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Reduction of methamphetamine-induced sensitization and reward in matrix metalloproteinase-2 and -9-deficient mice

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Abstract

Matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) function to remodel the pericellular environment. Their activation and regulation are associated with synaptic physiology and pathology. Here, we investigated whether MMP-2 and MMP-9 are involved in the rewarding effects of and sensitization to methamphetamine (METH) in animals, in which the remodelling of neural circuits may play a crucial role. Repeated METH treatment induced behavioural sensitization, which was accompanied by an increase in MMP-2 and MMP-9 activity in the brain. In MMP-2- and MMP-9-deficient mice [MMP-2-(–/–) and MMP-9-(–/–)], METH-induced behavioural sensitization and conditioned place preference, a measure of the rewarding effect, as well as METH-increased dopamine release in the nucleus accumbens (NAc) were attenuated compared with those in wild-type mice. In contrast, infusion of purified human MMP-2 into the NAc significantly potentiated the METH-increased dopamine release. The [^{3}H]dopamine uptake into striatal synaptosomes was reduced in wild-type mice after repeated METH treatment, but METH-induced changes in [^{3}H]dopamine uptake were significantly attenuated in MMP-2-(–/–) and MMP-9-(–/–) mice. These results suggest that both MMP-2 and MMP-9 play a crucial role in METH-induced behavioural sensitization and reward by regulating METH-induced dopamine release and uptake in the NAc.

Keywords: dopamine, drug dependence, matrix metalloproteinases, methamphetamine.

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Drug dependence is a complex phenomenon with important psychological and social causes and consequences, which result from adaptations in specific brain circuits caused by repeated exposure to drugs of abuse (Berke and Hyman 2000; Yamada *et al.* 2005). It has been proposed that cellular and molecular mechanisms for drug dependence involve processes similar to those operating in other forms of synaptic plasticity such as learning and memory (Shippenberg and Heidbreder 1995; Mizoguchi *et al.* 2004). Methamphetamine (METH), a typical drug of abuse, has both acute and long-lasting effects on psychomotor behaviours. The effects of METH are associated with an increase in extracellular dopamine levels in the brain through redistri-

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Abbreviations used: DAT, dopamine transporter; ECM, extracellular matrix; Fc, frontal cortex; GFAP, glial fibrillary acidic protein; METH, methamphetamine; MMP, matrix metalloproteinase; NAc, nucleus accumbens; NeuN, neuron-specific nuclear antigen; SDS-PAGE, sodium dodecyl sulfate - polyacrylamide gel electrophoresis; TIMP, tissue inhibitors of MMP; tPA, tissue plasminogen activator.

bution of dopamine from synaptic vesicles to the cytosol and promotion of reverse transport (Sulzer *et al.* 1995; Nakajima *et al.* 2004). Although the involvement of endogenous substances such as tumour necrosis factor (TNF)-α (Nakajima *et al.* 2004), tissue plasminogen activator (tPA: Nagai *et al.* 2004, 2005) and brain-derived neurotrophic factor (Nestler 2001), which are known to play a role in synaptic plasticity, has been implicated in the development of METH dependence (Robinson and Kolb 1997; Nestler 2001), the mechanism underlying the enduring brain dysfunction associated with the dependence are poorly understood.

Matrix metalloproteinases (MMPs) function to remodel the pericellular environment, primarily through the cleavage of extracellular matrix (ECM) proteins (Yong *et al.* 2001) and cell-surface components. MMPs constitute a family of enzymes with more than 20 members identified to date, which require Zn^{2+} for their enzymatic activity. Gelatinases (MMP-2 and MMP-9) are capable of cleaving collagens IV and V, laminin, and chondroitin sulfate proteoglycan, which were associated with cell adhesion, and have been implicated specifically in cerebral ischaemia (Lo *et al.* 2002), kainate-induced neuronal injury (Szklarczyk *et al.* 2002) and hippocampal long-term potentiation and memory (Nagy *et al.* 2006). MMP activity is regulated by interaction with tissue inhibitors of MMP (TIMPs), and thus the MMP/TIMP system may be involved in brain development (Vaillant *et al.* 1999; Wright *et al.* 2002). The consequences of proteolytic cleavage of target molecules by MMPs are varied and complex and are thought broadly to include both changes in physical constraints of the pericellular environment as well as signalling through liberation of normally sequestered molecules such as growth factors, or exposure of latent bioactive peptide fragments (Nagase and Woessner 1999).

We have recently demonstrated that tPA, an extracelluar serine protease, is involved in morphine and METH dependence (Nagai *et al.* 2004, 2005; Yamada *et al.* 2005). The tPA/plasmin system is one of the regulators of MMP (Wright *et al.* 2002; Wang *et al.* 2003) and could play a pivotal role in ECM degradation (Baricos *et al.* 1995). In this study, we investigated whether the MMP-2 and MMP-9 are involved in METH-induced behavioural sensitization and reward in an animal model. Here, we show that the expression of MMP-2 and MMP-9 is induced by repeated METH treatment in the brain, and that the MMP expression is involved in the development of METH-induced sensitization and place preference by regulating METH-induced dopamine release and uptake in the nucleus accumbens (NAc).

Materials and methods

Animals

Male Wistar rats (8 weeks old; Charles River Japan, Yokohama, Japan) weighing 270 ± 20 g at the beginning of experiments were used in the study. We also used MMP-2 heterozygous knockout [MMP-2-(+/−)], homozygous knockout [MMP-2-(−/−)] and their wild-type (C57BL/6 J) mice (10–12 weeks old). The mutant mice were backcrossed into the C57BL/6J strain more than 13 times, and the generation, genotyping, and characterization of MMP-2-(−/−) mice have been described (Itoh *et al.* 1997). MMP-9 homozygous knockout [MMP-9-(−/−)] mice (10–12 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Wild-type FVB/N [MMP-9-(+/+)] mice were obtained from the CLEA Japan (Fuji, Japan).

All experiments were performed in accordance with the 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 United States National Institutes of Health Guide for the Care and Use of Laboratory Animals.

METH treatment for behavioural sensitization

Rats were given saline or METH at a dose of 2 mg/kg (s.c.) for 5 days (Nakajima *et al.* 2004; Nagai *et al.* 2005). The animals were placed in their home cage for 1 h following injection and then locomotor activity was measured in an acrylic chamber (25 × 42 × 20 cm) for 1 h using behavioural analysis systems (SCANET SV-10; Neuroscience, Tokyo, Japan) on days 1, 3 and 5. In the experiments on mice, they were given saline or METH at a dose of 1 mg/kg (s.c.) for 7 days (Nakajima *et al.* 2004; Nagai *et al.* 2005). Locomotor activity was immediately measured for 1 h after METH treatment on days 1, 3, 5 and 7. After 7-day withdrawal of METH treatment (on day 14), the mice were given METH at a dose of 1 mg/kg (s.c.).

Conditioned place preference (CPP)

The apparatus used for the place conditioning task consisted of two compartments: a black Plexiglas box and a transparent Plexiglas box (both 15 × 15 × 15 cm high for mice) with a metal grid floor. The place conditioning paradigm was performed with a minor modification (Nagai *et al.* 2004; Nakajima *et al.* 2004). In the preconditioning test, the sliding door was opened and the animal was allowed to move freely between both boxes for 15 min once a day for 3 days. On the third day of the preconditioning test, we measured the pre-value using Scanet SV-10 LD (Tokyo Sangyo Co. Ltd, Toyama, Japan). On days 4, 6 and 8, a rat or mouse was given METH at 1 mg/kg and put on its non-preferred side for 20 min. On days 5, 7, and 9, the animal was given saline and placed on the opposite drug-conditioning side. In the post-conditioning test, the sliding door was opened, and we measured the post-value. Place conditioning behaviours were expressed as [(post-value)−(pre-value)].

Gel zymography

After the final administration of METH, rats were intracardially perfused with cold saline and then killed by rapid decapitation as described (Asahi *et al.* 2001; Lee *et al.* 2004). Various regions of the brain including the NAc and frontal cortex (Fc) were dissected out and immediately frozen and stored at −80°C until assayed. All dissection was made using brain matrix (NeuroScience Idea, Osaka, Japan) and based on the atlas of Paxinos and Watson (1982).

Sample preparation was made as described previously (Zhang and Gottschall 1997). Briefly, brain tissues were homogenized in

lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.05% Brij35, and 0.02% NaN_3, pH 7.6) with 1% TritonX-100 and centrifuged at 12 000 *g* for 10 min to pellet insoluble material. The protein concentration in the supernatant was determined using a Protein Assay Rapid Kit. The supernatant was incubated with gelatin-sepharose 4B (GE Healthcare Bio-Science, Piscataway, NJ, USA) that had previously been washed three times with the lysis buffer, with constant shaking, for 24 h at 4°C. After centrifugation at 500 *g* for 2 min, the pellet was re-suspended in 500 μL of the lysis buffer and washed three times. The pellet was re-suspended in 150 μL of lysis buffer containing 10% dimethyl sulfoxide and shaken for 2 h, and used for assaying gelatinase activity and for western blotting of MMP-2 and MMP-9.

The samples, together with human pro-MMP-2 (1 ng/lane; Amersham Pharmacia Biotech, Piscataway, NJ, USA) and cultured medium of the C6 cell line, were subjected to electrophoresis in 10% sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) containing 0.1% gelatin under non-reducing conditions. The human pro-MMP-2 and C6 cell medium were used as positive controls. To detect the active lower molecular weight forms of these proteases, samples were incubated with 400 mM *p*-aminophenylmercuric acetate (APMA; Amersham Pharmacia Biotech), which is known to induce the autocatalytic cleavage of gelatinases. After incubation for 5 h with APMA, samples were subjected to gel zymography. Gels were washed twice for 30 min in 2.5% Triton X-100 to remove SDS, washed for 30 min in incubation buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 2 μM $ZnCl_2$, 200 mM NaCl, and 0.02% Briji35, pH 7.4) at 25 ± 2°C, and further incubated for 24 h in the same buffer at 37°C. Gels were then stained for 3 h in Coomassie blue (1% Coomassie Brilliant Blue G-250, 30% methanol, 10% acetic acid) and destained in 40% methanol/7% acetic acid until clear bands of gelatinolysis appeared on a dark background. Total activity including pro-MMP was analysed with the ATTO Densitograph Software Library Lane Analyzer (Atto Instruments, Tokyo, Japan).

Western blotting

The sample preparation of brain tissues was made as described above. Brain tissues were homogenized in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.05% Brij35, and 0.02% NaN_3, pH 7.6) with 1% TritonX-100. The supernatant was boiled in 2 × sample buffer (0.25% bromophenol blue/12% 2-mercaptothanol/20% glycerol/4% SDS/0.1 M Tris-HCl, pH 6.8) and electrophoresed by SDS–PAGE on a 4.75% stacking gel and 10% separating gel, and then transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Bedford, MA, USA; Mizoguchi *et al.* 2004). The same amount of protein per lane was loaded for all western blotting. The membrane was incubated in the blocking solution (5% bovine serum albumin in Tris-buffered saline with 0.1% Tween 20) for 2 h at 25 ± 2°C and then incubated with primary antibodies. After washing, blots were incubated with the secondary antibodies. Immunoreactive materials on the membrane were detected using enhanced chemiluminescene (ECL) western blotting detection reagents (GE Healthcare Bio-Science) and exposed to X-ray film. The band intensities of the film were analysed by densitometry. The primary antibodies used in the present study were polyclonal rabbit anti-MMP-2 (1 : 3000; AB809; Chemicon, Temecula, CA, USA) and anti-MMP-9 (1 : 1000; AB19016; Chemicon) antibodies. The secondary antibodies, used at a 1 : 2000 or 1 : 5000 dilution, were horseradish peroxidase-linked anti-rabbit (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA).

In situ zymography

Rats were intracardially perfused with cold saline before being frozen at −80°C using O.C.T. Compound (Sakura Finetechnical, Tokyo, Japan). The brains were sectioned at 20 μm in a cryostat. We adapted an *in situ* zymography method to localize net gelatinolytic activity in brain sections as described previously (Szklarczyk *et al.* 2002). Non-fixed sections were incubated for 24 h at 37°C in a humid dark chamber with a reaction buffer containing 0.5 M Tris-HCl, 1.5 M NaCl, 50 mM $CaCl_2$, 2 mM sodium azide, pH 7.6 and 100 μg/mL FITC-labelled DQ-gelatin (Molecular Probes, Eugene, OR, USA) intramolecularly quenched. After incubation, sections were washed in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde and mounted on slides. Some sections were incubated with the broad spectrum MMP inhibitor 1,10-O-phenanthroline (1 mM; Molecular Probes) or with 200 ng/mL human recombinant TIMP-2 (Daiichi FineChem., Takaoka, Japan). Samples were observed with a FITC filter, and the images were analysed using AXIOVISION 3.0 systems (Carl Zeiss, Jene, Germany). Gelatin–FITC cleavage by tissue gelatinases releases quenched fluorescence representative of net proteolytic activity. Sections incubated without DQ-gelatin were not fluorescent.

Double immunostaining

Polyclonal rabbit anti-MMP-2 antibody (1 : 250; AB809, Chemicon), anti-MMP-9 antibody (1 : 250; AB19016, Chemicon), monoclonal mouse anti-neuron-specific nuclear antigen (NeuN) antibody (1 : 200; Chemicon) and anti-glial fibrillary acidic protein (GFAP) antibody (1 : 200; Chemicon) served as primary antibodies. Affinity-purified FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG served as secondary antibodies. Samples were observed with AXIOVISION 3.0 systems (Carl Zeiss).

In vivo microdialysis

Rats and mice were anaesthetized with sodium pentobarbital before stereotaxic implantation of a guide cannula into the NAc [AP +1.6, ML −1.0 from the bregma, DV −7 from the skull for rats (Paxinos and Watson 1982), AP −1.7, ML +0.8 from the bregma, DV −4 from the skull for mice (Franklin and Paxinos 1997)]. A dialysis probe (A1-8–1 or A1-6–1; 1-membrane length, Eicom, Kyoto, Japan) was inserted though the guide cannula and perfused with artificial cerebrospinal fluid (aCSF; 147 mM NaCl/4 mM KCl/2.3 mM $CaCl_2$) at a flow rate of 1.2 μL/min in rats and 2 μL/min in mice. Outflow fractions were collected every 20 min. After the collection of three baseline fractions, the animals were treated with METH (1 or 2 mg/kg, s.c.). Dopamine levels in the dialysates were analysed as described (Nakajima *et al.* 2004). To examine the effect of microinjection of MMP-2 into the NAc on METH-induced dopamine release in rats, a dialysis probe equipped with a microinjection tube (MI-A1-8–1; 1-mm membrane length, Eicom) was used (Nagai *et al.* 2004). After the collection of baseline fractions, a 10-ng dose of purified human MMP-2 (Chemicon) or its vehicle was injected into the NAc, at a volume of 1 μL during a 10-min period, through the

microinjection tube. METH at 2 mg/kg was injected s.c. 60 min after treatment with MMP-2. The probe placement was checked in each animal at the end of the *in vivo* microdialysis experiment, and the data of the mice in which tip of the probe was located outside the Nac were excluded for the data analysis.

Crude synaptosomal [^{3}H]dopamine uptake

Crude synaptosomal [^{3}H]dopamine uptake was determined as described (Nakajima *et al.* 2004). Tissue including the striatum and NAc was homogenized in ice-cold 0.32 M sucrose and centrifuged at 1000 *g* for 10 min at 4°C. The supernatant fractions were removed and centrifuged at 22 000 *g* for 15 min. The resulting pellet was re-suspended in an ice-cold modified Krebs–Ringer solution composed of (in mM) 125 NaCl, 4.8 KCl, 25 $NaHCO_3$, 1.2 $KH2PO_4$, 1.3 $MgCl_2$, 1.2 $CaCl_2$, 10 glucose, and 0.57 ascorbic acid gassed with 95% O_2 and 5% CO_2. Crude synaptosomal homogenate (100 μg synaptosomes protein) was contained 5 μM pargyline (Sigma, St Louis, MO, USA) in 200 μL of Krebs–Ringer solution. After pre-incubation for 10 min at 37°C, assays were initiated by the addition of [^{3}H]dopamine (60 Ci/mmol; Perkin-Elmer Life Sciences, Boston, MA, USA) in 1 mL of Krebs-Ringer solution. Samples were incubated at 37°C for 4 min, and then ice-cold Krebs-Ringer solution containing 10 μM GBR12909, a specific dopamine uptake inhibitor, was added. Samples were filtered through Whatman GF/B filters. Non-specific values were determined in the presence of 100 μM GBR12909. The radioactivity trapped on filters was measured using a liquid scintillation counter.

Statistical analyses

Results were expressed as the mean ± SE. The significance of differences was determined by one-way ANOVA, followed by the Student–Newman–Keuls test or by repeated ANOVA, followed by Sheffe's test for multigroup comparisons. Student's *t*-test was used for two-group comparison.

Results

METH increases MMP-2 and MMP-9 expression in the brain

Gelatinases, MMP-2 and MMP-9, have been implicated specifically in cerebral ischaemia and physiological tissue remodelling. For example, MMP-2 plays a principal role in establishing the growth-promoting properties of denervated peripheral nerve (Krekoski *et al.* 2002). MMP-9, but not MMP-2, is particularly involved in dendritic remodelling in the hippocampus of adult rat (Szklarczyk *et al.* 2002). Therefore, we examined whether repeated METH treatment altered the protein levels and enzymatic activities of MMP-2 and MMP-9 in the rat brain.

MMP-2 protein levels were increased in the Fc and NAc by repeated METH treatment for 5 days (Fig. 1a), which induced behavioural sensitization. In the Fc, the increase was evident as early as 2 h after the last administration of METH, whereas the change in the NAc was manifested 24 h after the last injection. Gel zymography revealed that MMP-2 activity

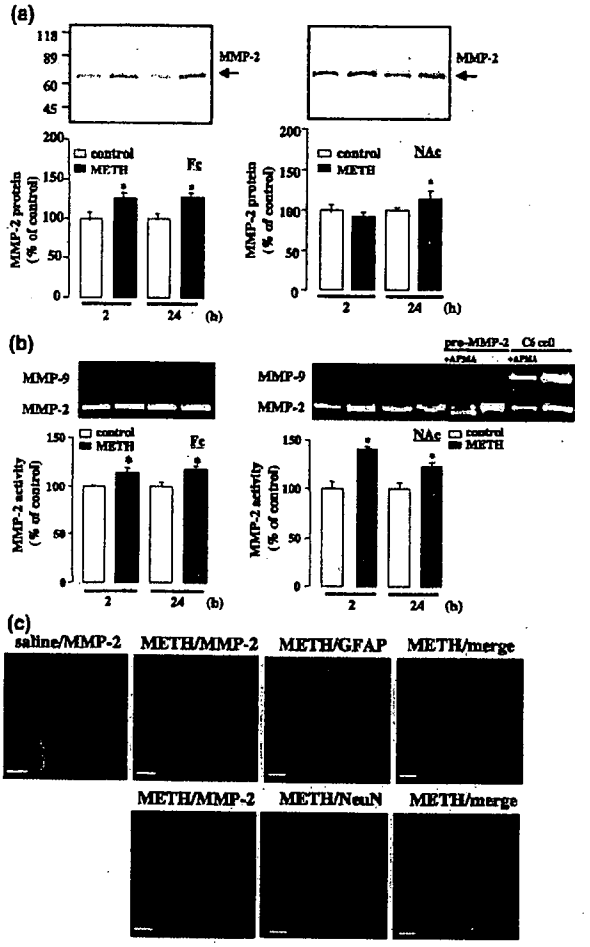

Fig. 1 METH-induced MMP-2 expression. Changes in protein levels (a) and MMP-2 activity (b) in Fc and NAc after repeated METH treatment. Rats were given METH at a dose of 2 mg/kg for 5 days and killed 2 or 24 h after the final administration. Control rats were given saline. The human pro-MMP-2 and C6 cell line was used as a positive control. Incubation for 5 h with APMA induced the autocleavage of pro-MMP-2 to active-MMP-2. $^*p < 0.05$ versus control group. Values are the means ± SE (a, n = 4–6; b, n = 3–5). Double immunostaining for MMP-2 and the neuronal marker NeuN or GFAP in the Fc (c). Rats were killed 2 h after the final administration of METH (2 mg/kg, for 5 days). Scale bar, 20 μm.

was enhanced in the Fc and NAc by repeated METH treatment 2 and 24 h after the last injection (Fig. 1b). The apparent discrepancy of the METH effects on MMP-2 protein and activity in the NAc observed 2 h after the last administration may be as a result of the different sensitivities of the methods used in the experiments. Treatment of pro-MMP-2 with APMA induced the autocatalysis of the gelatinases, resulting in lower molecular weight bands likely to represent active forms of gelatinases (Fig. 1b). Compared with the band of APMA-treated pro-MMP-2, no active-MMP-2 was detected in control or METH-treated rats.

Repeated METH treatment also increased MMP-9 protein levels and activity in the Fc and NAc (Figs 2a and b, and Fig. 1b showing the MMP-9 bands). It should be noted that repeated METH treatment produced a lower molecular weight band which may be an active MMP-9 form (Fig. 1b). Acute METH treatment had no effect on MMP-2 or MMP-9 activity in the Fc (MMP-2 100 ± 9.9 of control, $n = 5$–6, $p > 0.05$; MMP-9 100 ± 17.6 of control, $n = 5$–6, $p > 0.05$) and NAc (MMP-2 100 ± 7.2 of control, $n = 5$–6, $p > 0.05$; MMP-9 100 ± 7.3 of control, $n = 5$–6, $p > 0.05$) 2 h after the treatment.

To determine the cell types in which the expressions of MMP-2 and MMP-9 are induced by repeated METH treatment, double immunostaining for MMP-2 and MMP-9 with NeuN, a neuronal marker, or GFAP, a glial marker, was performed. The findings indicated the co-localization of MMP-2 immunoreactivity with NeuN and GFAP immunoreactivity, suggesting the neuronal and glial localization of MMP-2 (Fig. 1c). Similar results were found in double immunostaining for MMP-9 and NeuN or GFAP (Fig. 2c). These results indicate that MMP-2 and MMP-9 expression is induced by METH in both neuronal and glial cells in Fc.

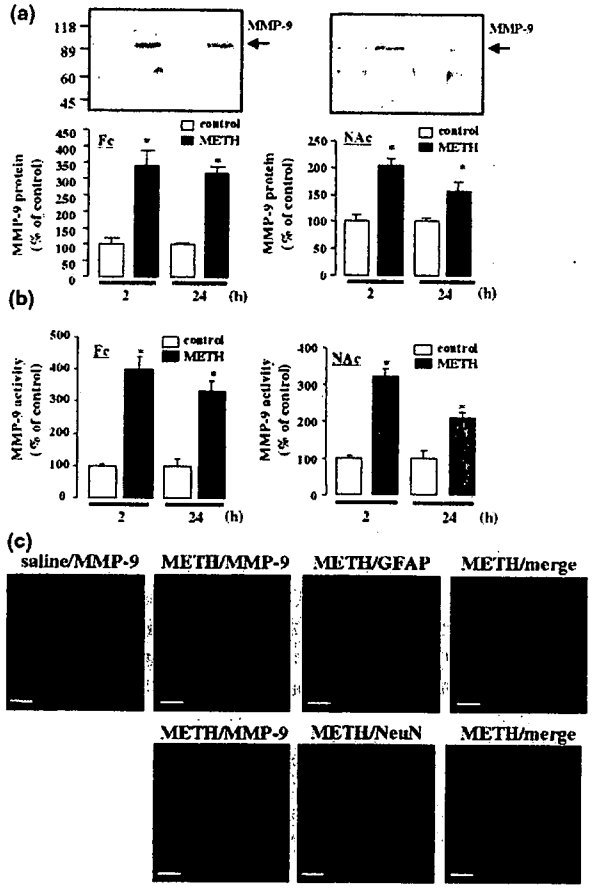

Fig. 2 METH-induced MMP-9 expression. Changes in protein levels (a) and MMP-9 activity (b) in Fc and NAc after repeated METH treatment. Rats were given METH at a dose of 2 mg/kg for 5 days and killed 2 or 24 h after the final administration. Control rats were given saline. The C6 cell line was used as a positive control. Incubation for 5 h with APMA induced the autocleavage of pro-MMP-9 to active-MMP-9 (Fig. 1b). *$p < 0.05$ versus control group. Values are the means ± S.E (a, $n = 4$–6; b, $n = 3$–4). Double immunostaining for MMP-9 and NeuN or GFAP in Fc (c). Rats were killed 2 h after the final administration of METH (2 mg/kg, for 5 days). Scale bar, 20 μm.

Spatial changes in MMP proteolytic activity in the brain after repeated METH treatment

We analysed the spatial changes in gelatinase activity in the brain following repeated METH treatment by *in situ* zymography. Brain sections were incubated with gelatin conjugated to a quenched fluorescence dye. Cleavage of gelatin results in an increase in fluorescence. The signal was inhibited by the zinc chelator phenantroline (Phe), broad spectrum MMP inhibitor, indicating that the fluorescence is associated with MMP activity (Fig. 3b compared with 3a). In control sections, gelatinase activity was observed in layer II–V of the cingulate and prelimbic cortex, the CA1–CA4 layers and dentate gyrus of the hippocampus, striatum and the shell of the NAc. Two

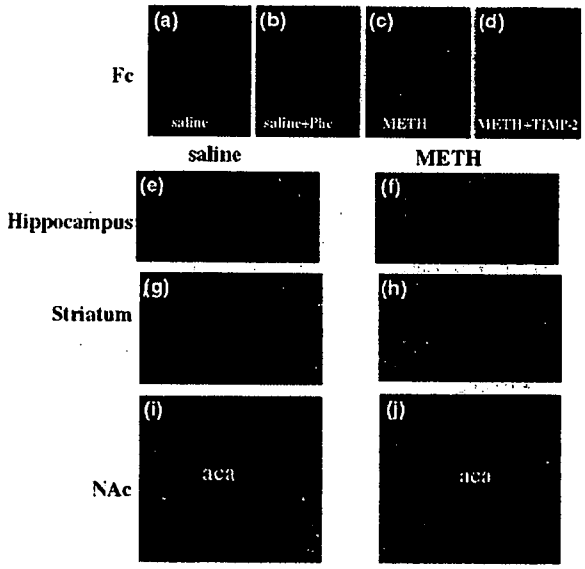

Fig. 3 Spatial changes in METH-induced MMP activity in the brain. *In situ* zymography detected gelatinase activity in Fc (a–d), hippocampus (e, f), striatum (g, h) and NAc (i, j). Rats were given saline (a, b, e, g, i) or METH (c, d, f, h, j) at a dose of 2 mg/kg for 5 days, and killed 2 h after the final administration. Brain sections were incubated with fluorescent gelatin. Cleavage of the gelatin by proteinases resulted in the unblocking of quenched fluorescence and an increase in fluorescence. Gelatinase activity was attenuated by either the zinc chelator 1,10-O-phenantroline (Phe, b) or TIMP-2 (200 ng/mL, d). Photomicrographs are representative of observations made in at least three animals per group. Photomicrographs were representative of observations made from at least three animals per group.

hours after the final administration of METH, a strong signal was visualized in the Fc compared with the saline group (Fig. 3c compared with 3a). METH-increased gelatinase activity in the Fc was inhibited by TIMP-2 at 200 ng/mL (Fig. 3d). In addition, the MMP activity was increased markedly by repeated METH treatment in the striatum and NAc, but moderately in the hippocampus (Figs 3e–j).

MMP-2-deficient [MMP-2-(–/–)] and MMP-9-deficient [MMP-9-(–/–)] mice show reduced responses to METH in behavioural sensitization, CPP and dopamine release in the NAc

We investigated a role of MMP-2 and MMP-9 in METH-induced behavioural sensitization and reward using MMP-2 and MMP-9 gene knockout mice. As shown in Fig. 4(a), there was no difference in spontaneous locomotor activity or single METH-induced hyperlocomotion among wild-type, MMP-2 heterozygous knock-out [MMP-2-(+/–)] and homozygous knock-out [MMP-2-(–/–)] mice. However, they showed significantly different responses to repeated METH treatment. Repeated ANOVA revealed that both MMP-2-(+/–) and MMP-2-(–/–) mice showed significantly reduced locomotor activity during repeated METH treatment compared with wild-type mice (Fig. 4a, $F_{5,68} = 17.8$; $p < 0.05$). On day 14 when METH (1 mg/kg) was challenged, the attenuation of the METH-induced sensitization in MMP-2 mutant mice was reconfirmed (Fig. 4a, $F_{5,68} = 43.5$; $p < 0.05$; one-way ANOVA). It was also indicated by repeated ANOVA that both METH-induced hyperlocomotion and repeated METH-induced sensitization were markedly decreased in MMP-9 homozygous knockout [MMP-9-(–/–)] mice compared with those in wild-type mice (Fig. 4b, $F_{3,49} = 50.8$; $p < 0.05$). Furthermore, the expression of METH-induced sensitization on day 14 was significantly attenuated in MMP-9-(–/–) mice compared with wild-type mice ($F_{3,49} = 57.6$; $p < 0.05$; one-way ANOVA).

Regarding METH reward, METH-induced CPP was significantly attenuated in MMP-2-(–/–) and MMP-9-(–/–) mice compared with wild-type mice (Fig. 4c, $F_{3,44} = 11.3$; $p < 0.05$; one-way ANOVA; Fig. 4d, $F_{3,46} = 5.45$; $p < 0.05$; one-way ANOVA). There was no difference in place preference between saline-treated wild-type mice and mutant mice.

Consistent with behavioural changes, METH-induced increase in extracellular dopamine levels in the NAc was markedly reduced in MMP-2-(–/–), MMP-2-(–/–) and MMP-9-(–/–) mice compared with wild-type mice (Fig. 4e, $F_{2,27} = 4.99$; $p < 0.05$; repeated ANOVA, and $p < 0.05$ by post-hoc test; Fig. 4f, $F_{1,11} = 8.45$; $p < 0.05$; repeated ANOVA, and $p < 0.05$ by post-hoc test. The gene dose-related changes in METH-induced increase in extracellular dopamine levels were evident among wild-type, MMP-2-(+/–) and MMP-2-(–/–) mice (Fig. 4e, $p < 0.05$).

Effect of MMP-2 infusion into the NAc on METH-induced dopamine release

We also investigated the effect of microinjection of MMP-2 into the NAc on the METH-induced increase in the extracellular dopamine levels in rats. Acute METH treatment increased the peak extracellular dopamine levels to approximately 500–600% of the basal levels in the NAc (Fig. 5). Infusion of purified human MMP-2 protein at a dose of 0.01 μg into the NAc had no effect on basal dopamine levels, but it significantly potentiated METH-induced increase in the extracellular dopamine levels compared with the response in vehicle-treated control group 40–60 min after acute METH treatment (Fig. 5, $p < 0.05$ by t-test). These results suggest that an increase in MMP-2 activity in the NAc leads to the potentiation of METH-induced dopamine release.

Dopamine transporter function in MMP-2-(–/–) and MMP-9-(–/–) mice

Finally, we examined the role of endogenous MMP on [^{3}H]dopamine uptake into a crude striatal synaptosome preparation. There was no difference in [^{3}H]dopamine uptake between wild-type mice and MMP-2-(–/–) or MMP-9-(–/–) mice, suggesting no changes in [^{3}H]dopamine uptake activity under normal conditions. The [^{3}H]dopamine uptake was markedly decreased in wild-type mice 1 h after the last administration of repeated METH treatment on day 7 (Figs 6a and b). In MMP-2-(–/–) mice, no changes in [^{3}H]dopamine uptake was observed after repeated METH treatment, and thereby a significant difference in [^{3}H]dopamine uptake was evident between two groups of mice (Fig. 6a, $F_{3,25} = 5.33$; $p < 0.05$; one-way ANOVA). A similar changes in [^{3}H]dopamine uptake was observed in MMP-9-(–/–) mice after METH treatment (Fig. 6b, $F_{3,19} = 21.8$; $p < 0.05$; one-way ANOVA). These results suggest that both mutant mice exhibit the resistance to the inhibitory effect of METH on dopamine transport activity, which may be associated with the impairment in METH-induced sensitization and CPP, as well as METH-increased dopamine release in MMP-2-(–/–) and MMP-9-(–/–) mice.

Discussion

Repeated treatment with psychostimulants such as cocaine and amphetamine produces changes in neural morphology and synaptic connectivity in the mesolimbic neuronal system (Nestler 2001). It has been hypothesized that alterations in synaptic connectivity in these structures might be involved in the long-lasting behavioural consequences of repeated treatment with drugs of abuse such as amphetamine psychosis and dependence, and dysphoria during drug withdrawal. Accordingly, various molecules that regulate synaptic structure and connectivity may play a crucial role in METH dependence. In this study, we demonstrated for the first time

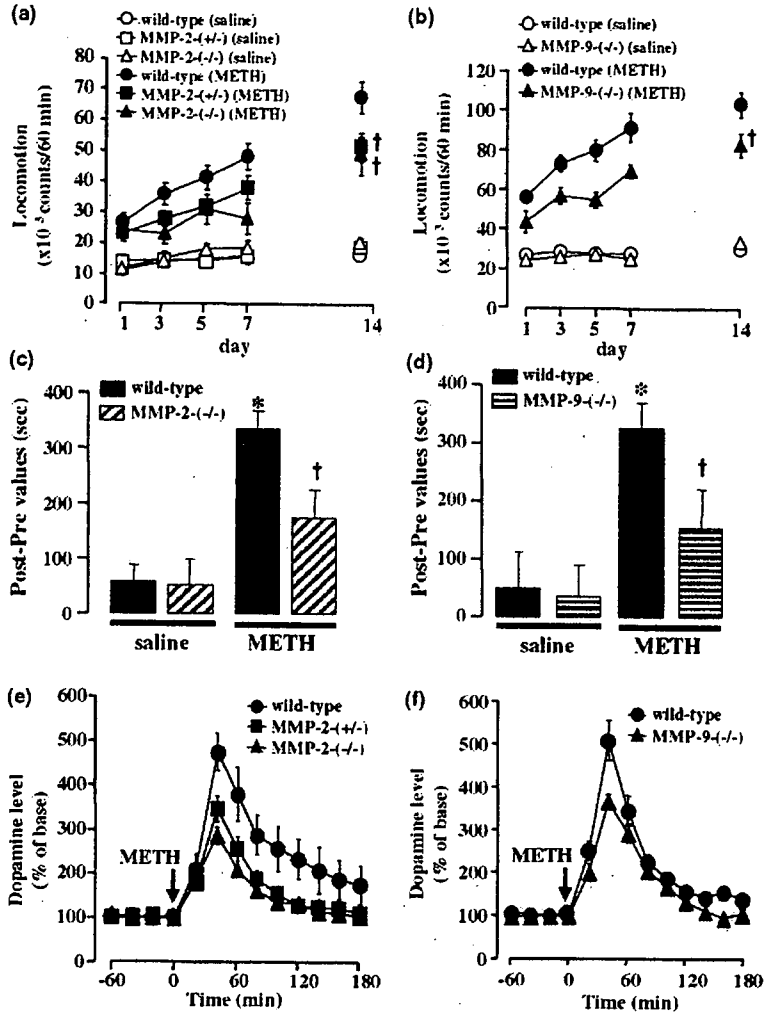

Fig. 4 Sensitization of METH-induced (a, b) hyperlocomotion, (c, d) reward and (e, f) dopamine release in the NAc in (a, c, e) MMP-2(–/–), (a, e) MMP-2(+/–) and (b, d, f) MMP-9(–/–). (a, b): Mice were given saline or METH at a dose of 1 mg/kg for 7 days and, after withdrawal for 7 days, they were given saline or METH (1 mg/kg) on day 14. Repeated-measures ANOVA revealed a significant difference in repeated METH-induced sensitization of MMP-2(+/–), MMP-2(–/–) and MMP-9(–/–) compared with wild-type mice. Values are the means ± SE (a, $n = 10$–14; b, $n = 13$–14). †$p < 0.05$ versus wild-type (METH). (c, d): Mice were trained for CPP to METH (1 mg/kg) to examine the METH reward in the MMP-2-(–/–) and MMP-9-(–/–) mice. Values are the means ± SE (c, $n = 12$; d, $n = 12$–13). *$p < 0.05$ versus wild-type (saline). †$p < 0.05$ versus wild-type (METH). (e, f): Mice were given METH at a dose of 1 mg/kg for 7 days. On day 7, extracellular dopamine release in the NAc was measured for 3 h after the administration of METH. Basal extracellular dopamine levels were 0.41 ± 0.06 nM for the wild-type, 0.50 ± 0.11 nM for MMP-2-(+/–) and 0.30 ± 0.06 nM for MMP-2(–/–). Basal extracellular dopamine levels were 0.42 ± 0.08 nM for the wild-type, 0.28 ± 0.03 nM for MMP-9(–/–). Values are the means ± SE (e, $n = 6$–8; f, $n = 6$–7). Repeated-measures ANOVA revealed a significant difference in METH-increased dopamine release in the NAc of MMP-2-(+/–), MMP-2-(–/–) and MMP-9-(–/–) mice compared with wild-type mice (Fig. 4e, $F_{2,27} = 4.99$; repeated ANOVA, and $p < 0.05$ by post-hoc test; Fig. 4f, $F_{1,11} = 8.45$; repeated ANOVA, and $p < 0.05$ by post-hoc test.

that repeated administration of METH led to behavioural sensitization which was accompanied by the induction of MMP-2 and MMP-9 expression in the Fc and NAc. No active-MMP-2 was detected, however, in METH-treated rats by gel zymography, indicating that repeated METH treatment increased the expression of pro-MMP-2. Although active-MMP-2 was not detected in the gel zymography, it is possible that the activity of MMP-2 may be affected at some point by repeated METH treatment.

Our data showed that MMP-2 and MMP-9 were expressed in neurons as well as glial cells in the Fc. Some previous studies suggested that the MMP/TIMP system is expressed by adult CNS neurons and glial cells, and the expression level and cellular localization may be regulated according to

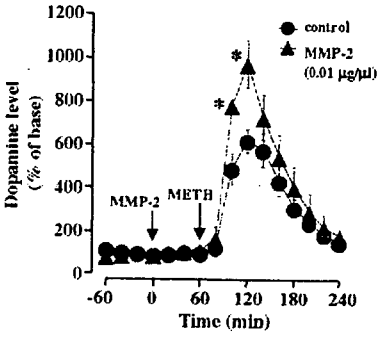

Fig. 5 Effect of MMP-2 on the METH-induced increase in extracelluar dopamine levels in the NAc of rats. Purified human MMP-2 (0.01 μg) was microinjected into the NAc in a volume of 1.0 μL 1 h before METH (2 mg/kg) treatment. Basal extracellular dopamine levels were 0.21 ± 0.07 nM for the control and 0.29 ± 0.11 nM for the MMP-2-infused group. Values are the means ± SE (n = 4–5). *p < 0.05 versus vehicle-treated control group (p < 0.05 by t-test).

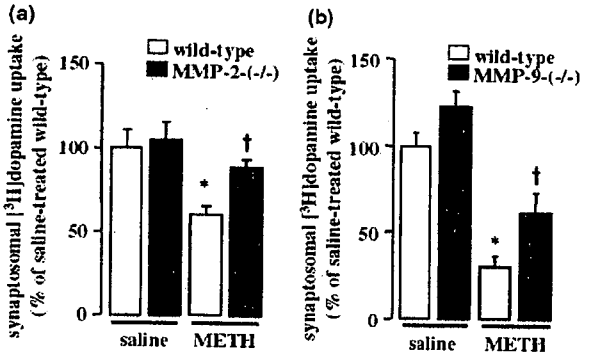

Fig. 6 Changes in METH-induced reduction of the activity of synaptosomal dopamine uptake in (a) MMP-2-(–/–) and (b) MMP-9-(–/–) mice. Mice were given saline or METH at a dose of 1 mg/kg for 7 days, and were killed 1 h after the final administration. (a) The [^{3}H]dopamine uptake activity in the saline-treated wild-type mice was 2.66 ± 0.2 pmol/μg protein/4 min. (b) The [^{3}H]dopamine uptake in the saline-treated wild-type mice was 1.34 ± 0.1 pmol/μg protein/4 min. Values are means ± SE (a, n = 7–8; b, n = 5–6). *p < 0.05 versus saline-treated wild-type mice. †p < 0.05 versus METH-treated wild-type mice.

the developmental and/or functional status of the brain (Vaillant *et al.* 1999; Szklarczyk *et al.* 2002). For instance, in the process of axonal extension, MMP is located at the growth cone tips, permitting attachment/detachment between the neurons and matrix substratum (Monard 1988), and oligodendrocytes use MMP to extend their processes (Uhm *et al.* 1998), suggesting that MMP regulation is likely to provide guidance during the proliferation of new synapses. Accordingly, it is plausible that the expression of MMP-2 and MMP-9 may take part in the structural and functional alterations in the brain following repeated exposure to METH.

We also demonstrated that METH-induced behavioural sensitization and CPP were markedly attenuated in MMP-2-(–/–) and MMP-9-(–/–) mice compared with those in wild-type, suggesting that METH-induced expression of the MMP in the brain plays a role in the development of METH-induced sensitization and CPP, which may be associated with neuronal plasticity and remodelling. Indeed, the inhibition of MMP alters functional and structural correlations of deafferentation-induced sprouting, such as remodelling in the dentate gyrus of the hippocampus (Reeves *et al.* 2003). In a behavioural study, MMP-9 knockout mice display impairments in long-term potentiation and hippocampal-dependent memory in a fear-conditioning memory task (Nagy *et al.* 2006). As learning/memory mechanisms are considered to overlap with and are involved in the development of drug dependence that occurs with chronic administration of drugs of abuse (Berke and Hyman 2000; Mizoguchi *et al.* 2005), MMP-2 and MMP-9 expression may play a crucial role in the acquisition of METH-induced CPP.

Behavioural changes induced by METH are linked to its capacity to elevate extracellular dopamine levels through the redistribution of dopamine from synaptic vesicles to the cytosol, promotion of reverse transport (Sulzer *et al.* 1995; Nakajima *et al.* 2004) and the internalization of dopamine transporter (DAT; Zahniser and Sorkin 2004; Cervinski *et al.* 2005). The microinjection of purified human MMP-2 directly into the NAc significantly potentiated the acute METH-induced increase in extracellular dopamine levels in NAc, without affecting basal dopamine levels. Moreover, in MMP mutant mice [MMP-2-(+/–), MMP-2-(–/–) and MMP-9-(–/–)], METH-induced dopamine release in the NAc was significantly decreased compared with the response in wild-type mice. These findings demonstrate a previously undescribed function of the MMP in the regulation of dopamine release in the NAc.

It is unlikely that changes in repeated METH-induced behavioural sensitization and dopamine release in the MMP-2-(–/–) and MMP-9-(–/–) mice are as a result of the alteration of basal DAT activity as there were no differences in basal [^{3}H]dopamine uptake *in vitro* between wild-type and MMP mutant mice. Furthermore, we observed no changes in dopamine D_1 agonist SKF-81297 or D_2 agonist quinpirole-stimulated [^{35}S]GTPγS binding between wild-type and MMP-2-(–/–) or MMP-9-(–/–) mice (data not shown), indicating no changes in dopamine D_1 and D_2 receptor activity in MMP-2-(–/–) and MMP-9-(–/–) mice. However, we cannot rule out a possibility that the observed behavioural and neurochemical changes induced by METH in the MMP-2-(–/–) and MMP-9-(–/–) mice may be as a result of the consequence of developmental compensation in the mutant mice.

The molecular mechanisms by which the MMP activation regulates METH-induced increase in extracelluar dopamine levels remain to be elucidated. It has been demonstrated that reverse activation and internalization of plasmalemmal DAT is involved in the METH-induced increase in extracellular dopamine levels (Sulzer *et al.* 1995; Khoshbouei *et al.* 2004). The degradation of ECM and other substrates by MMP may result in conformational and/or functional changes in plasma membrane proteins (Bixby *et al.* 1994; Sunderland *et al.* 2000; Kaczmarek *et al.* 2002). Recently, it was reported that association of membrane type 5-MMP with AMPA receptor binding protein can influence axon pathfinding or synapse remodelling through proteolysis of cadherins in neuron (Monea *et al.* 2006). Consistently, we demonstrated that [^{3}H]dopamine uptake into striatal synaptosomes was reduced in wild-type mice after repeated METH treatment, but METH-induced changes in [^{3}H]dopamine uptake were significantly attenuated in MMP-2-(–/–) and MMP-9-(–/–) mice (Fig. 6). These results suggest that the MMP-2 and MMP-9 may regulate the METH-induced increase in the extracellular dopamine levels by modulating DAT activity through the degradation of ECM. Alternatively, MMPs are reported to activate neurotrophic factors such as insulin-like growth factor-1 (Fowlkes *et al.* 1995) and fibroblast growth factor (Levi *et al.* 1996). Thus, it is possible that MMP-2 and MMP-9 may play a role in development of METH dependence by modulating the activity of these neurotrophic factors.

In conclusion, we have demonstrated for the first time that repeated but not acute METH treatment induces increased MMP-2 and MMP-9 expression in neurons and/or glial cells in the Fc and NAc. The net increase in MMP activity may be responsible at least in part for the development of METH-induced behavioural plasticity. Additionally, it is noticed that while the effects shown in the Fc and NAc are likely to represent METH-induced changes in MMP activity that are specific to reward areas, these changes might also be found in other brain regions, such as hippocampus and striatum, that are not specifically associated with METH reward, indicating the possibility that MMP changes are not specificity of the response to METH reward circuit. Further work would be required to completely resolve this important issue. Consistently, deletion of the MMP-2 or MMP-9 gene resulted in the attenuation of METH-induced behavioural sensitization and CPP in mice. We also provided pharmacological and genetic evidence that MMP-2 and MMP-9 are involved in the regulation of METH-induced changes in dopamine release and uptake in the NAc. These results, together with the well-known function of the MMPs to degrade ECM, suggest that repeated METH-induced overexpression of MMP-2 and MMP-9 plays a crucial role in functional and possibly structural changes in the Fc and NAc, which are related to METH-induced behavioural sensitization and reward.

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Role of matrix metalloproteinase and tissue inhibitor of MMP in methamphetamine-induced behavioral sensitization and reward: implications for dopamine receptor down-regulation and dopamine release

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Abstract

Matrix metalloproteinases (MMPs) and its inhibitors (TIMPs) function to remodel the pericellular environment. We have demonstrated that methamphetamine (METH)-induced behavioral sensitization and reward were markedly attenuated in MMP-2- and MMP-9 deficient [MMP-2-(–/–) and MMP-9-(–/–)] mice compared with those in wild-type mice, suggesting that METH-induced expression of MMP-2 and MMP-9 in the brain plays a role in the development of METH-induced sensitization and reward. In the present study, we investigated the changes in TIMP-2 expression in the brain after repeated METH treatment. Furthermore, we studied a role of MMP/TIMP system in METH-induced behavioral changes and dopamine neurotransmission. Repeated METH treatment induced behavioral sensitization, which was accompanied by an increase in TIMP-2 expression. Antisense TIMP-2 oligonucleotide (TIMP-AS) treatment enhanced the sensitization, which was associated with the potentiation of METH-induced dopamine release in the nucleus accumbens (NAc). On the other hand, MMP-2/-9 inhibitors blocked the METH-induced behavioral sensitization and conditioned place preference, a measure of the rewarding effect, and reduced the METH-increased dopamine release in the NAc. Dopamine receptor agonist-stimulated [^{35}S]GTPγS binding was reduced in the frontal cortex of sensitized rats. TIMP-AS treatment potentiated, while MMP-2/-9 inhibitor attenuated, the reduction of dopamine D2 receptor agonist-stimulated [^{35}S]GTPγS binding. Repeated METH treatment also reduced dopamine D2 receptor agonist-stimulated [^{35}S]GTPγS binding in wild-type mice, but such changes were significantly attenuated in MMP-2-(–/–) and MMP-9-(–/–) mice. These results suggest that the MMP/TIMP system is involved in METH-induced behavioral sensitization and reward, by regulating dopamine release and receptor signaling.

Keywords: dopamine, dopamine receptor, matrix metalloproteinase, methamphetamine, tissue inhibitor of matrix metalloproteinase.

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Abbreviations used: CPP, conditioned place preference; ECM, extracellular matrix; Fc, frontal cortex; GDP, guanosine-5′-diphosphate; METH, methamphetamine; MMP, matrix metalloproteinases; NAc, nucleus accumbens; TIMPs, tissue inhibitors of metalloproteinase.

Drug dependence is a complex phenomenon with important psychological and social causes and consequences, which may be associated with neural plasticity and remodeling of specific brain circuits caused by repeated exposure to drugs of abuse (Shippenberg and Heidbreder 1995; Berke and Hyman 2000; Yamada *et al.* 2005). Methamphetamine (METH), a common drug of abuse, has both acute and long-lasting effects on psychomotor behaviors. Although the involvement of various endogenous substances such as TNF-α (Nakajima *et al.* 2004), tissue plasminogen activator (tPA) (Nagai *et al.* 2004, 2005) and brain-derived neurotrophic factor (Nestler 2001), which are known to play a role in synaptic plasticity, has been implicated in the development of METH-induced behavioral sensitization and reward (Shippenberg and Heidbreder 1995; Robinson and Kolb 1997; Mizoguchi *et al.* 2004), the mechanism underlying psychostimulant-induced remodeling of synaptic structures remains to be determined.

Tissue inhibitors of metalloproteinase (TIMPs) belong to a family of multifunctional secreted proteins (TIMP-1–4) that regulate the proteolytic activity of matrix metalloproteinases (MMPs), and possess growth-promoting and cell cycle-regulating activities in various cell types (Mannello and Gazzanelli 2001). MMP and TIMP function to modulate functional and structural remodeling of cellular architecture in the context of pathophysiology primarily through the cleavage of extracellular matrix (ECM) proteins, bioavailability of growth factors and cytokines, and shedding of membrane receptors (Sternlicht and Werb 2001; Yong *et al.* 2001). It has been recently demonstrated that neuronal TIMP-1 (Rivera *et al.* 1997) and MMP-9 (Szklarczyk *et al.* 2002) are regulated by synaptic activity, suggesting that the balance between MMPs and TIMPs is important in the activity-dependent re-organization of the neuronal architecture with possible effects on synaptic physiology. Thus, the MMP/TIMP ratio and net MMP activity may be involved in brain development, because extensive cellular migration and remodeling of the ECM are necessary for neural development (Vaillant *et al.* 1999; Wright *et al.* 2002).

We have recently demonstrated that repeated but not acute METH treatment increases the expression of both MMP-2 and MMP-9 in neurons and/or glial cells in the frontal cortex (Fc) and nucleus accumbens (NAc), and that deletion of the MMP-2 [MMP-2-(−/−) mice] or MMP-9 gene [MMP-9-(−/−) mice] resulted in the attenuation of METH-induced sensitization and reward in mice. We also provided genetic evidence that the MMP expression is involved in the regulation of METH-induced changes in dopamine release and uptake in the NAc. Based on these findings, we have proposed that MMP-2 and MMP-9 are involved in METH-induced synaptic plasticity and related behavioral changes (Mizoguchi *et al.* 2007).

In the present study, we further investigated whether the MMP/TIMP system is involved in METH-induced behavioral sensitization and reward in rat. In this study, we show that TIMP-2 expression is induced by repeated METH treatment in the brain, and that the MMP/TIMP system is involved in the development of METH-induced behavioral sensitization and reward. Inhibition of TIMP-2 function enhanced whereas MMP inhibitors reduced the METH-induced dopamine release and the down-regulation of dopamine receptor agonist-stimulated [^{35}S]GTPγS binding.

Materials and methods

Animals

Male Wistar rats (8-weeks old; Charles River Japan, Yokohama, Japan) weighing 270 ± 20 g at the beginning of experiments were used in the study. We also used MMP-2 homozygous knock-out [MMP-2-(−/−)] and their wild-type (C57BL/6 J) mice (10–12 weeks old). The mutant mice were backcrossed into the C57BL/6 J strain more than thirteen times, and the generation, genotyping, and characterization of MMP-2-(−/−) mice have been described (Itoh *et al.* 1997). MMP-9 homozygous knock-out [MMP-9-(−/−)] mice (10–12-weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA), and crossed to FVB/N mice for five generations before being made homozygous. Wild-type FVB/N [MMP-9-(+/+)] mice were obtained from the CLEA Japan (Fuji, Japan).

All experiments were performed in accordance with the 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 United States National Institutes of Health Guide for the Care and Use of Laboratory Animals.

METH treatment for behavioral sensitization

Rats were given saline or METH at a dose of 2 mg/kg (s.c.) for 5 days (n = 7–8) (Nakajima *et al.* 2004; Nagai *et al.* 2005; Mizoguchi *et al.* 2007). Rats were placed in their home cage for 1 h following injection and then locomotor activity was measured in an acrylic chamber (25 × 42 × 20 cm) for 1 h using behavioral analysis systems (SCANET SV-10: Neuroscience, Tokyo, Japan) on day 1, 3 and 5.

Conditioned place preference

The apparatus used for the place conditioning task consisted of two compartments: a black Plexiglas box and a transparent Plexiglas box (both 27 × 22 × 26 cm high) with a metal grid floor. To enable the rat to distinguish easily the transparent box from the black one, the floors of the transparent and black boxes were covered with white plastic mesh and black frosting Plexiglas, respectively. Each box could be divided by a sliding door (10 × 26 cm high). The place conditioning paradigm was performed with a minor modification (Nagai *et al.* 2004; Nakajima *et al.* 2004). In the pre-conditioning test, the sliding door was opened and the rat was allowed to move freely between both boxes for 15 min once a day for 3 days. On the third day of the pre-conditioning test, we measured pre-value by using a Scanet SV-10 LD (Tokyo Sangyo Co Ltd, Toyama, Japan). On days 4, 6, and 8, a rat was given METH at 2 mg/kg and put in its

non-preferred side for 30 min. On days 5, 7, and 9, the rat was given saline and placed opposite the drug-conditioning side. In the post-conditioning test, the sliding door was opened, and we measured post-value. Place conditioning behaviors were expressed as [(post-conditioning preference)-(pre-conditioning preference)].

TIMP-2 antisense oligonucleotide treatment

Rats were anesthetized with pentobarbital (40 mg/kg, i.p.) and placed in a stereotaxic apparatus. The infusion cannula was connected to a miniosmotic pump (total capacity: 100 μL, Alzet 1007D, Alza, Palo Alto, CA, USA), which was filled with TIMP-2 antisense (TIMP-AS) or scramble oligonucleotides (TIMP-SC), and was implanted into the right ventricle [anteroposterior (AP) -0.8, mediolateral (ML) +1.5 from bregma, dorsoventral (DV) -4.0 from the skull, according to the atlas of Paxinos and Watson (1982)]. Phosphothioate oligonucleotides were custom-synthesized at Nisshinbo Biotechnology (Tokyo, Japan) and dissolved in artificial cerebrospinal fluid (aCSF; 147 mmol/L NaCl, 3 mmol/L KCl, 1.2 mmol/L $CaCl_2$, and 1.0 mmol/L $MgCl_2$, pH 7.2). The sequences of TIMP-AS and TIMP-SC were 5′-CC GCGGCGCCCAT-3′ and 5′-AGCGCGCGCCGTGCC-3′, respectively. The TIMP-AS and TIMP-SC were continuously infused into the cerebral ventricle at a dose of 3.6 nmol/day. The infusion of the oligonucleotide was maintained for 5 days (flow rate, 0.5 μL/h). One day after the start of oligonucleotide infusion, the rats (n = 7–11) were subjected to METH treatment for sensitization.

MMP inhibitor treatment

Doxycycline is a second-generation tetracycline antibiotic with an inhibitory effect on MMP (Uitto *et al.* 1994; Smith *et al.* 1996). Doxycycline at 10–100 μg/mL inhibits MMP-9 activity in brain tissue *in vitro* study (Hashimoto *et al.* 2005). MMP-2/-9 inhibitor III is a potent inhibitor of MMP-2 (IC_{50}=10 μmol/L) according to the manufacturer (Koivunen *et al.* 1999).

Rats were anesthetized with pentobarbital and placed in a stereotaxic apparatus. The infusion cannula was connected to a miniosmotic pump (total capacity: 200 μL, Alzet 2002; Alza, Palo Alto, CA, USA), which was filled with doxycycline (n = 18) (60 μg/12 μL/day) or MMP-2/-9 inhibitor III (n = 18) (2.4 μg/12 μL/day, Merck kGaA, Darmstadt, Germany), and was implanted into the right ventricle (AP −0.8, ML +1.5 from bregma, DV −4 from the skull) for CPP. MMP-2/-9 inhibitor III was dissolved in 2% DMSO in saline. Alternatively, the infusion cannula was connected to a miniosmotic pump (total capacity: 100 μL, Alzet 1007D), which was filled with aCSF (n = 10) or doxycycline (n = 10) (0.06 μg/12 μL/day), and was bilaterally implanted into the frontal cortex (AP +2.5, ML ± 0.6 from bregma, DV -3.0 from the skull). One day after the start of doxycycline infusion, the rats were subjected to METH treatment for sensitization.

Quantitative analysis of TIMP-2 mRNA by real-time PCR

After a final administration of METH, rats were intracardially perfused with cold saline and then decapitated as described (n = 4–10) (Asahi *et al.* 2001; Lee *et al.* 2004). Various regions of the brain including the Fc, NAc, striatum (St) and ventral tegmental area (VTA), were dissected from freshly perfused brains and immediately frozen and stored at −80°C until assayed. All dissection was made using brain matrix (NeuroScience Idea, Osaka, Japan) and based on the atlas of Paxinos and Watson (1982).

The sequences of forward and reverse primers for the rat TIMP-2 were 5′-GCAAGATGCACATTACCCTCTGT-3′ and 5′-CCATCT GGTACCTGTGGTTTAGG-3′, respectively, giving rise to a 92-bp PCR product. cDNA was synthesized from total RNA by reverse transcriptase using a superscript II kit. The cDNA was synthesized from 1 μg of total RNA for TIMP-2. For standardization of the quantitation, glyceraldehde-3-phosphate dehydrogenase was amplified simultaneously. The change in reporter fluorescence from each reaction tube was monitored with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

Gel zymography

Sample preparation was made as described previously (n = 4) (Zhang and Gottschall 1997; Mizoguchi *et al.* 2007). Briefly, brain tissues were homogenized in lysis buffer [50 mmol/L Tris–HCl, 150 mmol/L NaCl, 5 mmol/L $CaCl_2$, 0.05% Brij35, and 0.02% NaN_3, pH 7.6] with 1% TritonX-100 and centrifuged at 12 000 g for 10 min to pellet insoluble material. The protein concentration in the supernatant was determined using a Protein Assay Rapid Kit (Bio-Rad, Osaka, Japan). The supernatant was incubated with gelatin-sepharose 4B (GE Healthcare Bio-Science Corp., Piscataway, NJ, USA) that had previously washed three times with the lysis buffer, with constant shaking, for 24 h at 4°C. After centrifugation at 500 g for 2 min, the pellet was resuspended in 500 μL of the lysis buffer and washed three times. The pellet was resuspended in 150 μL of lysis buffer containing 10% dimethyl sulfoxide and shaken for 2 h, and used for assaying gelatinase activity of MMP-2 and MMP-9.

The samples were subjected to electrophoresis in 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.1% gelatin under non-reducing conditions. Gels were washed twice for 30 min in 2.5% Triton X-100 to remove SDS, washed for 30 min in incubation buffer [50 mmol/L Tris–HCl, 5 mmol/L $CaCl_2$, 2 μmol/L $ZnCl_2$, 200 mmol/L NaCl, and 0.02% Briji 35, pH 7.4] at 25 ± 2°C, and further incubated for 24 h in the same buffer at 37°C. Gels were then stained for 3 h in Coomassie blue (1% Coomassie brilliant blue G-250, 30% methanol, 10% acetic acid) and destained in 40% methanol/7% acetic acid until clear bands of gelatinolysis appeared on a dark background. Total activity including pro-MMP was analyzed with the ATTO Densitograph Software Library Lane Analyzer (Atto Instruments, Tokyo, Japan).

Western blotting

Brain tissues were homogenized in lysis buffer [20 mmol/L Tris–HCl, 100 mmol/L NaCl, 1 mmol/L $CaCl_2$, and 0.005% Brij35, p H7.4], and microwaved for 15 s according to the protocol for immunoblotting with monoclonal antibodies (n = 5). The homogenate was centrifuged at 13 000 g for 30 min to pellet insoluble material. The protein concentration in the supernatant was determined using a Protein Assay Rapid Kit (Bio-Rad Laboratories, Hercules, CA, USA). The sample was boiled in 2× sample buffer [0.25% bromophenol blue/12% 2-mercaptothanol/20% glycerol/4% SDS/0.1 mol/L Tris–HCl, pH 6.8] and electrophoresed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 4.75% stacking gel and 10 or 13% separating gel, and then transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The same concentration (20–40 μg) of protein per lane was loaded for all western blotting. The band intensities of the film were analyzed by densitometry. The amount of TIMP-2 was calculated versus the

amount of β-actin protein. The primary polyclonal rabbit or goat antibodies used in the present study were anti-TIMP-2 (1 : 1000; Chemicon, Temecula, CA, USA) and β-actin (1 : 2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibodies, used at a 1 : 2000 or 1 : 5000 dilution, were horseradish peroxidase-linked anti-rabbit or anti-goat IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA). Immunoreactive materials on the membrane were detected using ECL western blotting detection reagents (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) and exposed to X-ray film. The band intensities of the film were analyzed by densitometry.

Double immunostaining

Polyclonal rabbit anti-TIMP-2 antibody (1 : 250; Chemicon), monoclonal mouse anti-neuron-specific nuclear antigen (NeuN) antibody (1 : 200; Chemicon) and anti-glial fibrillary acidic protein (GFAP) antibody (1 : 200; Chemicon) served as primary antibodies. Affinity-purified FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG served as secondary antibodies. Samples were observed with AXIOVISION 3.0 systems (Carl Zeiss, Jene, Germany).

In vivo microdialysis

Rats were anesthetized with sodium pentobarbital before stereotaxic implantation of a guide cannula into the left Fc (AP +2.5, ML +0.8 from the bregma, DV -2 from the skull) or NAc (AP +1.6, ML +1.0 from the bregma, DV -7 from the skull). When the infusion cannula for doxycycline treatment was bilaterally implanted into the Fc (n = 7), a guide cannula was implanted into the NAc (20° from AP +1.6, ML +1.5 from the bregma, and DV −7.5 from the skull). One day after the surgery, a dialysis probe (AI-4–2 or AI-8–1; 1- or 2-membrane length, EICOM, Kyoto, Japan) was inserted though a guide cannula and perfused with aCSF (147 mmol/L NaCl/ 4 mmol/L KCl/ 2.3 mmol/L $CaCl_2$) at a flow rate of 1.2 μL/min. Outflow fractions were collected every 20 min. After the collection of three baseline fractions, rats were treated with METH (2 mg/kg, s.c.). Dopamine levels in the dialysates were analyzed as described (Nagai *et al.* 2004).

[^{35}S] GTPγS binding assay

For the membrane preparation, rats were repeatedly treated with METH at 2 mg/kg for 5 days. The TIMP-AS- (n = 8–11) and doxycycline- (n = 5–7) infused rats were treated with METH at 2 mg/kg for 3 days and 5 days, respectively. Wild-type mice, MMP-2-(−/−) mice (n = 6–7) and MMP-9-(−/−) mice (n = 5) were treated with METH at a dose of 1 mg/kg for 7 days. The Fc was dissected out and immediately frozen and stored at −80°C until assayed. All dissection was made using brain matrix and based on the atlas of Paxinos and Watson (1982) for rat and Frankin and Paxinos (1997) for mouse.

The animals were killed 2 h after the last treatment, and the Fc was homogenized in 20 volumes (w/v) of buffer containing 50 mmol/L Tris–HCl, 5 mmol/L $MgCl_2$ and 1 mmol/L EGTA, pH 7.4. The homogenate was centrifuged at 48 000 g for 10 min at 4°C and the pellet was resuspended in the assay buffer containing 50 mmol/L Tris–HCl, 5 mmol/L $MgCl_2$, 1 mmol/L EGTA, and 100 mmol/L NaCl (pH 7.4). The [^{35}S]GTPγS binding assay was performed according to the procedures described by Bowers *et al.* (2004) with a minor modification. Briefly, the membrane homogenate (10 μg protein) was incubated at 25°C for 2 h in 1 mL of the assay buffer with various concentrations of the agonists, 0.5 U of adenosine deaminase, 20 μmol/L guanosine-5′-diphosphate (GDP) and 100 pmol/L [^{35}S] GTPγS (Amersham Biosciences, Arlington Heights, IL, USA). The reaction was terminated by rapid filtration using Whatman GF/B glass filters. The filters were washed three times and then transferred to scintillation counting vials containing tissue solubilizer (Soluene-350, Perkin-Elmer Life Sciences, Japan) and scintillation cocktail (Hionic Fluor, Perkin-Elmer Life Sciences, Japan). The radioactivity in the samples was determined with a liquid scintillation analyzer. Non-specific binding was measured in the presence of 10 μmol/L of unlabeled GTPγS. The data are expressed as the percentage of basal [^{35}S]GTPγS binding measured in the presence of GDP and absence of agonist.

To investigate the effect of *in vitro* treatment with exogenous MMP-2 and TIMP-2 on [^{35}S]GTPγS binding, brain homogenates (100 μg) were incubated with vehicle (5 mmol/L Tris–HCl, 0.1 mmol/L $CaCl_2$, and 0.005% Brij35, pH 7.5) or purified human MMP-2 (Chemicon) at a dose of 0.4 μg in the activation buffer (50 mmol/L Tris–HCl, 100 mmol/L NaCl, 10 mmol/L $CaCl_2$, 0.05% Brij35 and 0.02% NaN_3, pH 7.4) in the presence or absence of 1 mmol/L EGTA at 37°C for 60 min (n = 5–7). Alternatively, brain homogenates (100 μg protein) were incubated with vehicle (PBS buffer) or recombinant human TIMP-2 protein (Daiichi FineChem., Takaoka, Japan) at a dose of 1 μg in the assay buffer without EGTA at 4°C for 120 min (n = 5–7). The sample was centrifuged at 48 000 g at 4°C for 10 min and the pellet was resuspended in the assay buffer with 1 mmol/L EGTA for [^{35}S]GTPγS binding.

Statistical analyses

Results were expressed as the means ± SE. The significance of differences was determined by one-way ANOVA, followed by the Student–Newman–Keuls test or by repeated ANOVA, followed by Sheffe's test for multi-group comparisons. Student's t-test was used for two-group comparison.

Results

METH increases TIMP-2 expression in the brain

In a DNA microarray screening, we have found that repeated METH treatment induces the gene expression of extracellular proteinase-related proteins, such as tissue plasminogen activator and TIMP-2 in the rat brain (Yamada *et al.* 2005). Additionally, repeated but not acute METH treatment induces MMP-2 and MMP-9 expression in neurons and glial cells in the Fc and NAc (Mizoguchi *et al.* 2007). Therefore, to further clarify the MMP/TIMP system on METH-induced behavioral sensitization and dependence, we examined whether single and repeated METH treatment altered the expression of TIMP-2 mRNA in the brain of rats, using a real-time reverse transcription-PCR method. Acute METH treatment at a dose of 2 mg/kg did not change TIMP-2 gene expression in any regions examined. When METH treatment was repeated for 5 days, METH-induced hyperactivity was significantly potentiated (sensitization, Fig. 1a),

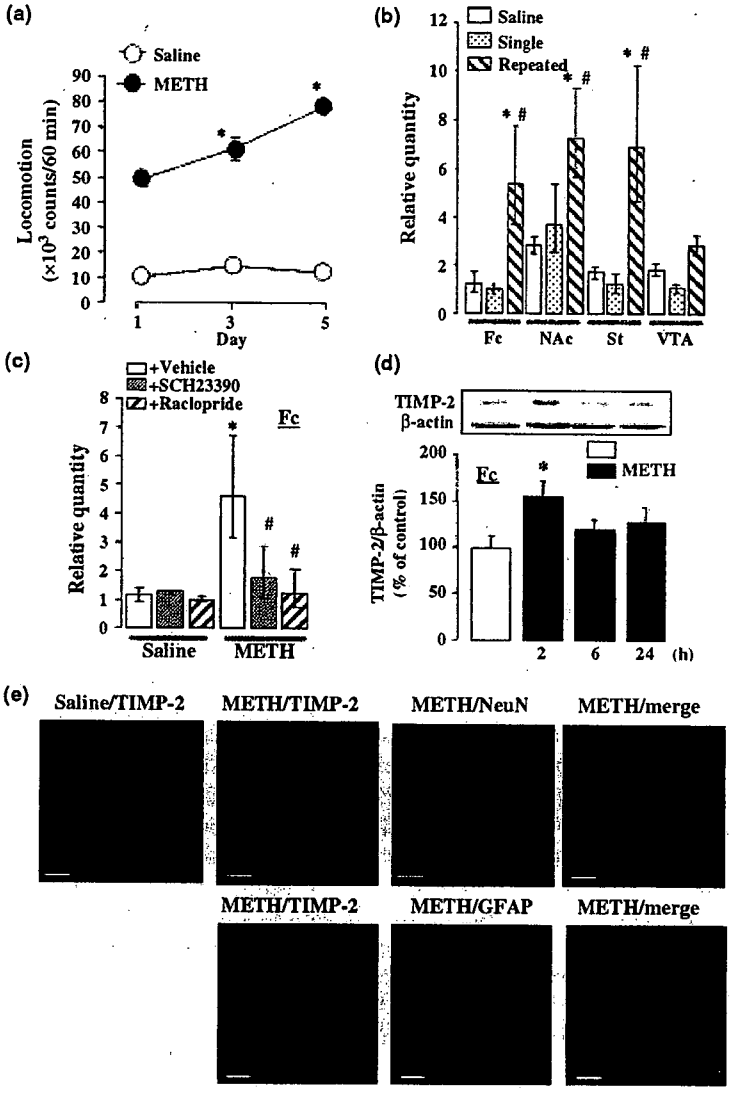

Fig. 1 METH-induced behavioral sensitization in rats (a): METH was administered at a dose of 2 mg/kg for 5 days. Values are means ± SE (n = 7–8). *p < 0.05 versus METH (day 1). Changes in the expression of TIMP-2 mRNA in the brain after METH treatment (b): rats were killed 2 h after a single or repeated METH treatment (2 mg/kg, s.c. for 5 days). Values are means ± SE (n = 5–10). *p < 0.05 versus saline, # p < 0.05 versus single METH treatment. The effects of the dopamine D1 receptor antagonist SCH23390 and D2 receptor antagonist raclopride on the METH-induced increase in TIMP-2 mRNA expression in the Fc (c): SCH23390 (0.1 mg/kg) or raclopride (2 mg/kg) was administered i.p 30 min before daily METH (2 mg/kg) treatment for 5 days. Values are means ± SE (n = 4–6 for C). *p < 0.05 versus saline+vehicle, #p < 0.05 versus METH+vehicle. Changes in the expression of TIMP-2 protein in the Fc after repeated METH treatment (d): Rats were given METH at a dose of 2 mg/kg for 5 days and killed 2, 6 or 24 h after the final administration. Control rats were given saline. *p < 0.05 versus control group. Values are means ± SE (n = 6–7). Double immunostaining for TIMP-2 and NeuN or GFAP reveals TIMP-2 expression in neurons of the Fc (e): rats were killed 2 h after repeated administration of METH (2 mg/kg, for 5 days). Scale bar: 20 μm.

and TIMP-2 mRNA levels were significantly elevated to 200–500% of the control level in the Fc ($F_{(2,17)}$ = 3.89, p < 0.05), NAc ($F_{(2,26)}$ = 5.96, p < 0.05) and St ($F_{(2,16)}$ = 4.25, p < 0.05), but not the VTA (Fig. 1b). Because it is well known that dopaminergic neuronal system is involved primarily in the pharmacological effects of METH, we examined whether the METH-induced increase in TIMP-2 mRNA levels is mediated by the activation of dopaminergic neurotransmission. The METH-induced increase in TIMP-2 mRNA expression was significantly and completely inhibited by pretreatment with either the dopamine D1 receptor antagonist SCH23390 ($F_{(3,16)}$ = 4.50, p < 0.05 in the Fc) or the dopamine D2 receptor antagonist raclopride ($F_{(3,37)}$ = 5.48, p < 0.05 in the Fc), suggesting that METH-induced gene expression of TIMP-2 in the Fc is attributable to dopamine D1 and D2 receptor activation (Fig. 1c). We also investigated TIMP-2 protein expression in the Fc by western blotting (Fig. 1d) and immunohistochemistry (Fig. 1e). Repeated METH treatment increased TIMP-2 protein expression transiently in the Fc (Fig. 1d, $F_{(3,23)}$ = 3.26, p < 0.05). In addition, to determine the cell types in which the expression of TIMP-2 is induced by repeated METH treatment, double immunostaining for TIMP-2 with NeuN, a neuronal marker, or GFAP, a glial marker, was performed. The findings indicated the co-localization of TIMP-2 immunoreactivity with NeuN, suggesting the neuronal localization of TIMP-2 in th Fc (Fig. 1e).

Role of TIMP-2 in METH-induced sensitization

To study the role of TIMP-2 in METH-induced behavioral sensitization, an antisense oligonucleotide strategy was used to inhibit TIMP-2 in the brain. TIMP-AS or TIMP-SC was continuously infused in the lateral ventricle. Acute METH

treatment induced hyperlocomotion and the METH-induced hyperlocomotion was augmented by repeated METH treatment in the aCSF-injected control group. Neither TIMP-AS nor TIMP-SC affected spontaneous locomotor activity and the acute METH-induced hyperlocomotion. However, TIMP-AS treatment accelerated the development of the sensitization, while TIMP-SC had no effect: TIMP-AS-treated animals showed a marked potentiation of METH-induced hyperlocomotion as early as the third day of the treatment (Fig. 2a, $F_{(5,46)} = 36.7$, $p < 0.05$ by one-way ANOVA). The increase in TIMP-2 protein levels evoked by repeated METH treatment

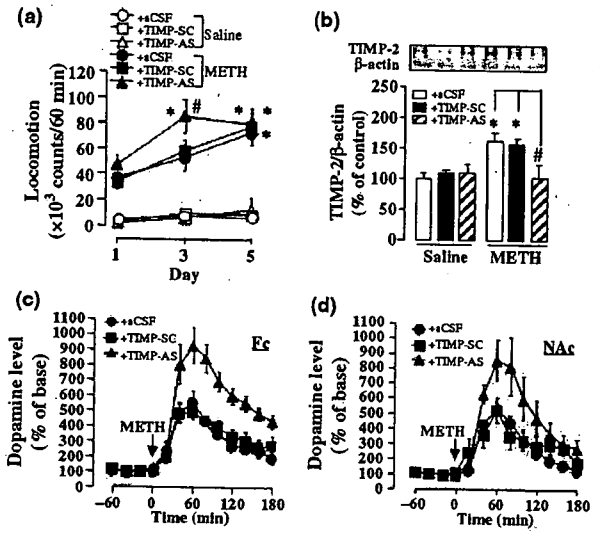

Fig. 2 Effects of TIMP-2 antisense oligonucleotide on repeated METH-induced behavioral sensitization (a), TIMP-2 protein expression in the Fc (b) and increased extracellular dopamine levels in Fc (c) and NAc (d). Rats were given saline or METH at a dose of 2 mg/kg for 5 days, and locomotor activity was measured on days 1, 3 and 5 (a): an osmotic minipump was used to deliver a continuous infusion of TIMP-AS (3.6 nmol/12 μL/day), TIMP-SC (3.6 nmol/12 μL/day) or aCSF into the right ventricle. Values are the means ± SE (n = 7–11). $^*p < 0.05$ versus day 1, $\#p < 0.05$ versus METH+aCSF, and METH+TIMP-SC. Rats were killed 2 h after saline or METH treatment on day 3 (b): values are the means ± SE (n = 5). $^*p < 0.05$ versus saline+aCSF, $\#p < 0.05$ versus METH+aCSF, and METH+TIMP-SC. Rats were given METH at a dose of 2 mg/kg for 3 days (c) and (d): On day 3, dopamine release was measured for 3 h after the administration of METH. Basal extracellular dopamine levels in Fc were 0.23 ± 0.03 nmol/L, 0.18 ± 0.04 nmol/L and 0.16 ± 0.04 nmol/L for aCSF-, TIMP-SC- and TIMP-AS-treated groups, respectively, and 0.44 ± 0.07 nmol/L, 0.44 ± 0.05 nmol/L and 0.62 ± 0.07 nmol/L in the NAc of aCSF-, TIMP-SC- and TIMP-AS-treated groups, respectively. Values are means ± SE (n = 5–7). In the TIMP-AS-treated group, the METH-induced increase in extracellular dopamine levels was significantly potentiated compared with the aCSF- or TIMP-SC-treated group in Fc and NAc ($F_{(2,13)}$=11.0; $p < 0.05$ by repeated ANOVA, and $p < 0.05$ by *post hoc*, $F_{(2,12)}$=4.13; $p < 0.05$ by repeated ANOVA, and $p < 0.05$ by *post hoc*). Fc: frontal cortex, NAc: nucleus accumbens.

in the Fc was significantly abolished by TIMP-AS although TIMP-SC had little effect (Fig. 2b, $F_{(5,24)} = 4.52$, $p < 0.05$ by one-way ANOVA). Neither TIMP-AS nor TIMP-SC affected TIMP-2 protein levels in saline-treated group. These results suggest that TIMP-2 is involved in the development of the behavioral sensitization induced by repeated METH treatment.

Effects of TIMP-AS on METH-induced increase in extracellular dopamine levels in the Fc and NAc

The pharmacological effects of METH are linked to its capacity to elevate extracellular dopamine levels in the brain through redistribution of dopamine from synaptic vesicles to the cytosol and promotion of reverse transport (Sulzer *et al.* 1995; Nakajima *et al.* 2004). Sensitization is related to the extracellular dopamine level in the NAc, which is regulated by the excitatory input from the Fc (Beyer and Steketee 2002). Accordingly, to explore the mechanisms by which TIMP-AS strengthened the METH-induced sensitization on day 3, we examined the effects of TIMP-AS on the METH-induced increase in extracellular dopamine levels in the Fc and NAc, by using a microdialysis technique. METH caused a marked increase in extracellular dopamine levels in the Fc of the aCSF-treated group on the third day (Fig. 2c). Peak extracellular dopamine was increased by METH treatment to approximately 600% of the baseline level in the aCSF-treated group. In the TIMP-AS-treated group, the METH-induced increase in extracellular dopamine levels was significantly potentiated compared with the aCSF- or TIMP-SC-treated group (Fig. 2c, $F_{(2,13)} = 11.0$, $p < 0.05$ by repeated ANOVA, and $p < 0.05$ by *post hoc*). Similar alterations of the METH-induced increase in extracellular dopamine levels were evident in the NAc of TIMP-AS-treated rats (Fig. 2d, $F_{(2,12)} = 4.13$, $p < 0.05$ by repeated ANOVA, and $p < 0.05$ by *post hoc*).

Role of MMP in METH-induced sensitization in the rat

To study the role of MMP in the Fc in behavioral responses to METH, we investigated the effect of an MMP-2/-9 inhibitor, doxycycline. It has been demonstrated that doxycycline binds directly to Zn^{2+} or Ca^{2+} associated with the enzyme, and blocks the active site or induces conformational changes that render the proenzyme susceptible to fragmentation during activation (Smith *et al.* 1996). It also inhibits the transcription of MMP mRNA (Uitto *et al.* 1994). Recently, it has been reported that doxycycline prevents the activation of pro-MMP-2 by MMP-14 *in vivo* (Strongin *et al.* 1995).

In the present study, doxycycline (0.06 μg/12 μL/day) or aCSF was continuously infused into Fc for 7 days. Acute METH treatment induced hyperlocomotion and the METH-induced hyperlocomotion was augmented by repeated METH treatment in the aCSF-injected control group. Doxycycline did not affect spontaneous locomotor activity or

acute METH-induced hyperlocomotion, but significantly inhibited the development of behavioral sensitization on day 5 (Fig. 3a, $F_{(3,15)} = 54.9$, $p < 0.05$ by one-way ANOVA). The increase in MMP-2 and MMP-9 activity after challenge administration of METH (1 mg/kg) on day 7 was significantly abolished in the Fc and NAc of doxycycline-treated rats (Fig. 3b, $F_{(4,15)} = 8.29$, $p < 0.05$ by one-way ANOVA; and Fig. 3c, $F_{(4,15)}=5.62$, $p < 0.05$ by one-way ANOVA), although the inhibitor had little effect on MMP-2 activity in the saline-treated control.

Effect of doxycycline infusion into the Fc on the METH-induced increase in extracellular dopamine levels in the NAc

Because doxycycline prevented the development of METH-induced behavioral sensitization, we compared the extracellular dopamine levels in the NAc after METH treatment on day 5 in the doxycycline-injected rats with those in the aCSF-treated control animals. Repeated METH treatment for 5 days increased the peak extracellular dopamine levels to about 800–900% of the basal levels in the NAc, which may be associated with behavioral sensitization (Sulzer *et al.* 1995; Nakajima *et al.* 2004) (Fig. 3b). Continuous infusion of doxycycline at 0.06 μg/day into the Fc significantly reduced the METH-induced increase in the extracellular dopamine levels on day 5 (Fig. 3d, $F_{(1,12)} = 5.01$, $p < 0.05$ by repeated ANOVA, and $p < 0.05$ by *post hoc*). From the results of the infusion of doxycycline into the Fc, it is suggested that MMP, possibly MMP-2 and/or MMP-9, in the Fc plays an important role in the METH-induced dopamine release in the NAc.

Next, the role of MMP in the rewarding effects of METH was examined in a CPP paradigm, in which animals learned to associate the environment paired with drug exposure. CPP is therefore considered a measure of the rewarding properties of drugs of abuse. When doxycycline or MMP-2/-9 inhibitor III was continuously infused into the right ventricle of rats, METH-induced CPP was significantly attenuated (Fig. 3e, $F_{(3,33)} = 5.77$, $p < 0.05$ by one-way ANOVA; Fig. 3f, $F_{(3,33)} = 6.48$, $p < 0.05$ by one-way ANOVA), although these inhibitors had no effect in saline-treated animals. These results suggest that MMP, especially MMP-2 and MMP-9, is involved in METH reward.

Regulation of dopamine receptor-stimulated G protein signaling by the MMP/TIMP system

As shown in Figs 2 and 3, over-expression of the MMP/TIMP system in the Fc may play a crucial role in the development of METH-induced sensitization. Behavioral sensitization to METH could also be associated with alterations in the responsiveness of post-synaptic dopamine receptors which belong to G protein-coupled receptors. In fact, G protein signaling in the Fc plays a crucial role in a potential pathological change contributing to cocaine sensitization and drug seeking (Bowers *et al.* 2004). The reduced signaling via Gi-coupled receptors may be an important neuroadaptation in cocaine addiction (Goldstein and Volkow 2002). Therefore, we focused on the dopaminergic signaling via the dopamine D1 and D2 receptors in the Fc. To assess potential changes in dopamine receptor-stimulated G protein signaling, we performed a [^{35}S]GTP γS binding assay. The ability of the dopamine D1 receptor agonist SKF81297 (0.01–0.1 mmol/L) and D2 receptor agonist quinpirole (0.5–1 mmol/L), to stimulate the binding of [^{35}S]GTP γS to Gα proteins in membranes of the Fc in METH-treated rats was compared with that in the saline-treated control group (Fig. 4a). Repeated METH treatment for 5 days reduced the [^{35}S]GTP γS binding stimulated by SKF81297 or quinpirole in membrane of the Fc, indicating a reduction of both dopamine D1 and D2 receptor-stimulated G protein signaling in the Fc (Fig. 4a, $p < 0.05$ by *t*-test). Interestingly, TIMP-AS treatment, which potentiated METH-induced behavioral sensitization and dopamine release on day 3 of the repeated METH treatment (Fig. 2), was accompanied by potentiation of the reduction of [^{35}S]GTPγS binding stimulated by quinpirole ($F_{(5,49)} = 5.00$, $p < 0.05$ by one-way ANOVA) although TIMP-SC had no effect (Fig. 4c). Neither TIMP-AS or TIMP-SC had effects on SKF81297-stimulated [^{35}S]GTPγS binding (Fig. 4b). In contrast, doxycycline treatment, which inhibited METH-induced behavioral sensitization and dopamine release on day 5 of the repeated METH treatment (Fig. 3a), was accompanied by blockade of the reduction of [^{35}S]GTPγS binding stimulated by quinpirole (Fig. 4e, $F_{(3,23)} = 3.03$, $p < 0.05$ by one-way ANOVA at 10^{-3} mol/L).

To further confirm the role of the MMP/TIMP system in the regulation of dopamine receptor-mediated G protein signaling in the Fc, the effects of *in vitro* treatment with purified human MMP-2 and recombinant human TIMP-2 protein on SKF81297- or quinpirole-stimulated [^{35}S]GTPγS binding were determined (Fig. 5). Purified human MMP-2 treatment at 37°C for 60 min *in vitro* significantly reduced the quinpirole-stimulated [^{35}S]GTPγS binding (Fig. 5b, $F_{(2,15)} = 5.51$, $p < 0.05$; $F_{(4,24)} = 4.02$, $p < 0.05$ by one-way ANOVA), without affecting the binding stimulated by SKF81297 (Fig. 5a). Co-treatment with EGTA (1 mmol/L) blocked the reduction of [^{35}S]GTPγS binding induced by purified human MMP-2 ($p < 0.05$ by *post hoc*). In contrast, the *in vitro* TIMP-2 treatment at 4°C for 120 min increased the quinpirole-stimulated [^{35}S]GTPγS binding (Fig. 5d, $F_{(2,18)} = 3.87$, $p < 0.05$; $F_{(2,17)} = 3.98$, $p < 0.05$ by one-way ANOVA), but had no effect on the SKF81297-stimulated binding (Fig. 5c). Thus, it is plausible that repeated METH treatment induces over-expression of MMP-2, MMP-9 and TIMP-2, leading to an increase in net MMP activity, which results in the potentiation of METH-induced dopamine release in the NAc as well as down-regulation of dopamine D2 receptor-mediated G protein signaling in the Fc.

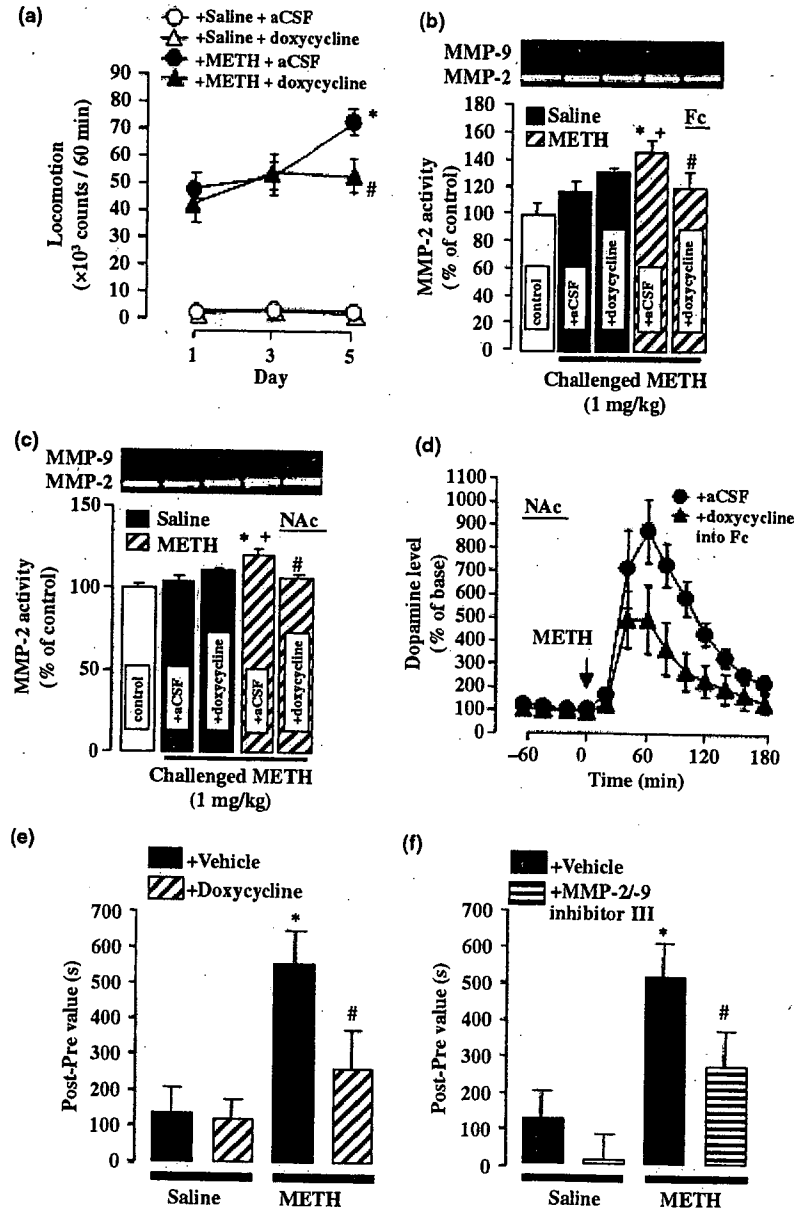

Fig. 3 Effect of continuous infusion of MMP inhibitor, doxycycline into Fc on repeated METH-induced behavioral sensitization (a), MMP-2 activity in Fc (b) and NAc (c), and dopamine release in NAc (d). (a): Rats were given saline or METH at a dose of 2 mg/kg for 5 days, and locomotor activity was measured on days 1, 3 and 5. An osmotic minipump was used to deliver a continuous infusion of doxycycline (0.06 μg/12 μL/day) or aCSF into the Fc for 7 days. Values are the means ± SE (n = 4–6). *p < 0.05 versus day 1, #p < 0.05 versus METH+aCSF. (b) and (c): Rats were given saline or METH at a dose of 2 mg/kg for 5 days. On day 7, rats were given METH at the challenge dose of 1 mg/kg and killed 2 h after the administration. Control rats were given saline. An osmotic minipump was used to deliver a continuous infusion of doxycycline (0.06 μg/12 μL/day) or aCSF into the Fc for 7 days. Values are the means ± SE (n = 4). *p < 0.05 versus control, +p < 0.05 versus saline+vehicle and # p < 0.05 versus METH+vehicle. (d): rats were given METH at a dose of 2 mg/kg for 5 days. Doxycycline (0.06 μg/12ul/day) was continuously infused by the osmotic minipump into the Fc. On day 5, dopamine release was measured for 3 h after the administration of METH in the NAc. Basal extracellular dopamine levels were 0.29 ± 0.07 nmol/L for the control and 0.18 ± 0.05 nmol/L for the doxycycline-infused group. Values are the means ± SE (n = 7). Continuous infusion of doxycycline at 0.06 μg/day into Fc significantly reduced the METH-induced increase in the extracellular dopamine levels on day 5 ($F_{(1,12)}$=5.01; p < 0.05 by repeated ANOVA, and p < 0.05 by *post hoc*). Effects of doxycycline (e) and MMP-2/-9 inhibitor III (f) on the rewarding effects of METH. Rats were trained for CPP to METH (2 mg/kg). Doxycycline or MMP-2/-9 inhibitor III was continuously infused into the right ventricle. Values are the means ± SE (n = 9–10). *p < 0.05 versus saline+vehicle. #p < 0.05 versus METH+vehicle. Fc: frontal cortex, NAc: nucleus accumbens.

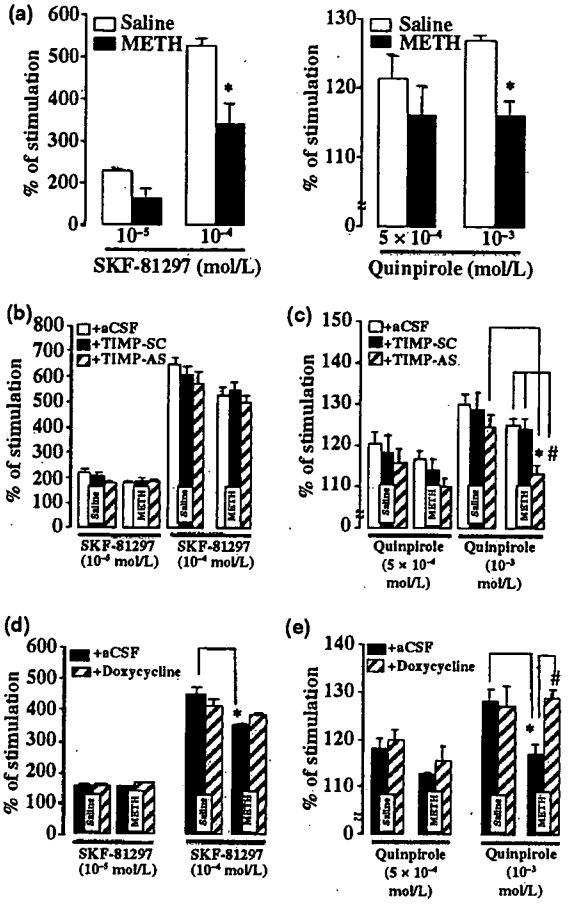

Fig. 4 Effects of repeated METH treatment on dopamine D1 agonist SKF81297- and D2 agonist quinpirole-stimulated [^{35}S]GTPγS binding in membranes of the Fc (a). Rats were given saline or METH at a dose of 2 mg/kg for 5 days and killed 2 h after the final administration. Values are means ± SE (n = 4). *p < 0.05 versus saline. Effects of TIMP-AS on SKF81297- (b) and quinpirole-stimulated (c) [^{35}S]GTPγS binding in membranes of saline- and METH-treated rats. Rats were given saline or METH at 2 mg/kg for 5 days and killed 2 h after the final administration. aCSF, TIMP-AS or TIMP-SC was continuously infused into the cerebral ventricle. Values are mean ± SE (n = 8–11 for b and c). *p < 0.05 versus saline+TIMP-AS. #p < 0.05 versus METH+TIMP-SC. Effects of doxycycline on SKF81297-(d) and quinpirole-(e) stimulated [^{35}S]GTPγS binding in membranes of saline- and METH-treated rats. Rats were given saline or METH at a dose of 2 mg/kg for 5 days and killed 2 h after the final administration. CSF or doxycycline was continuously infused into the cerebral ventricle. Values are means ± SE (n = 5–7 for d; n = 7 for e). *p < 0.05 versus saline+vehicle. #p < 0.05 versus METH+vehicle.

Reduction of dopamine receptor-stimulated G protein signaling in MMP-2-(−/−) and MMP-9-(−/−) mice

Previously, we have demonstrated that MMP-2-(−/−) and MMP-9-(−/−) mice show reduced responses to METH in behavioral sensitization, CPP and dopamine release in the NAc. TIMP-2 is not a specific inhibitor of MMP-2 and

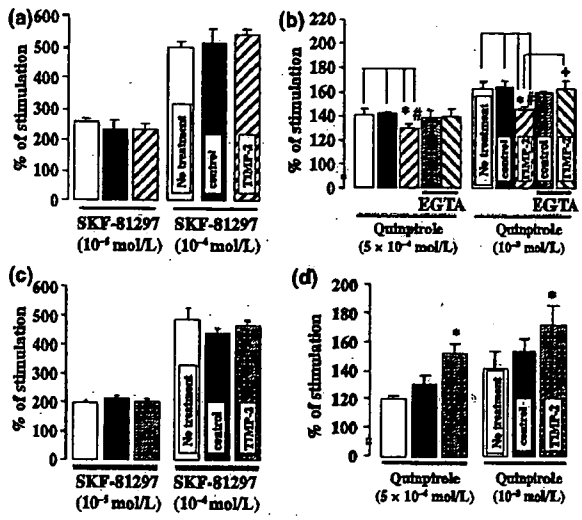

Fig. 5 Effects of *in vitro* treatment with purified human MMP-2 (a, b) or recombinant human TIMP-2 (c, d) on SKF81297- (a, c) and quinpirole-stimulated (b, d) [^{35}S]GTPγS binding in membranes of the Fc. Brain samples were incubated with activation buffer (control group) or purified human MMP-2 protein (MMP-2 group) in the presence or absence of 1 mmol/L EGTA at 37°C for 60 min. Alternatively, brain samples were incubated with activation buffer (control group) or recombinant human TIMP-2 protein (TIMP-2 group) for 120 min. Values are means ± SE (n = 5 for a; n = 5–7 for b; n = 5–6 for c; n = 6–7 for d). *p < 0.05 versus no treatment. #p < 0.05 versus control group. + p < 0.05 versus MMP-2 group.

tetracycline derivatives such as doxcycline inhibit most if not all of the MMPs that have been tested. Therefore, in addition to the pharmacological studies, we investigated a role of MMP-2 and MMP-9 in G protein signaling using MMP-2-(−/−) and MMP-9-(−/−) mice.

We observed no changes in SKF-81297 or quinpirole-stimulated [^{35}S]GTPγS binding between saline-treated wild-type and MMP-2-(−/−) or MMP-9-(−/−) mice, indicating no changes in dopamine D1 and D2 receptor activity in MMP-2-(−/−) and MMP-9-(−/−) mice (Fig. 6). Repeated METH treatment for 7 days reduced the [^{35}S]GTP γS binding stimulated by SKF81297, but not quinpirole, in membranes of the Fc of wild-type mice (Figs 6a and b, $F_{(3,16)}$ = 4.66, p < 0.05 by one-way ANOVA for SKF-81297 at 10^{-5} mol/L; $F_{(3,16)}$ = 5.00, p < 0.05 by one-way ANOVA for SKF-81297 at 10^{-4} mol/L). Such METH-induced reduction of [^{35}S]GTPγS binding stimulated by SKF81297 at 10^{-5} mol/L, but not 10^{-4} mol/L, was significantly attenuated in MMP-9-(−/−) mice (Fig. 6a). Repeated METH treatment-induced [^{35}S]GTPγS binding stimulated by quinpirole was increased in MMP-9-(−/−) mice compared with wild-type mice (Fig. 6b, p < 0.05 by t-test). On the other hand, in the MMP-2-(−/−) mice, repeated METH treatment failed to reduce the [^{35}S]GTP γS binding stimulated by SKF81297 significantly at 10^{-4} mol/L or quinpirole at 10^{-3} mol/L as

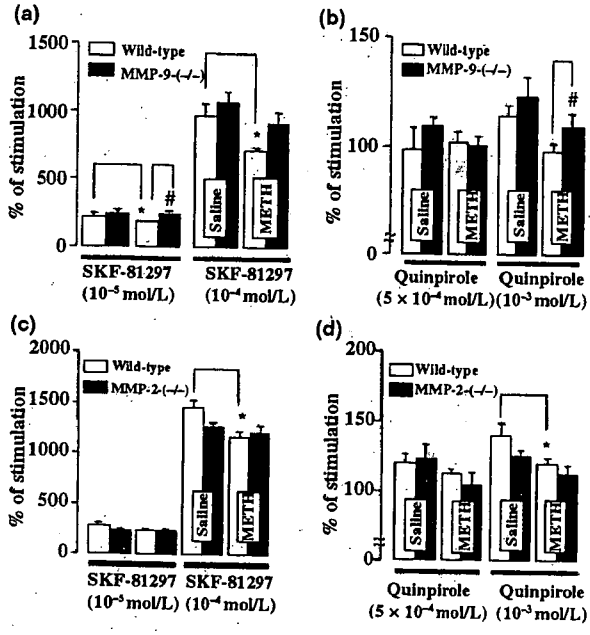

Fig. 6 Effects of repeated METH treatment on dopamine D1 agonist SKF81297- (a, c) and D2 agonist quinpirole-stimulated (b, d) [^{35}S]GTPγS binding in membranes of the Fc of MMP-9-(−/−) (a, b) and MMP-2-(−/−) (c, d) mice. Mice were given saline or METH at a dose of 1 mg/kg for 7 days and killed 2 h after the final administration. Values are means ± SE (n = 5 for A and B, n = 6–7 for c and d). *$p < 0.05$ versus saline-treated wild-type. *$p < 0.05$ versus saline-treated wild-type. #$p < 0.05$ versus METH-treated wild-type.

observed in wild-type mice, although there was no difference in [^{35}S]GTPγS binding stimulated by SKF81297 and quinpirole between two groups of mice (Fig. 6c, $F_{(3,24)} = 3.32$, $p < 0.05$ by one-way ANOVA at 10^{-4} mol/L; 6D, $F_{(3,21)} = 3.49$, $p < 0.05$ by one-way ANOVA at 10^{-3} mol/L). These results suggest that both mutant mice exhibit some resistance to the inhibitory effect of repeated METH treatment on dopamine receptor-mediated G protein signaling, which may be associated with the impairment in METH-induced behavioral sensitization and CPP, as well as METH-increased dopamine release in MMP-2-(−/−) and MMP-9-(−/−) mice (Mizoguchi *et al.* 2007).

Discussion

In the present study, we have demonstrated for the first time that repeated but not acute METH treatment leads to an increase in TIMP-2 expression in neurons in the Fc. The MMP inhibitor doxycycline prevented while TIMP-AS accelerated the development of the METH-induced behavioral sensitization. The net increase in MMP activity may be responsible at least in part for the development of METH-induced behavioral sensitization. Of note, MMP inhibitors also reduced the rewarding effect of METH. Further, we provided evidence that the MMP/TIMP system is involved in the regulation of METH-induced dopamine release as well as dopamine receptor-mediated G protein signaling. It is likely that MMP/TIMP system plays a role in METH-induced behavioral sensitization through the modulation of the function of plasma membrane protein such as dopamine receptors (The present study) and transporter (Mizoguchi *et al.* 2007). These results, together with the well known function of the MMP to degrade extracellular matrix proteins such as laminin and collagen IV, suggest that repeated METH-induced over-expression of the MMP-2, MMP-9 (Mizoguchi *et al.* 2007) and TIMP-2 (The present study) plays a crucial role in the structural and functional changes in the mesocorticolimbic dopamine system related to METH-induced behavioral sensitization and reward following repeated drug treatment.

Our data showed that repeated administration of METH led to behavioral sensitization which was accompanied by the induction TIMP-2 expression in the Fc. Additionally, we demonstrated that TIMP-2 was localized to neurons in the Fc. Previous study has revealed that MMP-2-like and TIMP-2-like immunoreactivities are found in cerebellar neurons but not glial cells (Vaillant *et al.* 1999). We have also shown that both MMP-2 and MMP-9 are highly expressed by neurons in the Fc of adult rat brain after repeated METH treatment (Mizoguchi *et al.* 2007). Taken together, it is suggested that the MMP/TIMP system is expressed by adult CNS neurons and the expression level and cellular localization may be regulated according to the developmental and/or functional status of neurons. In fact, MMP-7 disrupts dendritic spines in hippocampal neurons (Bilousova *et al.* 2006), and MMP-9 expression is regulated in the hippocampus by synaptic activity during dendritic remodeling (Zhang and Gottschall 1997), suggesting that MMP can influence the morphology of dendritic spines and hence synaptic stability. Although we do not directly examine the role of MMP/TIMP system in the METH-induced alterations of synaptic connectivity in the Fc, it is suspected that the expression of MMP/TIMP may take part in the structural and functional alteration in the brain following repeated exposure to METH.

The infusion of TIMP-AS into the ventricle exaggerated the behavioral sensitization induced by repeated METH treatment, while infusion of doxycycline and MMP-2/-9 inhibitor III inhibited the development of METH-induced behavioral sensitization and CPP. Consistent with the present findings, MMP-2-(−/−) and MMP-9-(−/−) mice exhibit the impairment of METH-induced behavioral sensitization and CPP (Mizoguchi *et al.* 2007). These findings suggest that METH-induced expression of the MMP/TIMP system in the brain plays a role in the development of METH-induced behavioral sensitization and reward, which may be associated with the METH-induced neuronal plasticity and remodeling. Indeed, cleavage of ECM induced by an imbalance of the MMP/TIMP ratio confers oncogenicity on the cell (Khokha *et al.* 1989), and excessive

