was strengthened by galantamine, at their noneffective doses, and antagonized by mecamylamine. Accordingly, it is plausible that galantamine ameliorates the $A\beta_{25-35}$-induced learning and memory deficits by activating nAChR, and thereby stimulates release of dopamine in the brain. Further, we found that the improving effects of galantamine were prevented by SCH-23390, a dopamine-D1 receptor antagonist, and sulpiride, a dopamine-D2 receptor antagonist. Taken together, our results suggest that these hippocampus-dependent performance in these tasks were impaired by $A\beta_{25-35}$ infusion as the result of failure of nAChR and dopamine responses, as the hippocampus is involved in the object recognition behavior (Rampon *et al*, 2000; Hammond *et al*, 2004) and the contextual fear conditioning (Daumas *et al*, 2004). These findings provide the first *in vivo* evidence that galantamine augments dopaminergic neurotransmission within the hippocampus through the allosteric activation of nAChR.

The deficit in the nAChR-dopaminergic systems is one of the facets of general degeneration in neurons induced by $A\beta_{25-35}$ treatment. The cognitive improving effects of galantamine at the present dose depend on the function of nAChR-dopaminergic systems, therefore the effects are prone to be blocked by the antagonism of the nAChR-dopaminergic systems. However, in the normal animals, neurons and their functions are almost intact: the functions and the homeostasis in neurons slightly impaired by the antagonists of the nAChR-dopaminergic systems at relatively low doses, as they can somewhat be restored by compensating mechanisms that are not very clear until now.

Because the improving-effects of galantamine on the cognitive dysfunction induced by $A\beta_{25-35}$ i.c.v. injection may be mediated through the activation of, at least in part, dopaminergic systems, we postulate that galantamine may activate dopaminergic neurotransmission in Alzheimer's disease by augmenting the activation of nAChR. This is supported by the fact that galantamine potentiated the hippocampal dopamine release in the $A\beta_{25-35}$-injected model of Alzheimer's disease, and the effects of galantamine on cognition and dopamine release was antagonized by an AChR antagonist, mecamylamine. Because the dopaminergic dysfunction has been implicated in the progress of Alzheimer's disease, dopaminergic agents may be beneficial in the treatment, and enhancement of dopamine release may be part of the mechanisms underlying the therapeutic benefit of galantamine. The results also suggest that in addition to Alzheimer's disease, galantamine may be valuable in the treatment of other diseases involving the dysfunction of dopaminergic systems, such as Parkinson's disease and neuropsychiatric dysfunctions including anxiety, depression, apathy, and psychosis.

ACKNOWLEDGEMENTS

We are grateful to the Janssen Pharmaceutical KK for donating galantamine. This work was supported, in part, by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (14370031) (15922139) (16922036) (17390018), for Scientific Research on Priority Areas on 'Elucidation of glia-neuron network mediated information processing systems' from Ministry of Education, Culture, Sports, Science and Technology (16047214), for Funds from Integrated Molecular Medicine for Neuronal and Neoplastic Disorders (21st Century COE program), for the Japan Brain Foundation, for the Mitsubishi Pharma Research Foundation and, for an SRF Grant for Biomedical Research.

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Neuroscience 146 (2007) 50–59

REINFORCING EFFECTS OF MORPHINE ARE REDUCED IN TISSUE PLASMINOGEN ACTIVATOR-KNOCKOUT MICE

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Abstract—Tissue plasminogen activator (tPA) plays a key role in neuroplasticity. We have recently demonstrated that the tPA-plasmin system is involved in the rewarding effects of drugs of abuse by regulating the release of dopamine in the nucleus accumbens. In the present study, we investigated whether tPA is involved in the reinforcing properties of morphine in a paradigm of drug self-administration. Eight-week-old tPA knockout and wild-type control mice were subjected to a single 24-h session of morphine self-administration under a fixed ratio (FR) 2 or a progressive ratio (PR) schedule of reinforcement after eight daily 30-min sessions of nose-poke training. tPA knockout mice responded significantly more often for morphine self-administration in a dose-dependent manner as compared with wild-type control mice. Under the PR schedule of morphine reinforcement, however, tPA knockout mice showed a lower breaking point than wild-type control mice. There was no significant difference in food-reinforced operant behavior, breaking points to food pellets, and saline self-administration between the two genotypes. The increased responding in tPA knockout mice under the FR2 schedule was significantly attenuated by the dopamine D1 receptor antagonist SCH23390 (0.3 mg/kg), whereas SCH23390, at a dose range of 0.03–2.0 mg/kg, demonstrated biphasic effects on morphine self-administration in wild-type control mice. Our findings suggest that the reinforcing effects of morphine are reduced in tPA knockout mice. Modulation of the tPA system in the brain may be a potential target against drugs of abuse. **© 2007 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: tPA knockout mice, drug self-administration, morphine, reinforcing properties, dopamine D1 receptor antagonist.

Tissue plasminogen activator (tPA) has been detected in the CNS (Teesalu et al., 2004). It has well been characterized that tPA, as an extracellular protease, converts zymogen plasminogen to the active protease plasmin and plays an important role not only in neuronal migration (Moonen et al., 1982; Seeds et al., 1999) and synaptic outgrowth (Krystosek and Seeds, 1981) during the development of the brain, but also in the synaptic plasticity and neurotoxicity of the mature brain, including visual cortical plasticity (Mataga et al., 2002), long-term potentiation (Frey et al., 1996; Huang et al., 1996; Baranes et al., 1998), stress-induced anxiety-like behavior (Pawlak et al., 2003), cerebellar motor learning (Seeds et al., 2003), excitotoxic cell death (Chen and Strickland, 1997; Nicole et al., 2001), and drug dependence (Ito et al., in press; Nagai et al., 2004, 2005a,b, 2006; Pawlak et al., 2005; Yamada et al., 2005). Thus, tPA has been suggested to be a neuromodulator in the CNS (Yamada et al., 2005).

In the nervous system, tPA was found to be packaged in and released directly from catecholamine storage vesicles in response to stimuli using PC12 cells in culture (Gualandris et al., 1996; Parmer et al., 1997; Lochner et al., 2006). Recently, it has been documented that the rapid and activity-dependent induction of tPA expression is controlled by an mRNA translation mechanism mediated by the metabotropic glutamate receptor (Shin et al., 2004). Different downstream cascades are involved in the distinct effects of tPA in the brain, including neuronal damage mediated by the NMDA receptor (Nicole et al., 2001) or protease-activated receptor 1 (PAR-1) (Junge et al., 2003), neurovascular toxicity controlled by activated protein C (APC) (Liu et al., 2004), neural remodeling related to annexin II (Siao and Tsirka, 2002), and neural plasticity via low density lipoprotein receptor-related protein (LRP) (Zhuo et al., 2000; Wang et al., 2003).

We have recently demonstrated that the tPA-plasmin system is involved in the rewarding effects of morphine (MOR) (Ito et al., in press; Nagai et al., 2004, 2005b), methamphetamine (Nagai et al., 2005a) and nicotine (Nagai et al., 2006). Accordingly, tPA knockout mice show a reduction in MOR-, methamphetamine- and nicotine-induced conditioned place preference and locomotor sensitization. Furthermore, exogenous recombinant human tPA or plasmin restored the defect of MOR-induced dopamine release in the nucleus accumbens and hyperactivity in tPA knockout mice (Nagai et al., 2004). Exogenous recombinant tPA and plasmin potentiate, whereas plasminogen activator inhibitor-1 (PAI-1) inhibits, the MOR-induced release of dopamine in the nucleus accumbens and dopamine-dependent hyperlocomotion in wild-type control mice (Nagai et al., 2005b). PAR-1 is involved in the regulation of MOR-induced dopamine release by the tPA–plasmin system (Ito et al., in press). Taken together, it is suggested that tPA plays an important role in the rewarding effects of MOR by regulating dopaminergic transmission (Nagai et al., 2004; Yamada et al., 2005). In the present study, we investigated whether tPA is involved in the reinforcing

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Abbreviations: ANOVA, analysis of variance; FR, fixed ratio; MOR, morphine; PAR-1, protease-activated receptor 1; PR, progressive ratio; tPA, tissue plasminogen activator.

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doi:10.1016/j.neuroscience.2007.01.011

properties of MOR using a paradigm of drug self-administration. Our findings suggest that the reinforcing effects of MOR are reduced in tPA knockout mice. Modulation of the tPA system in the brain may be a potential target against drugs of abuse.

EXPERIMENTAL PROCEDURES

Subjects and drug treatment

tPA knockout ($n=120$) and wild-type control mice ($n=138$) (male and 8 weeks old; Jackson Laboratory, Bar Harbor, ME, USA) weighing 20–25 g were used. All mice were kept in a regulated environment (23±0.5 °C; 50±0.5% humidity) with a reversed 12-h light/dark cycle (lights on at 9:00 A.M.). Water and food were available *ad libitum* in home cages, but unavailable in chambers for 24-h or 12-h test sessions. To minimize the number of mice used and their suffering, all experiments were performed in accordance with Guidelines for Animal Experiments of the Nagoya University School of Medicine, the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

MOR hydrochloride (Dainippon Pharmaceutical, Tokyo, Japan) was dissolved in sterile saline. Under standard experimental conditions, MOR was self-administered in a volume of 2.1 μl per infusion over 5 s. The unit dose for MOR self-administration was based on a previous report (Elmer et al., 2002) and our pilot studies. R-(+)-SCH23390 hydrochloride, a dopamine D1 receptor antagonist, was purchased from Sigma (Sigma-Aldrich Co., St. Louis, MO, USA) and dissolved in sterile saline immediately before injection. During a single 24-h session of self-administration, the mouse was disconnected from the operant system and administered i.v. with SCH23390 or vehicle via the catheter at two time points: immediately after the 5th and 12th h. The first time point was selected to make sure that before SCH23390 treatment, separate groups of mice demonstrated similar nose-pokeresponses after eight daily 30-min sessions of nose-poke training, whereas the second time point was selected to potentiate the pharmacologic effects of SCH23390 on MOR self-administration since the effects of the first infusion of SCH2390 on MOR self-administration were partial during a single 24-h session of MOR self-administration in our pilot studies.

Apparatus for operant self-administration and nose-poke training prior to MOR (or saline) self-administration

Food-reinforced operant behavior, nose-poke training, and MOR (or saline) self-administration were conducted in standard mouse operant conditioning chambers (ENV-307A, Med Associates, Georgia, VT, USA) located within a ventilated sound attenuation cubicle as described previously (Yan et al., 2006).

Except for the groups of tPA knockout and wild-type mice in the food-reinforced operant behavior, all naïve mice were initially subjected to eight daily 30-min sessions of nose-poke training prior to a single 24-h session of MOR (or saline) self-administration under a fixed ratio (FR) 2 schedule or three daily 12-h sessions of MOR/food-reinforced operant responding under a progressive ratio (PR) schedule. The nose-poke training sessions were conducted in the standard operant chamber mentioned above after food deprivation for 12 h. During the nose-poke training sessions, responses for a food pellet under the FR schedules were from FR1 (days 1–5), to FR2 (days 6–7), and then FR3 (day 8), in which active and inactive holes for nose-poke were assigned randomly throughout a session. After acquiring stable nose-poke responses for food pellets (the active responses account for more than 75% of the total number of nose-poke responses), the mice were returned to home cages for 48 h before implantation of the catheter.

Catheter implantation

After remaining in their home cages for 48 h following the nose-poke training, the mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). Indwelling catheters were constructed of micro-silicone tubing (inner diameter, 0.50 mm; outer diameter, 0.7 mm; IMG, Imamura Co., Ltd., Tokyo, Japan) and polyethylene tubing (inner diameter, 0.50 mm; outer diameter, 0.8 mm). Incisions were made on the skin of the head and ventral neck, and the right jugular vein was externalized. The end of the catheter was inserted into the jugular vein via a small incision and was secured to the vein and surrounding tissue with silk sutures. The exit port of the catheter passed s.c. to the top of the skull where it was attached to a modified 24-gauge cannula, which was secured to the mouse's skull with quick self-curing acrylic resin (Shofu Inc., Kyoto, Japan). To extend catheter patency, the catheters were flushed immediately after surgery, and in the morning and evening of the following days, with 0.03 ml of an antibiotic solution of cefmetazole sodium (20.0 mg/ml; Sankyo Co., Ltd., Tokyo, Japan) dissolved in heparinized saline (70 units/ml; Leo Pharmaceutical Products, Ltd., Japan). The catheter patency was confirmed by taking blood back from the catheter after the tests for operant behavior.

Experiment 1: food-reinforced operant behavior in tPA knockout and wild-type control mice

To investigate operant behavior for natural reinforcement in tPA knockout and wild-type control mice, naïve animals ($n=7$ for each genotype) were deprived of food for 12 h and then subjected to 15 daily 30-min sessions of food-reinforced operant responding under an FR1 schedule of reinforcement, in which active and inactive nose-poke holes were assigned randomly throughout a session. The house light was illuminated throughout the session. Nose-poke responses in the active hole resulted in a pellet being delivered from the dispenser and inactivation of the cue- and hole-lamps. After each delivery, there was a 5-s timeout period. Nose-poke responses in the inactive hole and in the active hole during the timeout period had no programmed consequences but were recorded by the software MED-PC for Windows (Med Associates). Food pellets (dustless precision pellets, 20 mg) were purchased from Holton Industries Co., Frenchtown, NJ, USA.

Experiment 2: dose-response curve for MOR self-administration under an FR2 schedule of reinforcement in tPA knockout and wild-type control mice

After eight daily 30-min sessions of nose-poke training and recovering from surgery to implant a catheter into the jugular vein for at least 2 days, each genotype was assigned randomly into four groups ($n=7$–8 for each group) according to a between-subjects design. tPA knockout and wild-type control mice were then subjected to MOR (or saline) self-administration in a single 24-h session under an FR2 schedule of reinforcement with a 5-s timeout period after each infusion (infusion volume, 2.1 μl), in which active and inactive nose-poke holes were fixed for each mouse throughout the 24-h session. During a single 24-h session, catheters were connected to liquid swivels via a joint FEP tube (inner diameter 0.25 mm, outer diameter 0.55 mm; Eicom Co., Ltd., Kyoto, Japan), which was encased in steel spring leashes (Instech, Plymouth Meeting, PA, USA). The swivels were suspended above the chamber and were connected to infusion pumps. The house light was illuminated throughout the session. Nose-poke responses in the active hole resulted in a 5-s activation of the infusion pump and inactivation of the cue- and hole-lamps. Nose-

poke responses in the inactive hole and in the active hole during the timeout period had no programmed consequences but were recorded by the software MED-PC for Windows (Med Associates).

Experiment 3: MOR and food-reinforced operant behavior under the PR schedule in tPA knockout and wild-type control mice

After eight daily 30-min sessions of nose-poke training and recovering from surgery to implant a catheter into the jugular vein for at least 2 days, according to a within-subjects design, separate groups of tPA knockout and wild-type control mice (n=7–8 for each genotype) were then subjected to three daily 12-h sessions of MOR self-administration under the PR schedule, in which nose-poke responses required to earn an infusion escalated according to the following series (Roberts and Bennett, 1993): 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492, 603, 737, etc. Each mouse was subjected to MOR self-administration as follows: 0.3 mg/kg/infusion (the first test: MOR 0.3 (the 1st test)), then 1.0 mg/kg/infusion (MOR 1.0), and 0.3 mg/kg/infusion once again (MOR 0.3 (the 2nd test)). The breaking point refers to the final ratio to earn the last infusion of MOR during the 12-h session.

To give a control for performance of tPA knockout mice under the PR schedule of MOR reinforcement, separate groups of tPA knockout and wild-type control mice (n=8 for each genotype) were subjected to three daily 12-h sessions of food-reinforced operant responding under the PR schedule as described above.

Experiment 4: effects of SCH23390 on MOR self-administration under the FR2 schedule in tPA knockout and wild-type control mice

After eight daily 30-min sessions of nose-poke training and recovering from surgery to implant a catheter into the jugular vein for at least 2 days, separate groups of tPA knockout and wild-type control mice were assigned randomly into 12 groups (n=6–7 for each group in each genotype) according to a between-subjects design. Both genotypes were then subjected to MOR (0.3 mg/kg/infusion) self-administration in a single 24-h session under the FR2 schedule. During a single 24-h session of self-administration, SCH23390 or vehicle was administered i.v. via the catheter at two time points: immediately after the 5th and 12th h by disconnecting a mouse from the operant system.

Experiment 5: effects of SCH23390 on saline self-administration under the FR2 schedule in tPA knockout and wild-type control mice

After eight daily 30-min sessions of nose-poke training and recovering from surgery to implant a catheter into jugular vein for at least 2 days, separate groups of tPA knockout and wild-type control mice were assigned randomly into four (tPA knockout) or six (wild-type control) groups (n=6–8 for each group) according to a between-subjects design. Both genotypes were then subjected to saline self-administration in a single 24-h session under the FR2 schedule. During a single 24-h session of self-administration, SCH23390 or vehicle was administered i.v. via the catheter at two time points: immediately after the 5th and 12th h by disconnecting the mouse from the operant system.

Statistical analysis

All data were expressed as mean±S.E.M. For food pellet, saline, and MOR self-administration, the differences between the two genotypes of mice were determined with a repeated measures two-way analysis of variance (ANOVA), and the differences between the active and the inactive nose-pokes in the same group were determined with Student's t-test. Statistical differences in the number or rate of active nose-poke responses at different doses of MOR self-administration in the two genotypes of mice, and the effects of SCH23390 on the active nose-poke responses for MOR self-administration in the two genotypes of mice were determined by one-way ANOVA, followed post hoc by the Bonferroni/Dunn tests. In all cases, the level of significance was set at $P<0.05$.

RESULTS

No significant difference in food-reinforced operant behavior was observed between tPA knockout and wild-type control mice

As shown in Fig. 1A, during the test for food-reinforced operant behavior, both genotypes of animals (n=7 for each genotype) failed to discriminate active from inactive nose-poke responses at the early phase. After 1 week, both genotypes could discriminate active from inactive nose-poke responses and then gradually showed stable responses for

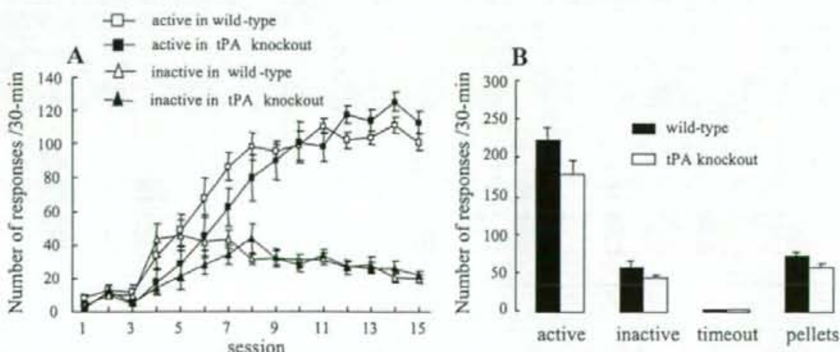

Fig. 1. No significant difference in natural reinforcement was observed between tPA knockout and wild-type control mice. A indicates active and inactive nose-poke responses under an FR1 schedule during 15 daily 30-min sessions of food-reinforced operant behavior between the two genotypes of animals (n=7 for each genotype). B indicates the total number of nose-poke responses under an FR3 schedule during the last session of nose-poke training prior to a single 24-h session of MOR self-administration between tPA knockout and wild-type control mice (n=7 for each genotype). Data are presented as mean±S.E.M.

food pellets. Importantly, there was no significant difference in active and inactive nose-poke responses between tPA knockout and wild-type control mice throughout the test.

Before a single 24-h session of MOR self-administration at a dose of 0.3 mg/kg/infusion, both tPA knockout and wild-type control mice (n=7–8 for each genotype) experienced the same nose-poke training for 8 days. As shown in Fig. 1B, during the last session of nose-poke training under an FR3 schedule, there was no significant difference in active, inactive, and timeout nose-poke responses as well as food pellets earned between the two genotypes, although tPA knockout mice tended to show a lower rate of nose-poke responses. Taken together, these observations suggest that there was no significant difference in nose-poke responses for natural reinforcement between tPA knockout and wild-type control mice.

tPA knockout mice showed an increase in nose-poke responding for MOR self-administration under the FR2 schedule in a bell-shaped manner

According to a between-subjects design, during saline self-administration, both genotypes of animals failed to discriminate active from inactive hole. Between tPA knockout and wild-type control mice, there was no difference in active or inactive nose-poke responses for saline self-administration (Fig. 2A). As shown in Fig. 2A, however, both genotypes of animals demonstrated significantly more active than inactive nose-poke responses for MOR self-administration at the dose of 0.1 mg/kg/infusion (Student's t-test, P<0.05 for tPA knockout and P<0.01 for wild-type mice), although there was no difference in active or inactive nose-poke responses between tPA knockout and wild-type control mice (two-way ANOVA, a main effect of genotype, $F_{(1, 27)}$=3.49, P=0.074). At higher doses of MOR (0.3 and 1.0 mg/kg/infusion), tPA knockout mice showed more active nose-poke responses than wild-type control mice (one-way ANOVA, P<0.05), whereas there was no significant difference in inactive (without MOR reinforcement) nose-poke responses between the two genotypes. The active nose-poke responses for MOR self-administration in tPA knockout mice were reduced at the dose of 1.0 mg/kg/infusion as compared with those at the dose of 0.3 mg/kg/infusion (one-way ANOVA, P<0.05). As shown in Fig. 2B, at the dose of 0.1 mg/kg/infusion, there was no difference in total MOR intake between tPA knockout and wild-type control mice during a single 24-h session of self-administration (8.90±1.77 mg/kg in tPA knockout versus 7.61±1.48 mg/kg in wild-type mice). In contrast, at higher doses (0.3 and 1.0 mg/kg/infusion), tPA knockout mice self-administered a much greater amount of MOR than wild-type control mice during a single 24-h session of self-administration (one-way ANOVA, P<0.05 at the dose of 0.3 mg/kg/infusion (57.56±11.12 versus 27.47±6.94 mg/kg); and P<0.01 at the dose of 1.0 mg/kg/infusion (77.71±10.16 versus 40.75±5.68 mg/kg)).

As shown in Fig. 3, either tPA knockout mice or wild-type control mice failed to discriminate active from inactive nose-poke responses for saline self-administration throughout a single 24-h session. In contrast, both genotypes of animals could discriminate active from inactive nose-poke responses for MOR self-administration throughout a single 24-h session at the dose of 0.3 mg/kg/infusion (Fig. 3B for wild-type control mice, two-way ANOVA with repeated measures, a main effect of nose-poke hole, $F_{(1, 335)}$=3.72, P<0.01; and Fig. 3D for tPA knockout mice, two-way ANOVA with repeated measures, a main effect of nose-poke hole, $F_{(1, 335)}$=14.38, P<0.001). Importantly, the increased nose-poke responses for MOR reinforcement in tPA

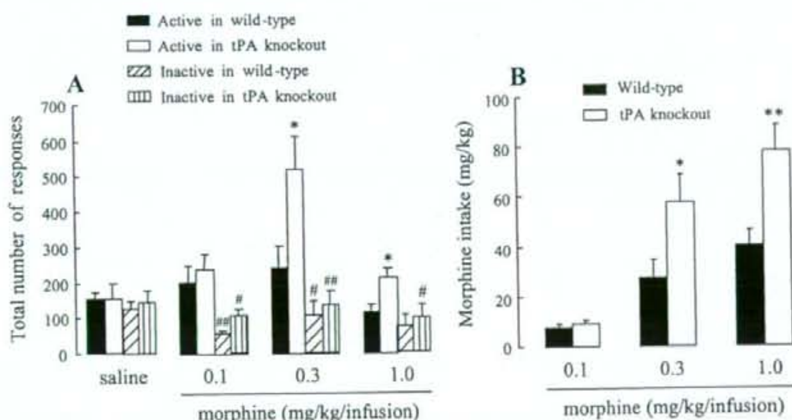

Fig. 2. tPA knockout mice showed an increase in active nose-poke responses for MOR self-administration in a bell-shaped manner. After eight daily 30-min sessions of nose-poke training, tPA knockout and wild-type control mice were subjected to saline or MOR self-administration in a single 24-h session under an FR2 schedule, according to a between-subjects design (n=6–8 for each genotype at one dose point). A indicates total active and inactive nose-poke responses for saline or MOR self-administration during the tests for dose-response curve between the two genotypes. B indicates total MOR intake during the tests for dose-response curve between the two genotypes. Data are presented as mean±S.E.M. * P<0.05 and ** P<0.01 versus corresponding wild-type control mice at the same dose point (one-way ANOVA). # P<0.05 and ## P<0.01 versus corresponding active nose-poke responses in wild-type and tPA knockout mice.

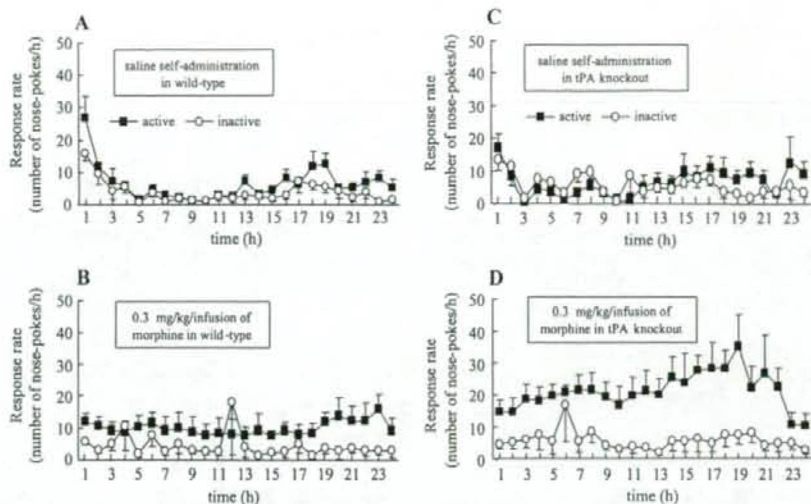

Fig. 3. Time course of changes in nose-poke responses for saline or MOR self-administration in a single 24-h session between the two genotypes. A and C indicate the response rate (nose-pokes per hour) for saline self-administration in wild-type and tPA knockout mice (n=7 for each genotype), respectively. B and D indicate the response rate (nose-pokes per hour) for MOR (0.3 mg/kg/infusion) self-administration in wild-type and tPA knockout mice (n=7 for each genotype), respectively. Data are presented as mean±S.E.M.

knockout mice appeared to be maintained throughout the single 24-h session (Fig. 3D). Taken together, the dose-response curve for MOR self-administration in a single 24-h session shifted upward in tPA knockout mice.

tPA knockout mice showed a decrease in breaking points for MOR self-administration, but not for food reinforcement, under the PR schedule

According to a within-subjects design, separate groups of animals (n=7–8 for each genotype) were subjected to three daily 12-h sessions of MOR self-administration under the PR schedule after daily 30-min sessions of nose-poke training for 8 days. As shown in Fig. 4A, during the first two sessions (at a dose of 0.3 or 1.0 mg/kg/infusion), there was no significant difference in breaking points for MOR self-administration under the PR schedule between the two genotypes of animals. When both genotypes of animals were subjected to the third 12-h session of MOR self-administration at the dose of 0.3 mg/kg/infusion, wild-type control mice showed a greater breaking point than tPA knockout mice to take MOR under the PR schedule (two-way ANOVA with repeated measures, a main effect of genotype, $F_{(1, 39)}=4.23$, $P<0.01$). Accordingly, total MOR intake was greater in wild-type control mice than in tPA knockout mice during the third

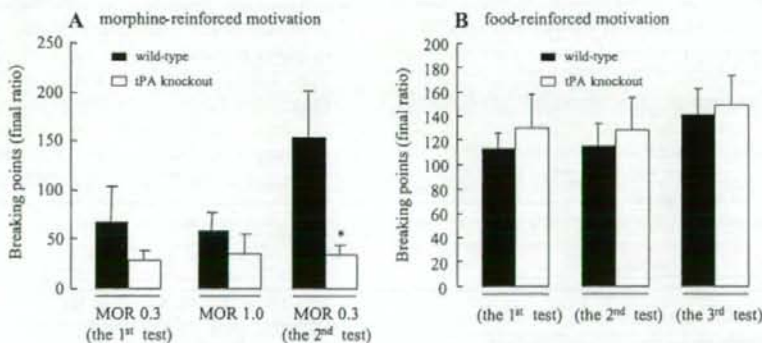

Fig. 4. Reduced breaking points for MOR self-administration, but not for food reinforcement, under the PR schedule in tPA knockout mice. After eight daily 30-min sessions of nose-poke training, separate groups of tPA knockout and wild-type control mice were subjected to MOR self-administration in a single 12-h session under the PR schedule, according to a within-subjects design (n=7 for wild-type and n=8 for tPA knockout mice). The mice were subjected to MOR self-administration at a dose of 0.3 mg/kg/infusion (1st test), then 1.0 mg/kg/infusion, and 0.3 mg/kg/infusion once again (2nd test). A indicates the breaking points for MOR reinforcement. B indicates the breaking points for food reinforcement. Data are presented as mean±S.E.M. * $P<0.05$ versus corresponding wild-type mice.

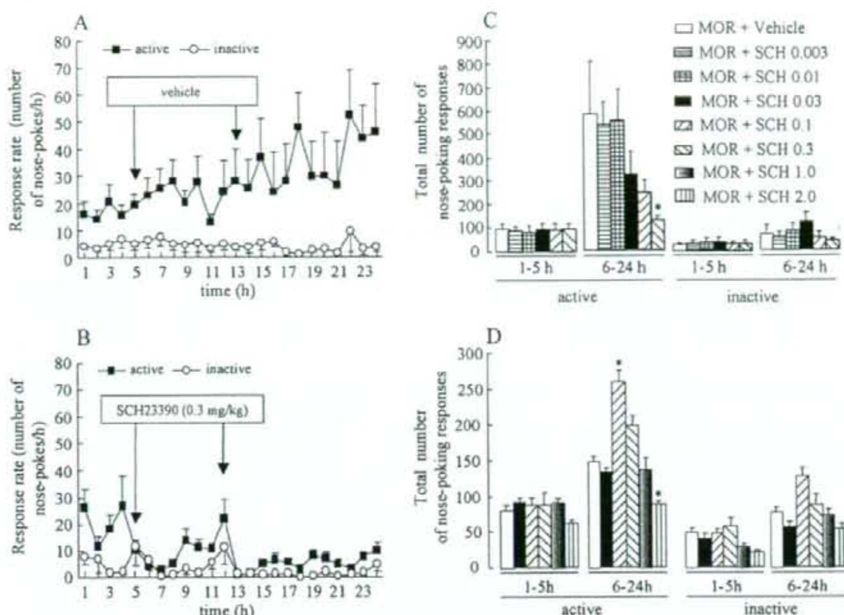

Fig. 5. Effects of the dopamine D1 receptor antagonist SCH23390 on nose-poke responses for MOR self-administration in tPA knockout and wild-type mice. After eight daily 30-min sessions of nose-poke training, separate groups of tPA knockout and wild-type control mice were subjected to MOR (0.3 mg/kg/infusion) self-administration in a single 24-h session under an FR2 schedule, according to a between-subjects design. *Arrows* in A and B indicate the time points for the i.v. injection of vehicle or SCH23390 during a single 24-h session. A indicates the time course of changes in the effects of vehicle on nose-poke responses for MOR (0.3 mg/kg/infusion) self-administration in tPA knockout mice. B indicates the time course of changes in the effects of SCH23390 (0.3 mg/kg) on nose-poke responses for MOR (0.3 mg/kg/infusion) self-administration in tPA knockout mice. C indicates the effects of SCH23390 on nose-poke responses for MOR self-administration in tPA knockout mice (n=6–7 for each dose point). D indicates the effects of SCH23390 on nose-poke responses for MOR self-administration in wild-type control mice (n=6 for each dose point). Data are presented as mean±S.E.M. * $P<0.05$ versus the group injected with vehicle injection.

session of MOR self-administration at the dose of 0.3 mg/kg/infusion (Student's t-test, $P<0.01$, data not shown). These observations suggest that tPA knockout mice had less motivation to take MOR than wild-type mice under the PR schedule of MOR reinforcement.

According to a within-subjects design, new groups of tPA knockout and wild-type control mice (n=8 for each genotype) were subjected to three daily 12-h sessions of food-reinforced operant behavior under the PR schedule using similar procedures. As shown in Fig. 4B, tPA knockout mice showed similar breaking points for food reinforcement to wild-type control mice throughout the three daily 12-h sessions. Taken together, these observations suggest that tPA knockout mice specifically showed lower breaking points for MOR self-administration under the PR schedule than wild-type control mice.

Dopamine D1 receptor antagonist SCH23390 inhibited the increased responding for MOR self-administration in tPA knockout mice, but demonstrated biphasic effects on MOR self-administration in wild-type control mice

After eight daily 30-min sessions of nose-poke training, according to a within-subjects design, separate groups of animals were subjected to a single 24-h session of MOR (0.3 mg/kg/infusion) self-administration. As shown in Fig. 5A and 5B, a dopamine D1 receptor antagonist SCH23390 was i.v. administrated via the catheter at two time points, immediately after the 5th and 12th hours. Prior to the administration of SCH2390 or vehicle (i.v.), tPA knockout mice (n=6–7 for each genotype) could discriminate active from inactive nose-poke responses (Fig. 5C, Student's t-test, $P<0.05$) and showed similar levels of nose-poke responses for MOR self-administration (1–5 h) (Fig. 5C, one-way ANOVA, $P=0.994$). However, the active nose-poke responses for MOR self-administration (6–24 h) in tPA knockout mice were attenuated by the dopamine D1 receptor antagonist SCH23390 at the dose of 0.3 mg/kg (Fig. 5B and 5C, one-way ANOVA, $P<0.05$), although much lower doses of SCH23390 (0.003 and 0.01 mg/kg) failed to affect active nose-poke responses for MOR self-administration in tPA knockout mice. Across the range of doses examined, SCH23390 treatment had little effect on inactive nose-poke responses. As shown in Fig. 5D, before the treatment with SCH23390 (1–5 h), wild-type control mice (n=6 for each group) showed no significant differences in active nose-poke responses for MOR self-administration (1–5 h). However, a lower dose of

SCH23390 (0.1 mg/kg) significantly increased active nose-poke responses for MOR reinforcement in wild-type mice (6–24 h, one-way ANOVA, $P<0.05$), whereas a higher dose of SCH23390 (2.0 mg/kg) significantly inhibited the active nose-poke responses in wild-type control mice (6–24 h, one-way ANOVA, $P<0.05$).

No significant effects of the dopamine D1 receptor antagonist SCH23390 on nose-poke responses for saline self-administration in the two genotypes of animals

After eight daily 30-min sessions of nose-poke training, according to a within-subjects design, separate groups of animals ($n=7-8$ for each group in tPA knockout mice and $n=6-7$ for each group in wild-type control mice) were subjected to a single 24-h session of saline self-administration. The dopamine D1 receptor antagonist SCH23390 was i.v. administrated via the catheter at two time points, immediately after the 5th and 12th hours. As shown in Fig. 6, SCH23390 (at doses of 0.03–2.0 mg/kg) had no significant effect on saline self-administration in either tPA knockout mice or wild-type control mice, suggesting that pharmacological effects of SCH23390 on active nose-poke responses for MOR self-administration in tPA knockout and wild-type mice are specifically attributable to changes in dopamine transmission, rather than general disruption of motor activity.

DISCUSSION

By using a novel single 24-h session of self-administration, we have demonstrated that tPA knockout mice showed an increase in active nose-poke responses for MOR self-administration under an FR2 schedule, but exhibited a decrease in breaking points (motivation) for MOR reinforcement under a PR schedule. In contrast, both genotypes of animals showed similar performance for food reinforcement under an FR or a PR schedule. The increased responding for MOR self-administration in tPA knockout mice was blocked by dopamine D1 receptor antagonist SCH23390 at the dose of 0.3 mg/kg, whereas the active nose-poke responses for MOR self-administration in wild-type control mice are attenuated by much higher dose of SCH23390 (2.0 mg/kg) but enhanced by a lower dose of SCH23390 (0.1 mg/kg). Our findings suggest that the reinforcing effects of MOR are reduced in tPA knockout mice.

In the present study, the dose-response curve for MOR self-administration under an FR2 schedule in tPA knockout mice shifted upward. This observation is consistent with a report that tPA knockout mice show an increase in active nose-poke responses for cocaine self-administration under a consecutive FR2 schedule of reinforcement (Ripley et al., 1999). In a paradigm of drug self-administration under an FR schedule of reinforcement, animals attempt to maintain a constant level of drug effect by increasing drug intake when drug impact is reduced and by decreasing intake when drug impact is increased. We have previously demonstrated, by using an *in vivo* dialysis technique, that the MOR-induced release of dopamine in the nucleus accumbens is reduced in tPA knockout mice as compared with wild-type control mice (Nagai et al., 2004). Thus, it is reasonable to postulate that in the present study, tPA knockout mice find a given dose of MOR (especially in the 0.3 mg/kg/infusion range) less reinforcing and therefore self-administer more of it, whereas at the lowest dose (0.1 mg/kg/infusion), neither wild-type nor tPA knockout mice found it particularly reinforcing, suggesting a subthreshold dose. In a paradigm of drug self-administration under a PR schedule, the "breaking point," expressed as the final ratio (the number of active nose-poke responses needed to earn the last infusion of MOR), may reflect the intensity of motivation for drug infusion or reinforcing effi-

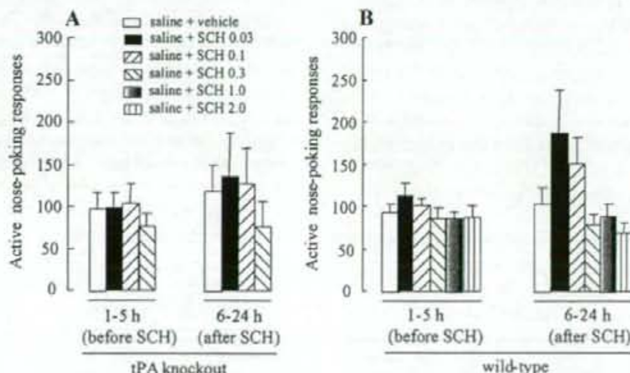

Fig. 6. Effects of the dopamine D1 receptor antagonist SCH23390 on nose-poke responses for saline self-administration in tPA knockout and wild-type mice. After eight daily 30-min sessions of nose-poke training, separate groups of tPA knockout and wild-type control mice were subjected to saline self-administration in a single 24-h session under an FR2 schedule, according to a between-subjects design. A indicates the effects of SCH23390 on nose-poke responses for saline self-administration in tPA knockout mice ($n=7-8$ for each dose point). B indicates the effects of SCH23390 on nose-poke responses for saline self-administration in wild-type control mice ($n=6-7$ for each dose point). SCH23390 was i.v. administered via a catheter at two time points as described above during a single 24-h session of saline self-administration. Data are presented as mean±S.E.M.

ciency of addictive drugs in animals (Roberts and Bennett, 1993). In our study, tPA knockout mice showed lower breaking points for MOR self-administration under the PR schedule than wild-type control mice (Fig. 4A). This observation further supports our hypothesis that reinforcing effects of MOR in tPA knockout mice are reduced as compared with wild-type control mice. It seem to be unlikely that the lower breaking points for MOR reinforcement in tPA knockout mice are derived from prior nose-poke training, since there was no difference in breaking points for natural reinforcement (food pellets) between the two genotypes of animals after the similar schedule of nose-poke training (Fig. 4B). These findings are consistent with our previous one that rewarding effects of MOR, which was assessed by a conditioned place preference test, are reduced in tPA knockout mice compared with wild-type control mice (Nagai et al., 2004).

It is well established that the dopamine transmission in the mesolimbic system in the brain plays a key role in the reinforcing properties of addictive substances such as MOR (e.g. Di Chiara and Imperato, 1988; Elmer et al., 2002; Self, 2004). It has been reported that, by the partial blockade of dopaminergic transmission, pretreatment with dopamine D1 receptor antagonists (SCH23390 and SCH39166) and a dopamine D2 receptor antagonist (eticlopride) leads to an increase in lever-presses for cocaine self-administration in rats (Caine and Koob, 1994; Barrett et al., 2004). In the present study, the low dose of SCH23390 (0.1 mg/kg) reduced the reinforcing effect of MOR to a level that supports an increase in nose-poke responses in order to regain the desired drug effects in wild-type control animals. As the dose of SCH23390 is increased (2.0 mg/kg) in wild-type control mice, dopamine D1 receptors are sufficiently blocked such that MOR is no longer reinforcing and the nose-poke responses are reduced. In tPA knockout mice, the increase in active nose-poke responses for MOR self-administration was attenuated by a lower dose of SCH23390 (0.3 mg/kg), suggesting that the combination of the dopamine D1 receptor antagonist and the mutation-induced reduction in the reinforcing effects of MOR (due presumably to a reduction in dopamine release in the nucleus accumbens, Nagai et al., 2004) resulted in a decrease in the reinforcing impact of MOR to a level that did not support nose-poke responses (hence SCH23390 dose-dependently decreased nose-poke responses). During the single 24-h session of MOR self-administration, the total number of active nose-poke responses in tPA knockout mice reached 600 or so (Fig. 5A and 5C). We therefore postulate that ceiling effects of MOR on active nose-poke responses in tPA knockout mice might mask an increase in active nose-poke responses induced by the lower doses of SCH23390. It is unlikely that the suppressive effect of SCH23390 on the increased responses for MOR self-administration in tPA knockout mice is due to a disruption of general operant behavior or nonspecific motor effects since, as compared with vehicle treatment, SCH23390 treatment had no inhibitory effects on saline self-administration in either genotype (Fig. 6). It has been reported that doses of up to and greater than 0.025 mg/kg of SCH23390 reduce cocaine- and amphetamine-induced hyperlocomotion in mice (O'Neill and Shaw 1999; O'Neill et al., 2003). Consistent with these findings, an impairment of general operant behavior by higher doses of SCH23390 (greater than 0.1 mg/kg, i.v.) was also observed immediately after the infusion of SCH23390 in our study. However, such a transient inhibition of operant performance is difficult to explain by the inhibitory action of SCH23390 on the increased responding for MOR self-administration in tPA knockout mice. One may argue that the doses of SCH23390 in our study are too high as compared with previous reports. For instance, in one report, the doses of SCH23390 to inhibit active responding for cocaine self-administration (0.01–0.1 mg/kg by pre-treatment; 1–3 h session; Corrigall and Coen, 1991; Caine and Koob, 1994) were lower than those used in the present study (0.3 mg/kg in tPA knockout mice and 2.0 mg/kg in wild-type controls during a single 24-h session; by post-treatment). The differences may be explained by the timing of administration (pre- versus post-administration of the antagonist) as well as the experimental conditions (a session of a few hours versus 24 h).

There is a body of evidence to suggest that tPA is involved in some forms of learning and memory. tPA knockout mice show deficits in hippocampal-dependent and cerebellar motor learning (Madani et al., 1999; Seeds et al., 2003). During the test for food-reinforced operant behavior (Fig. 1A) and the period of nose-poke training prior to a single 24-h session of MOR self-administration (Fig. 1B), no significant difference in active nose-poke responses for natural reinforcement was observed between tPA knockout and wild-type control mice. This observation further suggests that the increased active nose-poke responses for MOR self-administration in tPA knockout mice are related to the alteration of MOR-induced reinforcing effects, not to general deficits in learning and memory. In the present study, the single 24-h sessions of MOR self-administration were performed after eight daily 30-min sessions of nose-poke training. At the beginning of the single 24-h sessions, active nose-poke responses for MOR reinforcement might be facilitated by the habitual operant behavior or food extinction effects. Such habitual nose-poke responses or food extinction effects may mask the difference in the ability to acquire MOR self-administration behavior between the two genotypes. However, it is unlikely that, throughout the single 24-h session, the increased nose-poke responses for MOR self-administration in tPA knockout mice contributed to the extinction effects derived from the nose-poke training, since with the same procedure, no such pattern of nose-poke responses was observed for saline self-administration in the two genotypes (Fig. 2A). In both genotypes, the active nose-poke responses for saline self-administration were greater only at the beginning of the single 24-h session, but rapidly declined and then remained lower level (Fig. 3A and 3C). In contrast, active nose-poke responses for MOR self-administration were maintained at higher levels throughout the single 24-h session in both genotypes (Fig. 3B and D).

A previous report has demonstrated that a single administration of cocaine altered synaptic plasticity in the ventral tegmental area for several days (Ungless et al., 2001). Furthermore, rats given a single 2-h session of cocaine self-administration exhibit drug-seeking behavior for up to 1 year after the intake of cocaine (Ciccocioppo et al., 2004). Together with the single-session cocaine self-administration reported by Olsen and Winder (2006), the single 24-h session of self-administration used in the present study may provide an alternative experimental design to investigate genetic factors involved in the reinforcing properties of addictive substances by using genetically modified strains of mice.

CONCLUSION

In conclusion, our findings suggest that tPA is critically involved in the reinforcing properties of MOR in mice. The reinforcing effects of MOR are reduced in tPA knockout mice. The modulation of the tPA system in the brain may be a potential target against drug abuse.

Acknowledgments—We thank Dr. Tsuneyuki Yamamoto and Dr. Masayuki Nadai for technical assistance in the establishment of a mouse self-administration system in our laboratory.

This study was supported in part by a Grant-in-aid for Scientific Research and Special Coordination Funds for Promoting Science and Technology, Target-Oriented Brain Science Research Program, 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 Dementia and Fracture from the Ministry of Health, Labor and Welfare of Japan; by the Japan Society for the Promotion of Science Joint Research Project as part of the Japan-Korea Basic Scientific Cooperation Program; by a Smoking Research Foundation Grant for Biomedical Research; by a Grant-in-aid for Scientific Research (B) and Young Scientists (A); by Uehara Memorial Foundation Research Fellowship; and in part by the 21st Century Center of Excellence Program "Integrated Molecular Medicine for Neuronal and Neoplastic Disorders" from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and by the Brain Research Center from 21st Century Frontier Research Program funded by the Ministry of Science and Technology, Republic of Korea.

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(Accepted 11 January 2007)
(Available online 20 February 2007)

Molecular Psychiatry (2008), 1–13
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www.nature.com/mp

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

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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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] 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

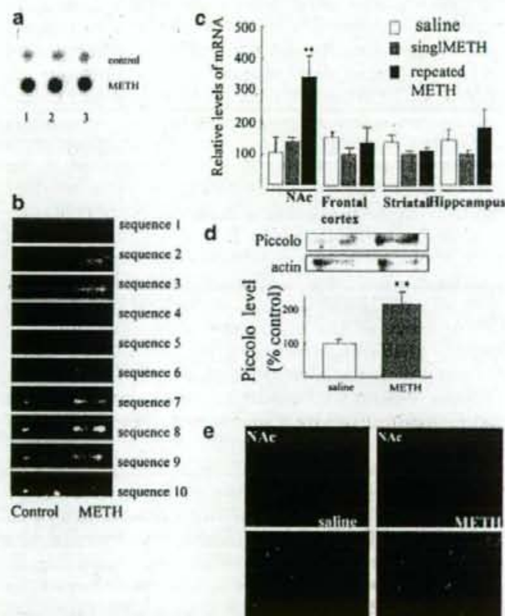

Figure 1 Piccolo expression in nucleus accumbens (NAc) was upregulated by repeated methamphetamine (METH) administration. (**a**) Piccolo overexpression in the NAc of a presentative mouse after daily METH administration for 5 days was shown. (**b**) RT–PCR analysis revealed a significant increase in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

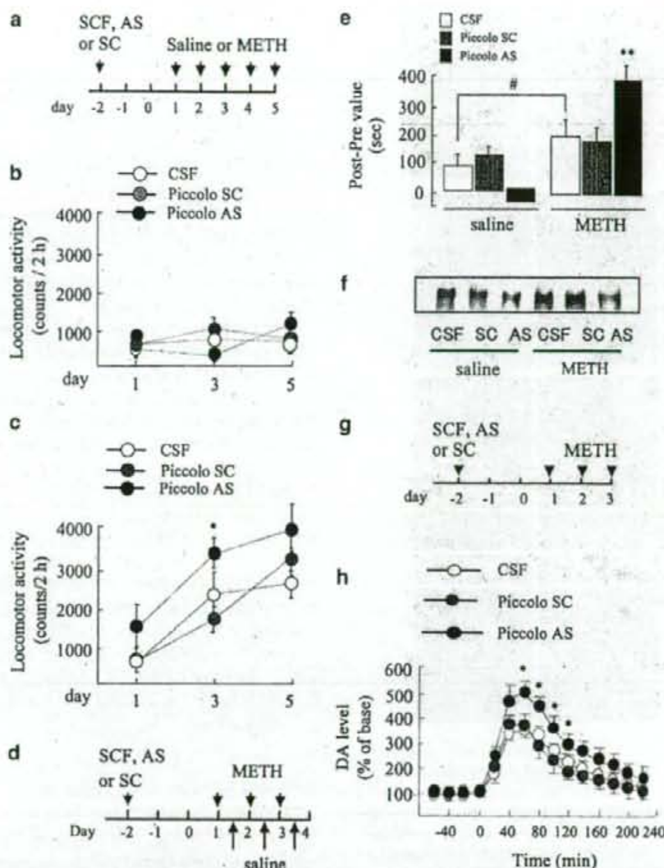

Figure 2 Downregulation of Piccolo expression with antisense oligodeoxynucleotide (AS) promoted methamphetamine (METH)-induced behavioral and synaptic plasticity. (**a–c**) Mice were infused intracerebroventricularly with Piccolo AS, scramble oligodeoxynucleotide (SC) or cerebrospinal fluid (CSF) for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

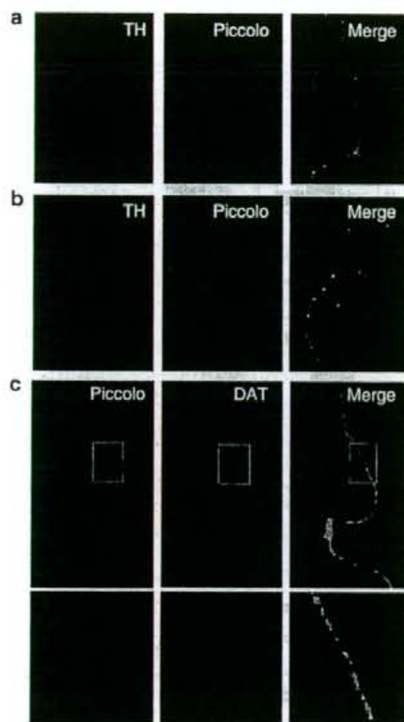

Figure 3 Expression of Piccolo in dopaminergic neurons. (**a**) Double immunostaining showed that Piccolo is expressed in tyrosine hydroxylase (TH)-positive neurons. (**b**) Abundant expression of Piccolo is present at the presynaptic component of cultured dopaminergic neurons. (**c**) Dopamine transporter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

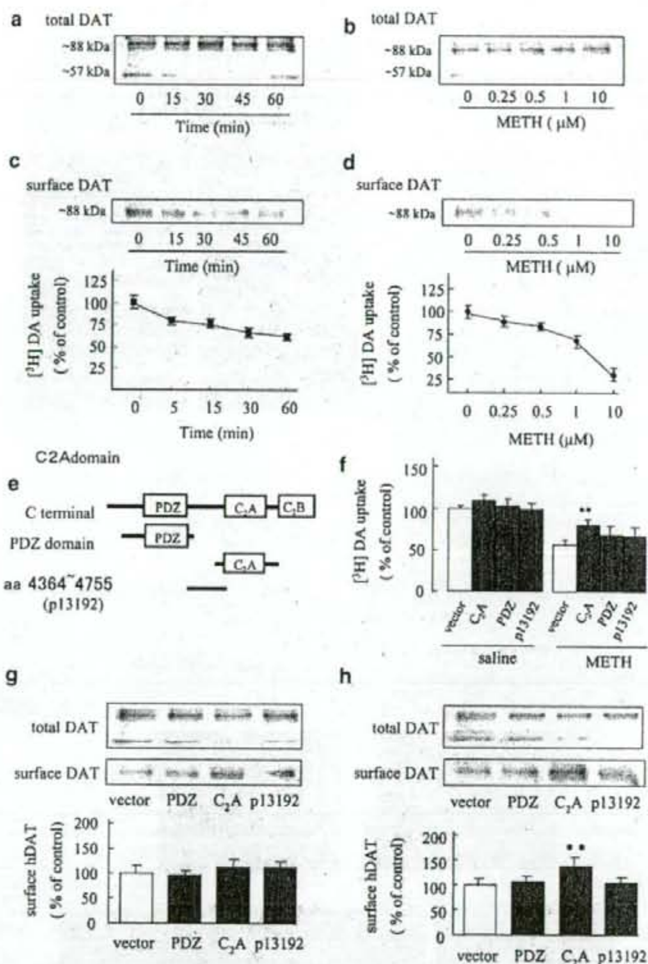

Figure 4 Piccolo C_2A domain increased dopamine (DA) uptake and dopamine transporter (DAT) surface expression. (**a**, **b**) Methamphetamine (METH) could not alter the total DAT expression levels in hDAT-PC12 cells in both time- (**a**) and dose-dependent studies (**b**). (**c**) METH (1 μM) decreased 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

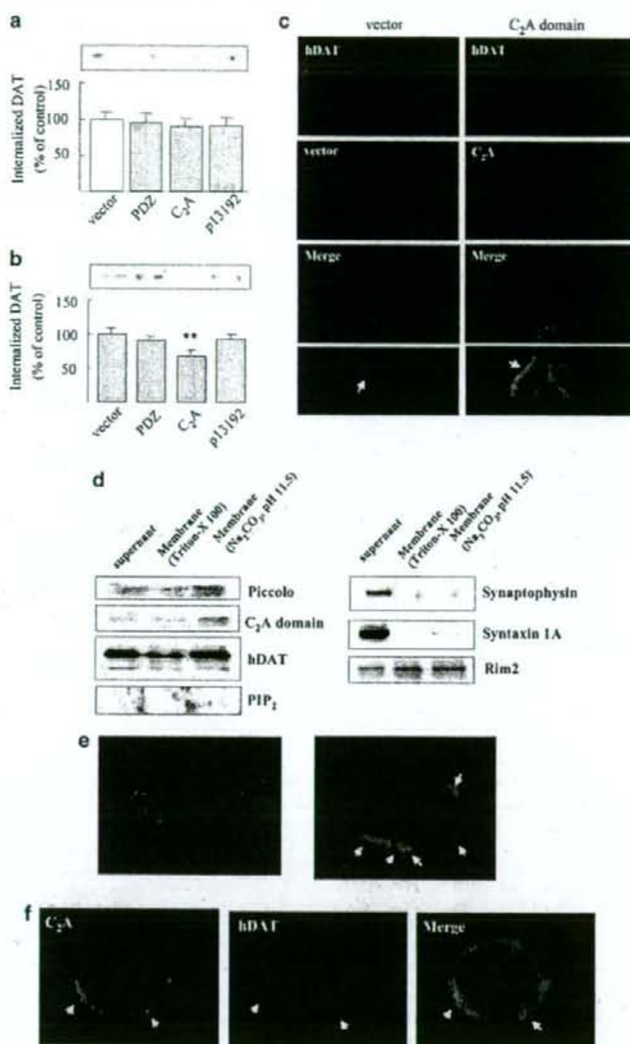

Figure 5 Piccolo modulates dopamine transporter (DAT) internalization by a mechanism of membrane association. (**a, b**) After transfection with various vectors the hDAT-PC12 cells were biotinylated and treated with either saline (**a**) or 1 μM methamphetamine (METH) (**b**) for 30 min to initiate endocytosis. 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