibasaki Takao (Division of Cellular and Molecular Medicine, Kobe University Graduate School of Medicine, Japan) for the kind gifts of pCMV-HA-Piccolo-PDZ, pCMV-Myc-Piccolo-C₂A and pGEX4T-GST-p13192. We thank Mrs Nobushi Hamada and Yoshiyuki Nakamura radioisotope Center Medical Branch, Nagoya University School of Medicine for technical support. This study was supported in part by a Grant-in-Aid for Science Research and Special Coordination Funds for Promoting Science and Technology, Target-Oriented Brain Science Research Program and 21st Century Center of Excellence Program 'Integrated Molecular Medicine for Neuronal and Neoplastic Disorders' and 'Academic Frontier Project for Private Universities (2007–2011), from the Ministry of Education, Culture, Sports, Science and Technology of Japan; by a Grant-in-Aid for Health Science Research on Regulatory Science of Pharmaceuticals and Medical Devices, and Comprehensive Research on Aging and Health from the

Ministry of Health, Labor and Welfare of Japan; by a Smoking Research Foundation Grant for Biomedical Research and Takeda Science Foundation.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (<http://www.nature.com/mp>)

**Restraining tumor necrosis factor-alpha by thalidomide prevents the Abeta-induced
impairment of recognition memory in mice**

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The number of: text pages: 34

tables: 0

figures: 4

The number of words in abstract: 209/250

introduction: 327/500

discussion: 686/1500

Abstract

No effective remedy has currently been realized to prevent the cognitive impairments of Alzheimer's disease (AD). The interruption of the toxic pathways of amyloid beta peptide ($A\beta$) still remains promising for the treatment. The involvement of tumor necrosis factor-alpha ($TNF-\alpha$) in the toxicity of $A\beta_{1-40}$ in recent reports provide a fresh target for the interruption. In the current study, we evaluated the feasibility of a strategy that target $TNF-\alpha$ to prevent the impairment of memory induced by $A\beta$. The *i.c.v.*-injection of $A\beta_{25-35}$ increased the hippocampal mRNA expression of both $TNF-\alpha$ and inducible nitric oxide synthase (iNOS), of which the former was stronger. The knock-out of $TNF-\alpha$ ($TNF-\alpha$ (-/-)) in mouse prevented the increase of iNOS mRNA induced by $A\beta_{25-35}$. Not only the inhibition of iNOS activity but also $TNF-\alpha$ (-/-) prevented the nitration of proteins in the hippocampus and the impairment of recognition memory in mice induced by $A\beta_{25-35}$. Daily treatment with thalidomide (20mg/kg), a preferential degrader of $TNF-\alpha$ mRNA, or *i.c.v.*-injection of an anti- $TNF-\alpha$ antibody (10ng/mouse) prevented the nitration of proteins in the hippocampus and the impairment of recognition memory induced by $A\beta_{25-35}$ or $A\beta_{1-40}$ in mice. These results suggested the practicability of targeting $TNF-\alpha$ as a preventive strategy against $A\beta$ -mediated cognitive impairments.

Key words: Amyloid beta (25-35), tumor necrosis factor alpha, protein nitration, recognition memory, thalidomide

Introduction

Treating the cognitive impairments of Alzheimer's disease (AD) is a complex practice due to its multifactoral pathology that including the increase of toxic amyloid beta peptide ($A\beta$), neuroinflammation, oxidative damage, and neurodegeneration in critical brain regions involved in memory and cognition (18,42,49,53,63,66). Although there have been many recent advances in the understandings of the pathological process of AD, therapeutic approaches still remain limited to targeting the toxic pathways of $A\beta$, the assumed central player in the pathogenesis of the disease (15,19,30,38,39,41,50).

The neurotoxicity of $A\beta$ has recently been implicated by the involvement of tumor necrosis factor - alpha ($TNF-\alpha$), a multifunctional cytokine that triggers a wide range of cellular responses (35). $TNF-\alpha$ is found upregulated in AD patients (8,17,43,56), and involved in $A\beta_{1-40}$ -induced inhibition of long-term potentiation in hippocampal slices and the impairment of spatial memory in mice (36,65). $A\beta_{1-40}$ induces toxicity by a mechanism of $TNF-\alpha$ dependent overexpression of inducible nitric oxide synthase (iNOS) (2,13,69). In general, $TNF-\alpha$ is regarded as an inducer of the expression of iNOS (11,23,51). The induced iNOS produces high amount of NO (25,59) which interacts rapidly with superoxide to form proxynitrite (6,46). Proxynitrite mediates the extensive nitration of proteins in the brain of AD and evidently correlated with the increased level of cerebral $A\beta$ and the severity of cognitive impairment (27,28,54,57). The impairments of memory induced by $A\beta$ in mice are prevented

by the genetic deletion of TNF- α receptor or iNOS as well as by scavenging of peroxynitrite (3,36,40). The implications of the involvement of TNF- α in iNOS-driven peroxynitrite and memory failure prompt a fresh strategy of targeting the synthesis of TNF- α for the prevention of A β -induced cognitive impairment. In this study, we have reconfirmed the critical involvement of TNF- α in the neurotoxicity of A β ₂₅₋₃₅, the most toxic A β fragment that detected in the brain of AD patient (29,44,68), by utilizing TNF- α (-/-) mouse and evaluated the usefulness of targeting TNF- α by using thalidomide to prevent the A β -induced impairment of memory in mice.

2. Material and methods

2.1. Animals

Male C57BL/6 (wild type, WT) mice, male TNF- α knock out (TNF- α (-/-)) mice (55), and male ICR mice (Nihon SLC Co., Shizuoka, Japan), were used. The animals were housed in a controlled environment (23 \pm 1°C, 50 \pm 5% humidity) and allowed food and water ad lib. The room lights were kept on between 8:00a.m and 8:00p.m. All experiments were performed in accordance with the Guidelines for Animal Experiments of Nagoya University Graduate School of Medicine. The procedures involving animals and their care conformed to the international guidelines set out in "Principles of Laboratory Animal Care".

2.2. Treatment and experimental design

A β ₂₅₋₃₅ and A β ₃₅₋₂₅ (Bachem, Bubendorf, Switzerland) were dissolved in sterile double-

distilled water in a concentration of 1mg/ml and stored at -20 °C before use and incubated for aggregation at 37 °C for 4 days before the injection. A β ₁₋₄₀ and A β ₄₀₋₁ (Bachem, Bubendorf, Switzerland) were dissolved in 35 % acetonitrile / 0.1 % trifluoroacetic acid to a stock concentration of 1mg/ml and stored at -20 °C before use. Peptides including A β ₂₅₋₃₅ and A β ₃₅₋₂₅ were *i.c.v.*- injected at a volume of 3 μ l. Peptides including A β ₄₀₋₁ and A β ₁₋₄₀ were *i.c.v.*-injected at a volume of 5 μ l. A β ₃₅₋₂₅ and A β ₄₀₋₁ were injected as the control. All peptides were injected as described previously (3,34). Aminoguanidine (Wako, Saitama, Japan) was dissolved in saline and *i.p.*-injected at the dose of 100mg/kg/day as described previously (36,59). Thalidomide (Sigma, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) and *p.o.*-administrated at the dose of 20mg/kg/day that was selected according to the previous literature on its pharmacodynamic study in mice (12). Anti-TNF- α antibody (α -TNF- α) (R&D Systems, Minneapolis, MN) was *i.c.v.*-injected at the dose of 10 μ g that followed by the injection of A β peptides with 15 minutes interval as described previously (36). The schedule of administration of peptides and drugs as well as biochemical and behavioral investigations are shown in Fig. 1.

2.3. Real-time reverse transcription-polymerase chain reaction

Mice were decapitated at the indicated time-points after the *i.c.v.*-injection of A β ₂₅₋₃₅ (Day 0). The hippocampi were removed on ice-cold glass plate and stored at -80°C. The hippocampal tissue was homogenized and total RNA was extracted using an RNeasy total

RNA isolation kit, following the supplier's protocol (Qiagen, Valencia, CA). cDNA was synthesized by using a SuperscriptTM reverse transcriptase kit (Invitrogen, Carlsbad, CA). The primer sequences were given below: for iNOS (Gene Bank access: NM_010927), forward primer: 5'-GGGCAGCCTGTGAGACCTT-3'; reverse primer: 5'-GCATTGGAAGTGAAGCGTTTC-3'; TaqMan probe: TGTC CGAAGCAAACATCACATTCAGATCC; For TNF- α (Gene Bank access: NM_023517), forward primer: 5'-CTTTCGGTTGCTCTTTGGTTGAG-3'; reverse primer: 5'-GCAGCTCTGTCTGTTGGATCAG-3'; TaqMan probe: TGCGACAGCACAAAGTCACAGCCCC; for the brain-derived neurotrophic factor (BDNF) (Gene Bank access: BC034862), forward primer: 5'-GCAAACATGTCTATGAGGGTTCG-3'; reverse primer: 5'-ACTCGCTAATACTGTCACACACG-3'; TaqMan probe: ACTCCGACCCTGCCCGCCGT; for glial cell-derived neurotrophic factor (GDNF) (Gene Bank access: NM_010275), forward primer: 5'-GAAGAGAGAGGAATCGGCAGG-3'; reverse primer: 5'-TGGCCTCTGCGACCTTTC-3'; TaqMan probe : AGCTGCCAGCCCAGAGAATTCCAGAG; For all, the experimental amplification protocol consisted of a first round at 95°C for 3 min and then 30 cycles of denaturation at 95°C for 60 s, annealing at 60°C for 60 s, and extension at 72°C for 1 min, with a final extension reaction carried out at 72°C for 10 min. PCR was carried out on Bio-Rad iCycler iQTM real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). The signal was detected according to the manufacturer's instructions. The expression levels were calculated