levels in the NAc through potentiation of plasmalemmal and vesicular DA uptake *via* induction of TNF-α expression (Fig. 7), although the mechanism by which TNF-α is regulated by shati remains to be elucidated.

Motif analyses have revealed that shati contains sequences of GNAT (Niwa *et al.* 2007a). Docking simulations with acetyl-CoA or ATP conducted using Molecular Operating Environment software reveal possible acetyl-CoA- and/or ATP-binding sites, since there is low potential energy for these interactions, in contrast with the prohibitively high energy of docking with DA, DNA or nuclear localization signals (Niwa *et al.* 2007a). These results suggest shati to have a physiological role in producing acetylcholine or the metabolic action of ATP. Accordingly, we have to investigate the mechanism by which shati regulates the production of acetylcholine or metabolic roles of ATP in subsequent studies.

In conclusion, we hypothesized that TNF-α expression induced by shati inhibits the METH-induced increase in extracellular DA levels in the NAc by promoting DA uptake and finally inhibits sensitization to and the rewarding effects of METH (Fig. 7). Targeting the shati-TNF-α system would provide a new therapeutic approach to the treatment of METH dependence.

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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 administrated with METH. Piccolo downexpression by antisense technique augmented METH-induced behavioral sensitization, conditioned reward and synaptic dopamine accumulation in NAc. Expression of Piccolo C_2A 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 C_2A 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 C_2A domain may act as a cytoskeletal regulator for plasmalemmal DAT internalization, which may underlie its contributions in behavioral plasticity.
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Keywords: Piccolo; dopamine transporter; methamphetamine; behavioral plasticity; C_2A 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 Ca^{2+}/phospholipid binding (C_2A and C_2B) domains, each of which exhibits

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distinctive features.[10,12] PDZ domain may interact with other presynaptic molecules involving molecule anchoring and assembly at AZ.[13] C_2A domain shows an unusual ability to sense intracellular changes of Ca^{2+} levels and then trigger the association with membrane phospholipids (PIs) via electrostatic interaction.[14] Notably, it interacts with phosphatidylinositol 4,5-bisphosphate (PIP_2),[15] a critical molecule for actin dynamics and endocytosis. It is well established that PIP_2 coordinates membrane fusion with actin filament to promote membrane movement, and recruits accessory adaptors for clathrin-coated pits.[16] Therefore, modulation of plasmalemmal PIP_2 may affect PIP_2-dependent biological processes like membrane trafficking and endocytosis.

In this study we find that Piccolo servers as a negative presynaptic modulator for behavioral hypersensitivity and blunts excessive dopaminergic synaptic plasticity by regulating plasmalemmal DAT internalization. Moreover, Piccolo C_2A domain may contribute to such distinct effects by targeting membrane PIP_2.

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-C_2A (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); PIP_2 (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′-GGATAGCGCACAAGGTTTTCC-3′ (base pair 4180–4200) with reverse being 5′-TTCAACCGAAT CATAGGATGCTC-3′ (base pair 4257–4279), and the dye probe was 5′-CACAAAGAGAATCCTGAGCTGG TCGATGA-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′-CTCTGCC AAAACTTC-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 ($1\,\mu l\,h^{-1}$, $10\,nmol\,ml^{-1}$), 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 ($2.0\,\mu l\,min^{-1}$). 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 Na_2CO_3, 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 [^{3}H]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 $\mu g\,ml^{-1}$ 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). [^{3}H]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,-^{3}H)-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 [^{3}H]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 C_2A domain were generated using the amino-acid sequence data from Protein Data Bank (Gi:42543545). The C_2A 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 Ca^{2+} 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

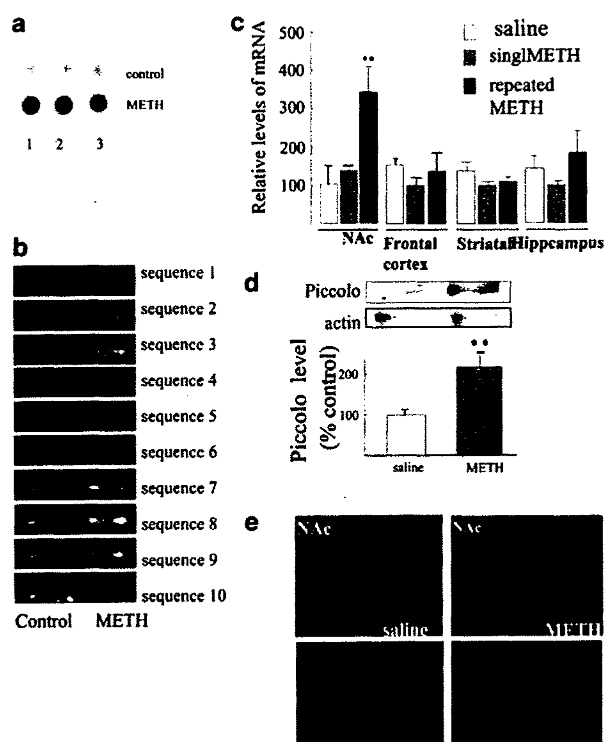

Figure 1 Piccolo expression in nucleus accumbens (NAc) was upregulated by repeated methamphetamine (METH) administration. **(a)** Piccolo overexpression in the NAc of a presentative mouse after daily METH administration for 5 days was shown. **(b)** RT–PCR analysis revealed a significant increase in 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

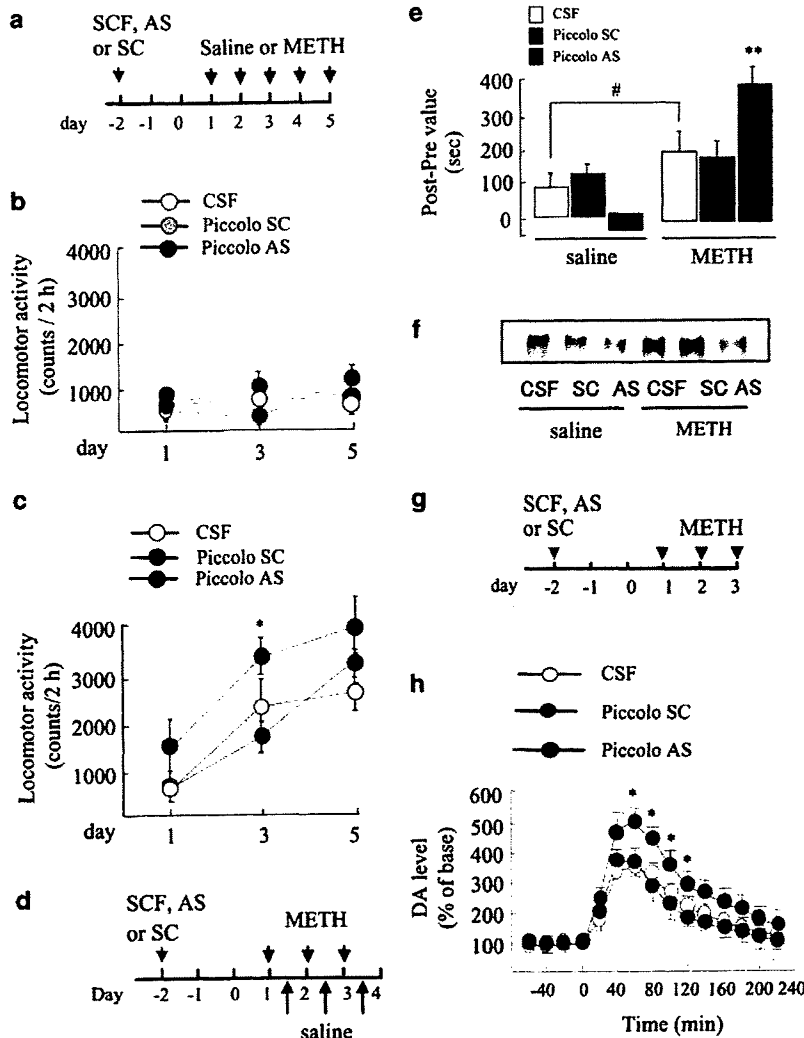

Figure 2 Downregulation of Piccolo expression with antisense oligodeoxynucleotide (AS) promoted methamphetamine (METH)-induced behavioral and synaptic plasticity. (**a–c**) Mice were infused intracerebroventricularly with Piccolo AS, scramble oligodeoxynucleotide (SC) or cerebrospinal fluid (CSF) for 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 presentative 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.

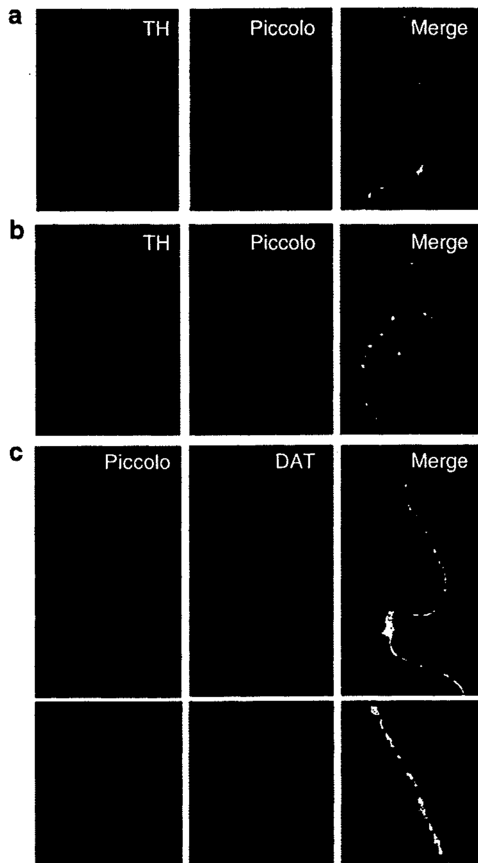

Figure 3 Expression of Piccolo in dopaminergic neurons. (a) Double immunostaining showed that Piccolo is expressed in tyrosine hydroxylase (TH)-positive neurons. (b) Abundant expression of Piccolo is present at the presynaptic component of cultured dopaminergic neurons. (c) Dopamine transporter (DAT)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

Piccolo C_2A 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 μM) 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 [^{3}H]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 [^{3}H]DA uptake responding to various concentrations of METH ($F_{(4,15)}=73.0$, $P<0.001$; Figure 4d).

The schematic representations of C_2A domain, PDZ domain and a fragment between C_2A domain and PDZ domain are shown in Figure 4e. The C_2A domain, PDZ domain or the fragment were expressed in hDAT-PC12 cells to investigate the changes in [^{3}H]DA uptake. We found that the cells transfected with C_2A domain showed a slight, but not significant, increase in [^{3}H]DA uptake in response to saline; moreover, transfection of PDZ domain or p13192 did not alter [^{3}H]DA uptake, either (Figure 4f, left panel). We then pretreated the cells with 1 μM METH for 30 min, followed by [^{3}H]DA uptake assay. METH obviously inhibited [^{3}H]DA uptake, and importantly, C_2A domain-transfected cells showed a higher level of [^{3}H]DA uptake compared with empty pCMV (Stratagene, La Jolla, CA; $F_{(3,20)}=18.68$, $P<0.01$), indicating that the C_2A 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 C_2A domain, PDZ domain or p13192 in basal conditions (Figure 4g). When the cells were pretreated with 1 μM METH for 30 min, C_2A 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 [^{3}H]DA uptake shown in Figure 4f, indicating that Piccolo C_2A domain may attenuate the METH-induced inhibition of DA uptake and maintain DAT expression at cell surface.

Piccolo C_2A domain modulates DAT internalization by a mechanism of membrane association

Given that DAT can be internalized and/or recycled, we speculated that the decreased lose of membrane DAT induced by METH in C_2A 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 C_2A 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 C_2A domain expression when the cells were exposed to 1 μM 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 C_2A domain showed the similar findings that the cells transfected with C_2A 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 C_2A domain attenuates METH-induced DAT internalization, which accounts for the decrease in the loss of DAT at cell surface.

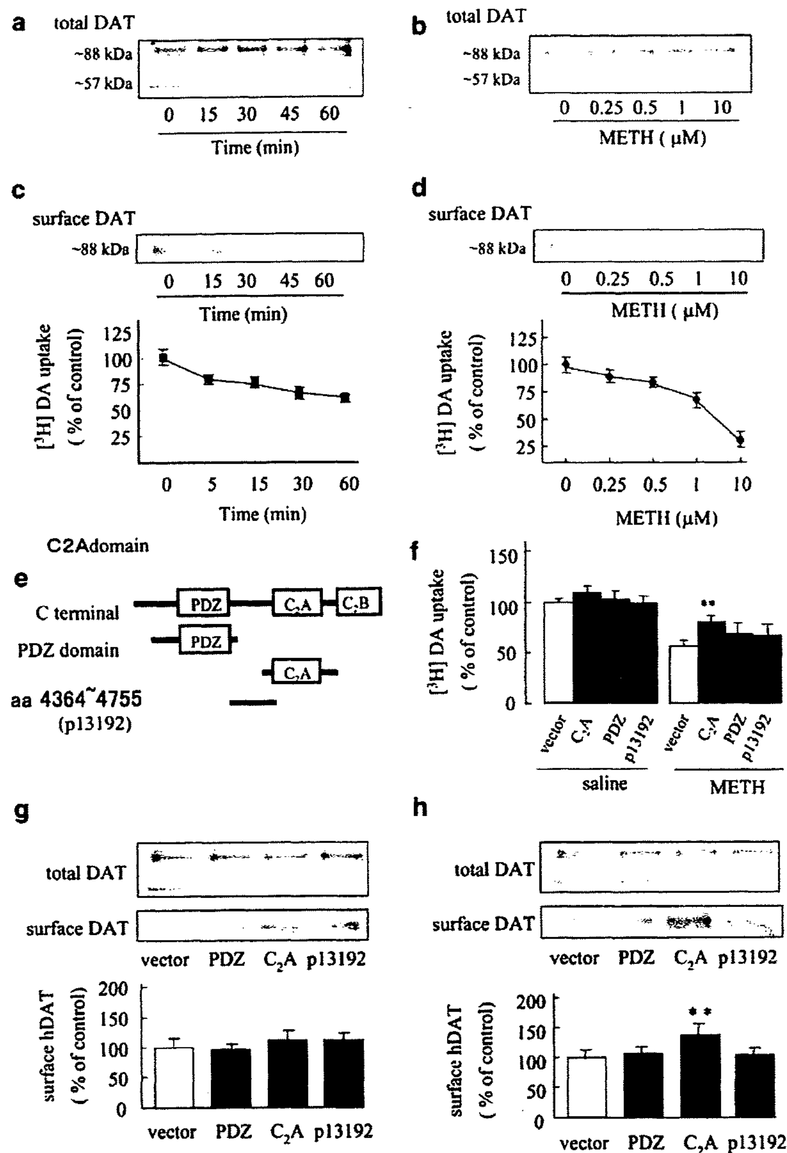

Figure 4 Piccolo C_2A domain increased dopamine (DA) uptake and dopamine transporter (DAT) surface expression. (**a, b**) Methamphetamine (METH) could not alter the total DAT expression levels in hDAT-PC12 cells in both time- (**a**) and dose-dependent studies (**b**). (**c**) METH (1 μM) decreased plasmalemmal DAT expression (top) in time-dependent manner, which was paralleled with the decrease in [^{3}H]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 [^{3}H]DA uptake (bottom). $^{**}P<0.01$ and $^{*}P<0.05$, compared with the basal level. (**e**) Schematic representations of C_2A domain, PDZ domain and a fragment (amino acid 4364–4755). (**f**) Piccolo C_2A domain attenuated the METH-induced inhibition of [^{3}H]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 C_2A 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 C_2A domain on DAT internalization, we introduced C_2A domain into hDAT-PC12 cells and then analyzed membrane subcellular distributions of Piccolo, C_2A domain, hDAT as well as PIP_2. 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 Na_2CO_3 (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 C_2A domain and PIP_2 did not fractionate like a soluble cytosolic protein but was mainly found in membrane sediment extracted by Na_2CO_3, indicating that a substantial

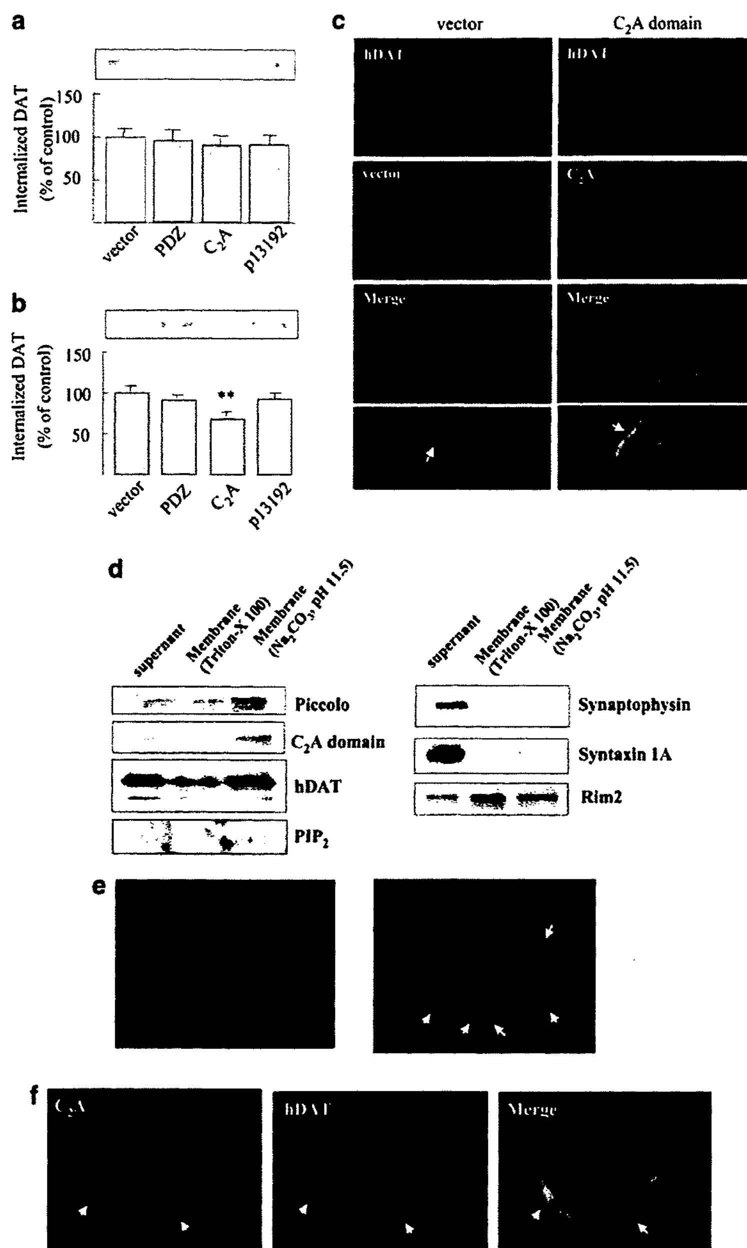

Figure 5 Piccolo modulates dopamine transporter (DAT) internalization by a mechanism of membrane association. (**a, b**) After transfection with various vectors the hDAT-PC12 cells were biotinylated and treated with either saline (**a**) or 1 μM methamphetamine (METH) (**b**) for 30 min to initiate endocytosis. 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 μM 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-C_2A domain (green) reveal the strong plasmalemmal hDAT immunoreactivity (right panel). Internalized hDAT is depicted. (**d**) Distributions of Piccolo, c-Myc-tagged C_2A domain, hDAT, PIP_2 and other presynaptic proteins in hDAT-PC12 cells. The cells and membrane fractions were extracted with RIPA buffer containing 0.1 M Na_2CO_3 (pH 11.5) or not. (**e**) The transfected Piccolo C_2A domain specially targets plasma membrane in hDAT-PC12 cells. (**f**) Piccolo C_2A domain shows a paralleled immunoreactivity pattern at plasmalemmal rafts with hDAT (arrowhead).

fraction of membrane-bound Piccolo, C_2A domain and PIP_2 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 Na_2CO_3, indicating that a relatively major part of membrane DAT is localized at the same subcellular fraction with Piccolo C_2A domain and PIP_2. The similar distributions of these

components in lipid raft fractions hint that C_2A domain–PIP_2 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 C_2 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 C_2A domain. To get an insight into the interplay among DAT, Piccolo C_2A domain and PIP_2, double immunostaining was performed. We found that Piccolo C_2A domain mainly anchored nonuniformly to the inner leaflet of plasma membrane (Figure 5e), which is consistent with its property of targeting membrane PIP_2. Notably, the distribution pattern of C_2A domain resembled that of hDAT, as revealed by the paralleled immunoreactivities at membrane microdomains (Figure 5f).

Internalization of plasmalemmal DAT is PIP_2-dependent

The concept of PIP_2 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 PIP_2 for recruiting endocytic adaptors through PIP_2-binding modules. To test this idea, hDAT and PIP_2 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 PIP_2 in the cytosolic compartment (Figure 6, bottom panel), whereas the saline-treated cells only showed the constitutively internalized PIP_2 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 PIP_2.

Interaction of Piccolo C_2A domain and PIP_2

Although Piccolo C_2A domain binding to PIP_2 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 PIP_2 accumulation using double immunostaining. The clusters of Piccolo immunoreactivities in dendrite profile colocalized precisely with those of PIP_2 in the primary cultured dopaminergic neurons (Figure 7a). Moreover, the localization of transfected C_2A domain in hDAT-PC12 cells was similar with that of PIP_2, which revealed a patchy staining pattern at plasma membrane (Figure 7b). Importantly, the clusters with strong immunoreactivity of C_2A domain also showed substantially larger and stronger labeling macroscopic of PIP_2 clusters, indicating that C_2A domain may sequester PIP_2, thus augmenting the formation of microscopically detectable plasmalemmal PIP_2 clusters.

To better understand the interaction of the two molecules, we generated a PIs binding model of Piccolo C_2A domain with Ca^{2+} docking. As show in Figure 7c, the three-dimensional structure indicated that the predicted PIs binding sites are Ca^{2+}-binding loops at the top of C_2A domain, which shows the similar binding residues for phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP) and PIP_2. Notably, the crystal packing contacts for PIP_2 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 C_2A domain showed that PIP_2 binding sites are positively charged (Figure 7d), further indicating that clustering PIs by C_2A domain depends on electrostatic interactions between the

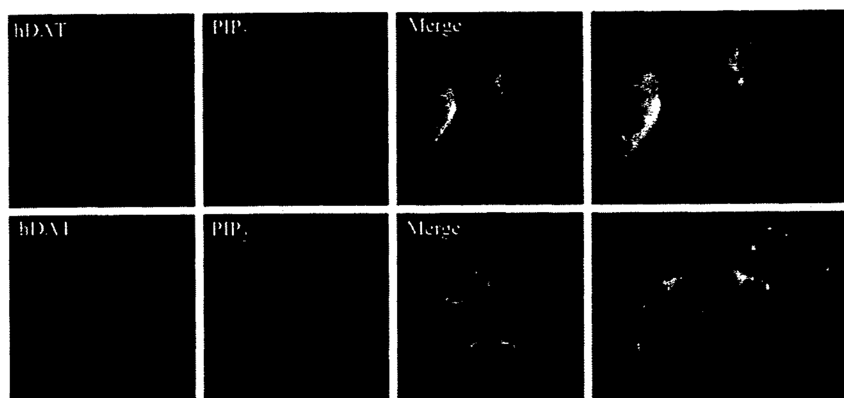

Figure 6 Piccolo C_2A domain attenuates dopamine transporter (DAT) internalization responding to methamphetamine (METH). Double-immunostaining of PIP_2 (red) and hDAT (green) in hDAT-PC12 cells. The internalization of hDAT was promoted by METH, which is accompanied by PIP_2 (bottom panel). The saline-treated cells show strong immunoreactivities of both hDAT and PIP_2 (top panel).

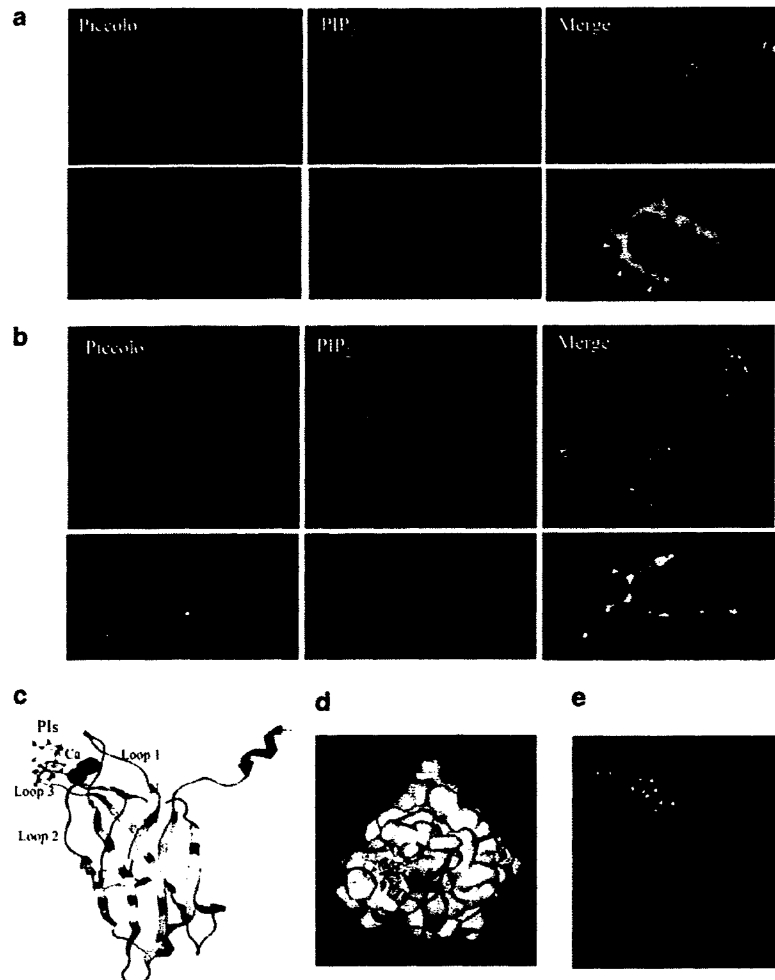

Figure 7 Interaction of Piccolo and PIP_2. (a) PIP_2 (red) colocalizes precisely with Piccolo (green) along the presynaptic terminal in primary cultured dopaminergic neurons (arrowed). (b) PIP_2 (red) accumulates at plasmalemmal rafts, where it colocalizes with Piccolo (green) in hDAT-PC12 cells. Arrowheads point to regions of intense staining of PIP_2 and Piccolo. (c) Model of Piccolo C_2A domain with three bound Ca^{2+} ions on top (green spheres). The top surface of C_2A domain shows the binding sites for the headgroups of PIs. (d) Surface plot showing the electrostatic potential of C_2A domain. Blue, positive; red, negative charge; white, neutral. PIP_2 is pointed. (e) Space-filling model of PIP_2 is shown on top in pink C_2A domain, which provides a cupped shape of polybasic region to accommodate PIP_2.

positively charged residues in proteins and the negatively charged headgroups of PIs. The lowest binding energies of Piccolo C_2A domain for PI, PIP and PIP_2 with Ca^{2+} docking were −59.491, −93.229 and −102.642 Kcal, respectively, suggesting a specific interaction between PIP_2 and C_2A domain. Furthermore, the space-filling model showed that PIP_2 is tightly packed against the top surface of C_2A domain, which forms a favorable pocket to accommodate the moiety of PIP_2 (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

[^{3}H]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 [^{3}H]DA uptake in the cells treated with saline. Although Bont/C1 slightly elevated [^{3}H]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, [^{3}H]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 C_2A, 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 C_2A domain-transfected cells responding to METH.

It is well established that PIP_2 functions in regulating cytoskeleton, channels and transporters, and membrane trafficking at presynaptic terminal.[16,28,29] Especially, PIP_2 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 PIP_2 to assemble various molecules to form endocytic compartment. Significantly, we found that PIP_2 exhibits a similar distribution pattern with DAT at membrane microdomains. Furthermore, internalized DTA triggered by METH is accompanied with PIP_2 in endocytic compartments. These results indicate that PIP_2 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 PIP_2, thus potentially modulating the endocytic process.[32,33] In this study we obtained several evidences further supporting the notion that Piccolo can electrostatically sequester PIP_2. Firstly, Piccolo C_2A domain may laterally bind membrane PIP_2, and augment PIP_2 clusters in hDAT-PC12 cells. In principle, the augmented clusters could represent the sequestration of phospholipids like PIP_2 at the plasma membrane.[34] Secondly, the crystal

packing contacts for PIP_2 were the clusters of basic/aromatic residues, which exhibit a universal capability of sequestering membrane PIP_2.[35] Thirdly, the space-filling model showed that Piccolo C_2A domain may pocket PIP_2 by a cupped shape of polybasic region, where the local positive potential electrostatically attracts the negatively charged PIP_2. Finally, C_2A domain shows stronger interacting potential with PIP_2 than PI or PIP. Our results are consistent with previous investigations indicating that PIs binding with Piccolo C_2A domain is largely driven by electrostatic interaction.[15]

Based on these findings, we speculated that Piccolo C_2A domain may regulate METH-triggered DAT internalization through sequestering PIP_2, and the findings in immunostaining strongly support this prediction. Piccolo C_2A domain mainly anchors nonuniformly to the inner leaflet, which is accompanied with the retention of DAT and PIP_2 at membrane microdomains; moreover, it clearly attenuated METH-triggered DAT and PIP_2 internalization in cytosol. These results show that Piccolo may sequester or 'control' locally PIP_2 by C_2A domain in membrane raft and suppress PIP_2-dependent endocytic process, thus leading to the attenuated DAT internalization.

How does the Piccolo C_2A domain–PIP_2 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 PIP_2 sequestration. Given the strong dependence of the endocytic machinery on PIP_2, more membrane PIP_2 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 PIP_2, 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 C_2A 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 PIP_2 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 PIP_2 or other PIs through C_2A domain and disturb PIP_2-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 C_2A domain, because synaptotagmin C_2A domain which shares a great structural similarity with Piccolo C_2A 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 C_2A 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 C_2A domain–PIP_2 interplay in DAT internalization remains to be determined. No matter which mechanism could be more reasonable, sequestration of PIP_2 in lateral domains through C_2A domain appears to be important for Piccolo to regulate DAT internalization. Therefore, a greater understanding of the molecular regulators for PIP_2, which governs DAT trafficking, would shed light on the modulation of DAT surface presentation. Further investigation measuring membrane fluorescence resonance energy transfer and PIP_2 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 PIP_2 by its C_2A 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.

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

COE

RT2 Profiler PCR Array (Neurotrophocs and Re(	PAMM-031A	1	363,825	363,825
RT2 Real-Time PCR SYBR Green/ROX	PA-012-12	1	264,600	264,600
RT2 First Strand Kit	C-03	2	66,150	132,300

1研

AteloSiLence in vivo用(siRNA) 10nmol	1	50600	50600
19塩基スクランブル配列解析+21塩基siRNA合成 10nmol	1	62100	62100

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IMAGE

Piccolo regulates dopamine transporter internalization via PIP_2

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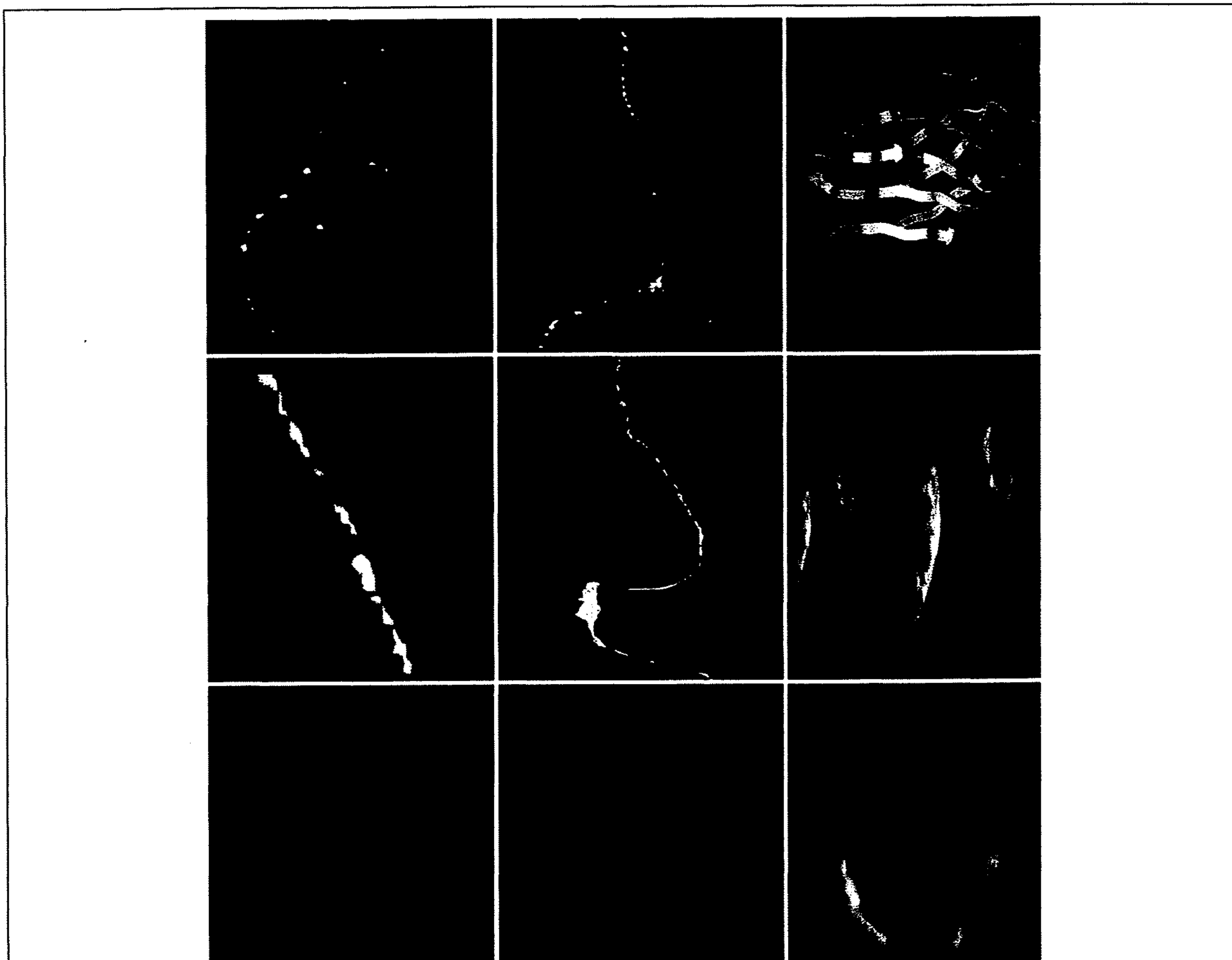

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Two left upper panels show that piccolo is not colocalized with tyrosine hydroxyls (TH)-positive neurons. Two left middle panels show that piccolo is colocalized with dopamine transporter (DAT)-positive neurons. Bottom three panels show piccolo C2A domain (left; green), dopamine transporter (middle; red) and merge image (right). Most upper right panel is docking simulation between piccolo C2A domain and PIP_2 under Ca^{2+} present condition. The right middle panel shows the piccolo C2A domain is colocalized with dopamine transporter in normal condition. For more information on this topic see the paper by Cen *et al.* on pages 451–463.

脳由来神経栄養因子およびグリア細胞由来神経栄養因子と神経精神疾患との関係

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略語

BDNF: Brain-derived neurotrophic factor, **CREB**: cAMP response element-binding protein, **GDNF**: glial cell line-derived neurotrophic factor, **HDAC**: histone deacetylase, **Hsc70**: heat shock cognate protein 70, **Hsp90**: heat shock protein 90, **Leu-Ile**: leucyl isoleucine, **MPTP**: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, **NMDA**: N-methyl-D-aspartate

要約：神経精神疾患には、器質的な変化で誘発されるものが多い。アルツハイマー病やパーキンソン病にとどまらず、最近ではうつ病などにおいても神経変性や神経新生の障害が一因として示されている。一方で、個体の成熟後も脳においてシナプス可塑性や神経新生など、様々な機能的・構造的変化が起こっていることが明らかになってきており、このような可塑的変化を促進または調節することにより神経精神疾患の治療が可能ではないかと考えられる。脳由来神経栄養因子（BDNF）およびグリア細胞由来神経栄養因子（GDNF）は、神経回路形成やシナプス可塑性に重要な役割を持つ因子である。その機能解明については細胞内シグナル伝達機構の解析から個体レベルにおける記憶や情動の制御まで多岐にわたり様々な研究が行われ、これらの因子が神経精神疾患の治療の鍵となることが明らかとなりつつある。BDNF および GDNF そのものを脳内に投与し増加させる試みが神経精神疾患に有効であることも示されている。また、これらの神経栄養因子は、低分子化合物の末梢投与により脳内で産生誘導することも可能である。我々は、疎水性ジペプチド Leu-Ile が BDNF および GDNF の産生を増大させることを見いだし、薬物依存などに対する薬理効果について追究しており、その結果も含めて BDNF および GDNF の産生誘導による神経精神疾患の治療の可能性について考察する。

キーワード： BDNF, GDNF, 神経精神疾患, Leu-Ile, クロマチン制御

序論

情動、記憶、認知機能や運動機能など様々な活動に障害が起きる神経精神疾患には、神経細胞の脱落など器質的な変化が原因で発症するものが多く、特にアルツハイマー病やパーキンソン病などがよく知られている。さらに、これまで器質的な変化よりもセロトニンの減少などモノアミン系の異常によると考えられていたうつ病においても海馬の萎縮が認められるなど（Campbell et al., 2004; Videbech and Ravnkilde, 2004)、現在では神経変性や神経新生の障害などがその一因として示されている。一方、シナプスの可塑性や神経新生など、脳の機能と構造は成熟後にも可塑的に変化していることが明らかとなってきており、炎症反応や酸化ストレスその他細胞傷害性要因による変性からの神経細胞の保護や神経新生の促進は様々な神経精神疾患の治療手段として重要視され、治療に向けて開発も盛んに行われている。脳由来神経栄養因子（brain-derived neurotrophic factor；BDNF）は神経細胞の生存や分化に影響を及ぼし、シナプス形成や神経細胞の保護や新生に重要な働きをしている。また、シナプス可塑性にも大きく影響する（Lu and Gottschalk, 2000）ことから、記憶や情動との関わりも様々解析されている。グリア細胞由来神経栄養因子（glial cell line-derived neurotrophic factor；GDNF）もまた神経細胞保護に重要な働きを持っている。本稿では、BDNF および GDNF と神経精神疾患との関連、および予防や治療への応用の可能性について考察する。

BDNF

BDNF は神経分化や生存、維持に大きく影響することから、その機能異常は様々な神経疾患に関与すると考えられている。BDNF には疾患と関連する一塩基多型（single nucleotide polymorphism；SNP）が存在し、その中でも Val66Met 遺伝子多型がよく知られている。この変異では前駆体 BDNF の分泌が抑制される（Egan et al., 2003; Chen et al., 2006）ため、成熟型 BDNF の発現が減少する。この Val66Met の多型を持つ人にはエピソード記憶の低下や海馬活性化の異常が観察される（Egan et al., 2003）。また、ホモ型で Val66Met を持つノックインマウスは不安・うつ様行動が増加し、抗うつ薬フロキセチンでも回復しない（Chen et al., 2006）。また、統合失調症ではコントロール群に比較してこの多型が有意に多く検出され、疾患との関連性が示されている（Neves-Pereira et al., 2005）。

切片培養した神経細胞の樹状突起に BDNF を作用させると、後シナプスの長期増強が誘導され、蛋白合成などが誘導される（Kovalchuk et al., 2002）。また、BDNF は神経終末から取り込まれ、細胞体へ輸送されて神経の増殖分化や生存を促進する。Guillin ら（2001）はドパミン神経由来の BDNF が発達過程および成体脳両方の側坐核におけるドパミン D3 受容体の発現を誘導することを報告している。BDNF はセロトニン神経の正常な発達や機能にも重要であり、BDNF ヘテロノックアウトマウスでは前頭皮質や海馬、視床下部におけるセロトニン遊離反応の低下や、前頭皮質のセロトニンレセプターの発現が上昇するなどセロトニン神経系に変化が見られ、また攻撃性の亢進などセロトニン神経系の機能障害と関係した行動異常を示す（Lyons et al., 1999）。BDNF は記憶にも深く関わっており、Mizuno らは BDNF が空間記憶の形成および保持に重要な役割を果たすことを示した（Mizuno et al., 2000）。また、BDNF は学習後の海馬における長期間の記憶保持に必要であることも示されている(Bekinschtein et al., 2008)。このように、BDNF の減少は神経の発達と機能の維持に異常をもたらし、情動障害や認知、記憶の低下など神経精神疾患を誘発する要因となっていると考えられる。

BDNF 発現量の低下はうつ病や慢性ストレス負荷マウスの海馬で検出されている一方、BDNF の産生を増大させる因子には学習訓練や豊かな環境下での飼育が知られている。Mizuno ら（2000）は、八方向迷路を用いた空間記憶試験を行うと海馬で BDNF mRNA が増加することを見いだした。豊かな環境下で飼育したマウスにおける BDNF 産生増大の報告も数多くなされており、本マウスで観察される虚血時の認知能力維持、ストレス抵抗性および記憶力の向上に BDNF の発現上昇が重要であることも示されている（Zhao et al., 2001）。このように BDNF の発現上昇が脳神経機能の障害の保護に重要であることから、人工的に脳内 BDNF の産生を増大させる事によって神経精神疾患の治療を目指す試みも行われている。Shirayama ら（2002）は、ラット脳海馬に BDNF を注入すると、注入後 3 日から 10 日にかけて強制水泳試験などのうつ様行動試験において抗うつ様効果が現れることを報告している。また、細胞移植により BDNF を連続的に供給することによって、神経の生存やシナプス可塑性を促進できることも報告されている（Rodrigues Hell et al., 2008）。