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 (AI-8–1 or AI-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-AI-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

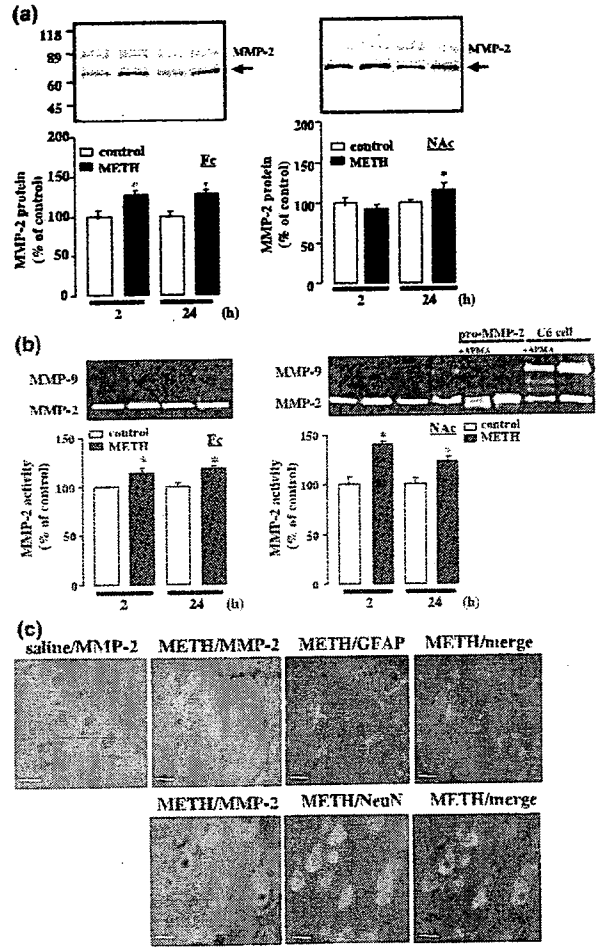

Fig. 1 METH-induced MMP-2 expression. Changes in protein levels (a) and MMP-2 activity (b) in Fc and NAc after repeated METH treatment. Rats were given METH at a dose of 2 mg/kg for 5 days and killed 2 or 24 h after the final administration. Control rats were given saline. The human pro-MMP-2 and C6 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.

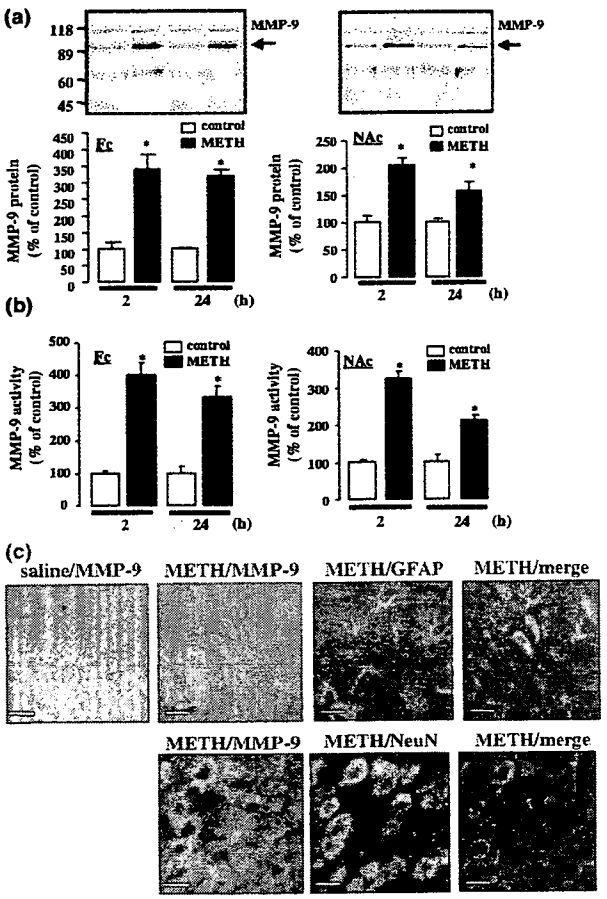

Fig. 2 METH-induced MMP-9 expression. Changes in protein levels (a) and MMP-9 activity (b) in Fc and NAc after repeated METH treatment. Rats were given METH at a dose of 2 mg/kg for 5 days and killed 2 or 24 h after the final administration. Control rats were given saline. The C6 cell line was used as 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

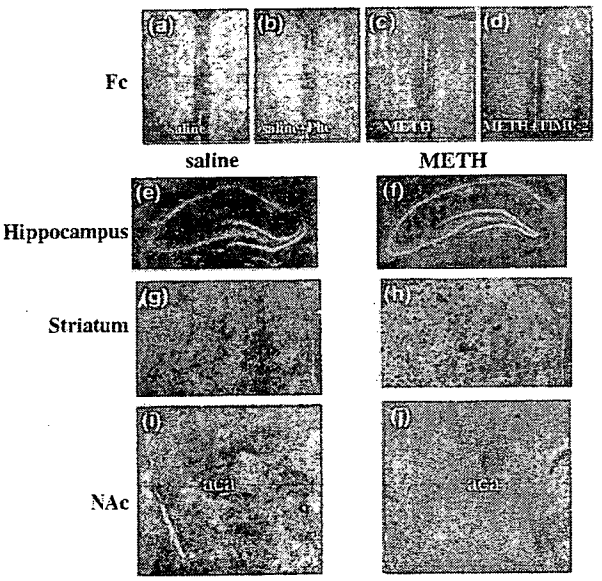

Fig. 3 Spatial changes in METH-induced MMP activity in the brain. *In situ* zymography detected gelatinase activity in Fc (a–d), hippocampus (e, f), striatum (g, h) and NAc (i, j). Rats were given saline (a, b, e, g, i) or METH (c, d, f, h, j) at a dose of 2 mg/kg for 5 days, and killed 2 h after the 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

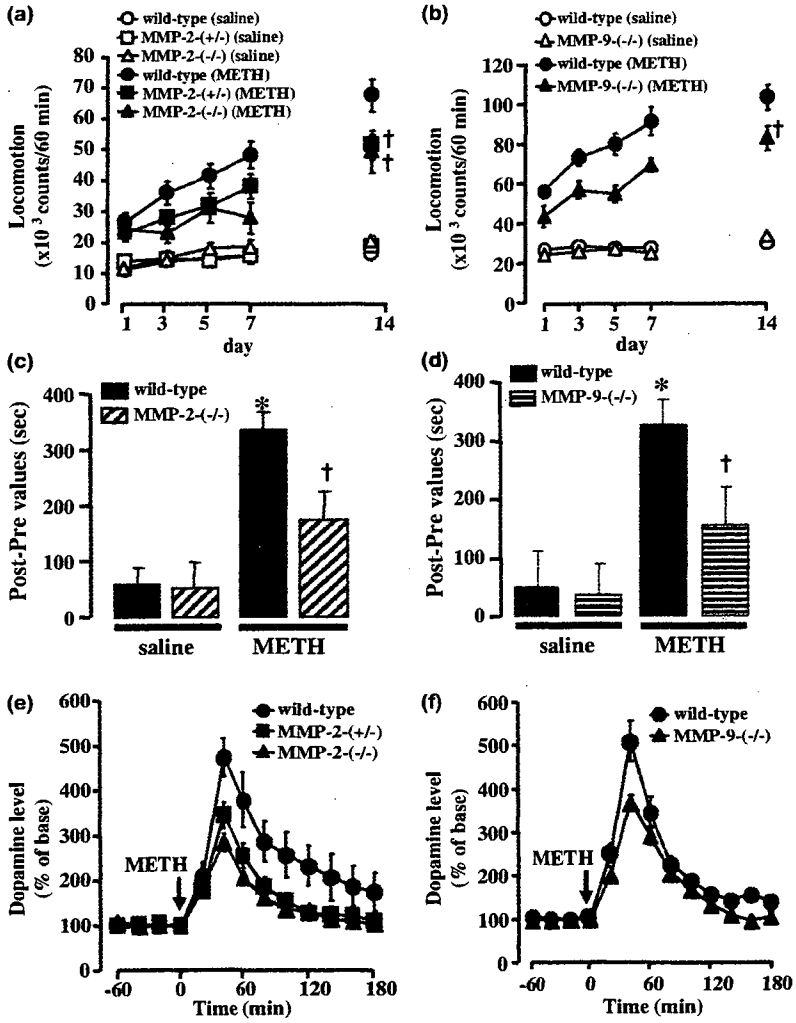

Fig. 4 Sensitization of METH-induced (a, b) hyperlocomotion, (c, d) reward and (e, f) dopamine release in the NAc in (a, c, e) MMP-2(–/–), (a, e) MMP-2(+/–) and (b, d, f) MMP-9(–/–). (a, b): Mice were given saline or METH at a dose of 1 mg/kg for 7 days and, after withdrawal for 7 days, they were given 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

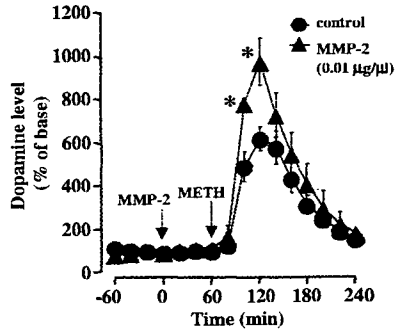

Fig. 5 Effect of MMP-2 on the METH-induced increase in extracelluar dopamine levels in the NAc of rats. Purified human MMP-2 (0.01 μg) was microinjected into the NAc in a volume of 1.0 μL 1 h before METH (2 mg/kg) treatment. Basal extracellular dopamine levels were 0.21 ± 0.07 nM for the control and 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).

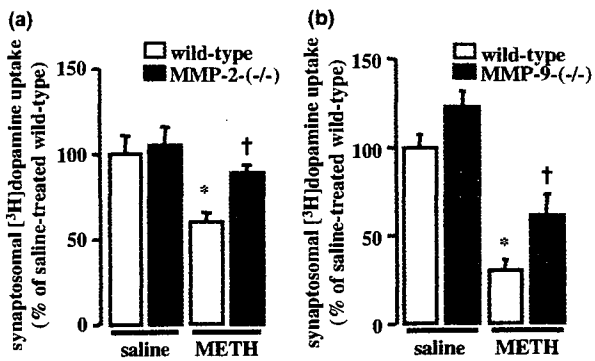

Fig. 6 Changes in METH-induced reduction of the activity of synaptosomal dopamine uptake in (a) MMP-2-(–/–) and (b) MMP-9-(–/–) mice. Mice were given saline or METH at a dose of 1 mg/kg for 7 days, and were killed 1 h after the final administration. (a) The [^{3}H]dopamine uptake activity in the saline-treated 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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Synergistic effect of galantamine with risperidone on impairment of social interaction in phencyclidine-treated mice as a schizophrenic animal model

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Abstract

Social withdrawal is the first sign and key component of the negative symptoms of schizophrenia. The efficacy of risperidone, an atypical antipsychotic, on the symptom is practically limited by dose-dependent side effects in clinical trials, therefore there is the need for adjuvant treatments. In the present study, we aimed to investigate the synergistic effect and mechanism of risperidone and galantamine, which is a nicotinic acetylcholine receptor (nAChR)-allosteric modulator and a modest cholinesterase inhibitor, on phencyclidine (PCP)-treated mouse model of social withdrawal. At non-effective doses by themselves, co-administration of galantamine (0.05 mg/kg) and risperidone (0.05 mg/kg) showed synergistic effects on PCP-induced impairments of social interaction and dopamine release in the medial prefrontal cortex (mPFC). The behavioral synergistic effect was abolished by the administration of a dopamine-D_1 receptor antagonist, SCH 23390 (0.02 mg/kg, systemic; or 0.02 μg/0.5 μL/mouse, intra-mPFC), and a nAChR antagonist, mecamylamine (3 mg/kg), but not a muscarinic receptor antagonist, scopolamine (0.1 mg/kg). Mecamylamine (3 mg/kg) also abolished the synergistic effect on dopamine release in the mPFC. We conclude that galantamine may have synergistic effect with risperidone on the negative symptom of social withdrawal in schizophrenia, which is mediated by dopamine-D_1 receptors in the mPFC through nAChR activation-increased dopamine release.

Keywords: Schizophrenia; Social withdrawal; Galantamine; Risperidone; Synergistic effects

1. Introduction

Schizophrenia is characterized by three broad types of symptoms, positive or psychotic (e.g., hallucination, delusion), negative (e.g., anhedonia, social withdrawal or poor social interaction) and cognitive symptoms (e.g., deficits in attention, working memory, mental flexibility), most of which are related with dopaminergic aberration. Positive symptoms involving the loss of contact with reality are thought to arise from

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a subcortical hyperstimulation of dopamine-D_2 receptors, especially in striatal areas (Abi-Dargham, 2004). Negative symptoms are more pervasive and fluctuate less over time than positive symptoms, and are strongly associated with poor psychosocial functioning (Mueser and McGurk, 2004). Negative symptoms are thought to arise from a dopaminergic hypofunction that results in the hypostimulation of dopamine-D_1 receptors in the dorsolateral prefrontal cortex (PFC) in schizophrenia patients, an area for which the corresponding brain region in rodents is the medial PFC (mPFC) (Abi-Dargham, 2004; Albert et al., 2004; Kolb, 1990).

Phencyclidine (PCP), a non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, induces psychotomimetic states in humans and rodents. Since PCP psychosis incorporates not only the positive symptoms (e.g., hallucinations, paranoia) but also the negative symptoms (e.g., social withdrawal, motor retardation) and cognitive impairments (e.g., impairment of attention and working memory), PCP-treated animals have been proposed as a preclinical model of schizophrenia (Castner et al., 2004; Javitt and Zukin, 1991; Morris et al., 2005; Noda et al., 1995, 2000, 2001).

Risperidone is an atypical antipsychotic drug with antagonistic properties at D_2, 5-HT_{2A} and α_1 receptors. Risperidone is more effective than conventional neuroleptics on the positive symptoms of schizophrenia (Khan, 1997). It also has some effects on the negative symptoms, however, these effects are practically limited by various dose-dependent side effects in clinical trials. Therefore, there is a need for adjuvant drugs or new drug-treating strategies for the negative symptoms (Arnold et al., 2004). Interestingly, a number of studies have indicated that there is a deficit in nicotinic acetylcholine receptor (nAChR) in the PFC of schizophrenia patients (Arnold et al., 2004; Deutsch et al., 2005), which has been postulated to be related with the negative symptoms (Kumari and Postma, 2005). In addition, the activation of nAChRs may ameliorate the extrapyramidal side effects (Kumari and Postma, 2005). In a clinical survey, nicotine-containing cigarettes have been found to reduce negative symptoms without affecting positive symptoms compared with nicotine-free cigarettes, and the effects are supposed to reflect nicotine's ability to raise dopamine level in the PFC (Kumari and Postma, 2005).

Galantamine, a medicine for the treatment of Alzheimer's disease, is an allosteric potentiating ligand at nAChRs and also displays the weakest acetylcholinesterase (AChE)-inhibiting effect among the clinically used AChE inhibitors (Samochocki et al., 2003; Sharp et al., 2004). In a clinical case report, galantamine showed adjuvant therapeutic effect on apathy in a schizophrenia patient (Arnold et al., 2004).

The present study was designed to test the hypothesis that co-administration of risperidone and galantamine synergistically attenuates negative symptom-like behavioral impairment in a PCP-treated animal model of schizophrenia, and the synergistic effect of galantamine and risperidone is mediated via the activation of nAChR-dopaminergic systems, by (1) comparing the effects of individual and co-administration of risperidone and galantamine on the impairments of social interaction and dopamine release in the mPFC induced by repeated PCP treatment, and (2) investigating whether the synergism of galantamine with risperidone is abrogated by acetylcholine and/or dopamine receptor antagonists in the mice repeatedly treated with PCP.

2. Methods and materials

2.1. Animals

Male mice of the ICR strain (Japan SLC Inc., Shizuoka, Japan), 6 weeks old at the beginning of experiments, were used. They were housed in plastic cages, five mice per cage, received food (CE2; Clea Japan Inc., Tokyo, Japan) and water ad libitum, and were maintained on a 12/12-h light/dark cycle (lights on from 8:00 AM to 8:00 PM). Behavioral experiments were carried out in a sound-attenuated and air-regulated experimental room, to which mice were habituated for at least 1 h. All experiments were performed in a blind manner and in accordance with the Guidelines for Animal Experiments of Nagoya University Graduate School of Medicine, which conformed to the international guidelines set out in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

2.2. Drugs

Phencyclidine HCl was synthesized by the authors according to the method of Maddox et al. (1965) and was checked for purity. Risperidone and galantamine were supplied by Janssen Pharmaceutical K.K. (Tokyo, Japan). SCH 23390, mecamylamine hydrochloride and (−)-scopolamine hydrobromide were purchased commercially from Sigma–Aldrich (St. Louis, MO, USA). PCP was dissolved in saline. An oral solution of risperidone and/or galantamine was freshly prepared by dissolving in dilute tartaric acid solution (final pH 3.2).

2.3. Drug treatment

The doses of risperidone and galantamine were referred to the clinical doses and determined in our preliminary experiments. The doses of antagonists were referred to other related researches in the laboratory (Wang et al., in press), and determined in preliminary experiments.

The mice were administered PCP (10 mg/kg/day s.c.) or saline once a day for 14 consecutive days. Risperidone (0.025, 0.05 and 0.1 mg/kg) and galantamine (0.05, 0.1, 0.2, 0.3 and 3 mg/kg) were p.o. administered 1 h before the social interaction test, or immediately after baseline collections in microdialysis experiment. Mecamylamine at the dose of 3 mg/kg and scopolamine at the dose of 0.1 mg/kg were s.c. injected 20 min after the treatment with risperidone and galantamine. SCH 23390 at the dose of 0.02 mg/kg was s.c. injected 30 min after the treatment with risperidone and galantamine.

For mPFC-local microinjection, mice were anesthetized with diethyl ether and fixed on the stereotactic apparatus (Narishige, Tokyo, Japan) at least 20 min before the social interaction test. An injection cannula (27 gauge) with a bevel tip was clipped on a pincers and implanted into the mPFC (−0.3 mm mediolateral from the midpoint on the line linking the rear canthi, −2.5 mm in depth). The microinjection position crossed the second motor, cingulate and prelimbic cortices, which were the same subregions as those in the microdialysis experiment. Fifteen minutes before the social interaction test, SCH 23390 at the dose of 0.02 μg/0.5 μL/mouse was infused into the mPFC over 45 s using a Hamilton microsyringe connected to the cannula via a Teflon tube, and the connection was held for another 45 s after the injection. After the behavioral experiments, the mice were decapitated, and the brains were taken out and dissected to confirm that injection site crossed the second motor, cingulate and prelimbic cortices in the mPFC, but not any other brain regions, according to the atlas of Franklin and Paxinos (1997). Misinjected mice were excluded from subsequent data analysis.

2.4. Social interaction test

The protocol of Qiao et al. (2001) was adopted for evaluation of the negative schizophrenic symptom-like behavior, which was modified from

the original social interaction test in that the aggressive behaviors (biting, boxing) and passive contact (sitting or lying with bodies in contact) were not included in the social interaction score (Corbett et al., 1993; Qiao et al., 2001). The apparatus used for the social interaction test consisted of a square open arena (25 × 25 × 30 (*H*) cm) made of gray non-reflecting acrylics, illuminated with lamps that could not be seen by the mice directly. The light was diffused to minimize shadows in the arena.

Two days after final administration of PCP, each mouse was placed in the test box for 10 min to habituate to the testing environment. One day after habituation, every mouse was randomly assigned to an unfamiliar partner in each drug-treated group. Each pair of unfamiliar mice was placed in the apparatus for 10 min and the time that a pair spent in social interaction (sniffing and grooming the partner, following, mounting, and crawling under or over the partner) was recorded by an observer who was blind to the drug treatments.

2.5. *In vivo microdialysis*

In vivo microdialysis was performed 3 days after the final PCP injection. One day before the microdialysis, mice were anesthetized with sodium pentobarbital and a guide cannula (MI-AG-6; Eicom Corp., Kyoto, Japan) was implanted in the PFC (−1.9 mm anteroposterior, −1.0 mm mediolateral from the bregma, −1.5 mm dorsoventral from the skull, −15° angle from vertical) according to the atlas of Franklin and Paxinos (1997). One day after the operation, a dialysis probe (A-I-6-01; 1 mm membrane length; Eicom Corp., Kyoto, Japan) was inserted through the guide cannula, and perfused with an artificial cerebrospinal fluid (aCSF; 147 mM NaCl, 4 mM KCl, and 2.3 mM $CaCl_2$) at a flow rate of 1.2 μL/min (Shintani et al., 1993). Following the collection of three stable baseline fractions, risperidone and/or galantamine were p.o. administered to the mice, and dialysate sampling was started 60 min after the co-administration for 10 min (Fig. 1), which was identical to the time schedule in the social interaction test. Dopamine levels in the dialysates were analyzed using an HPLC system equipped with an electrochemical detector (Nagai et al., 2004).

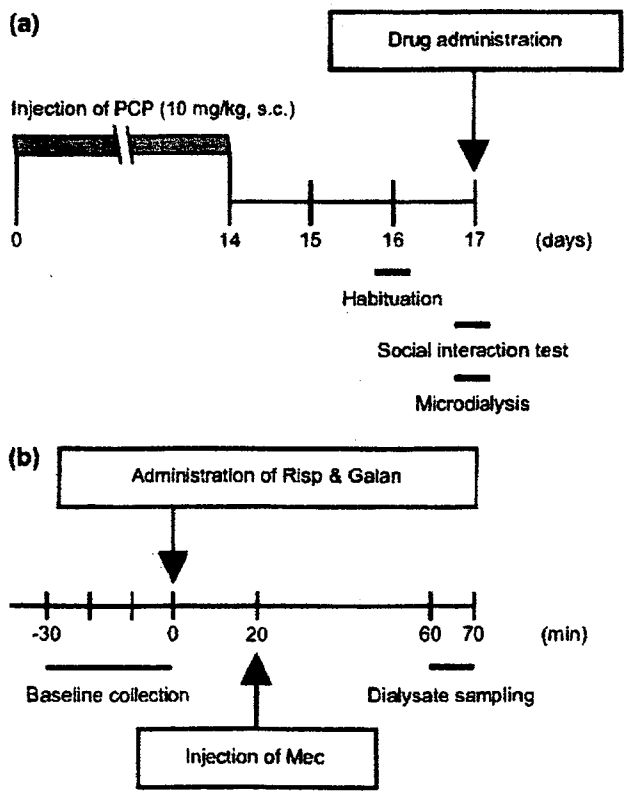

Fig. 1. Experimental schedule. (a) Schedule of drug treatment, behavioral experiment and microdialysis. (b) Schedule of microdialysis experiment.

2.6. *Statistical analyses*

Statistical difference among the experimental groups was tested using a one-way analysis of variance (ANOVA). The modified Tukey's test (Keselman and Rogan, 1978) was applied for multiple comparisons. A p value less than 0.05 was accepted as significant.

3. Results

3.1. *Individual effects of risperidone and galantamine on impairment of social interaction in PCP-treated mice*

Social interaction time in the unfamiliar mice pairs was shortened by repeated s.c. administration of PCP at the dose of 10 mg/kg for 14 days ($p < 0.01$). The impairment of social interaction in PCP-treated mice was ameliorated by individual treatments with risperidone and galantamine in a dose-dependent manner. The treatments with risperidone at the dose of 0.1 mg/kg and galantamine at doses of 0.3 and 3 mg/kg significantly increased the interaction time in PCP-treated mice (Fig. 2). The treatment with galantamine at 3 mg/kg was not more effective than 0.3 mg/kg (Fig. 2). Risperidone at the dose of 0.1 mg/kg and galantamine at the dose of 0.3 mg/kg did not significantly affect the performance in saline-treated mice (Fig. 2).

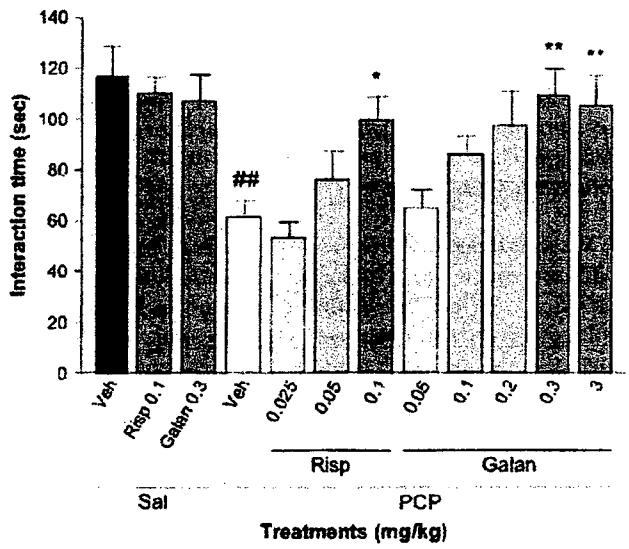

Fig. 2. Individual effects of risperidone and galantamine on impairment of social interaction in PCP-treated mice. Results are expressed as means ± S.E.M., $n = 14$–16. $F_{12,157} = 5.003$ ($p < 0.01$), analyzed by a one-way ANOVA, followed by the modified Tukey's test for multiple comparisons. $^{**}p < 0.01$, compared to Sal/Veh-treated group. $^{*}p < 0.05$, $^{**}p < 0.01$, compared to PCP/Veh-treated group. Veh: dilute tartaric acid solution, pH 3.2; Sal: saline; PCP: phencyclidine; Risp: risperidone; Galan: galantamine.

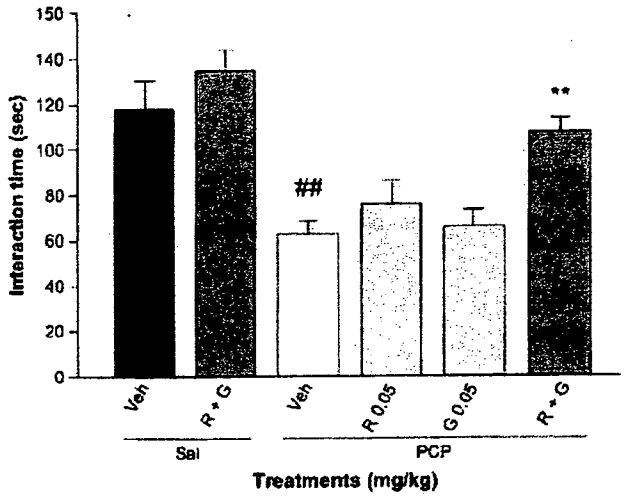

Fig. 3. Synergistic effect of galantamine with risperidone on impairment of social interaction in PCP-treated mice. Results are expressed as means ± S.E.M., $n = 12$–17, $F_{5,85} = 9.423$ ($p < 0.01$), analyzed by a one-way ANOVA, followed by the modified Tukey's test for multiple comparisons. $^{\#\#}p < 0.01$, compared to Sal/Veh-treated group. $^{**}p < 0.01$, compared to PCP/Veh-treated group. Veh: dilute tartaric acid solution, pH 3.2; Sal: saline; PCP: phencyclidine; R: risperidone; G: galantamine; R + G: co-administration of risperidone and galantamine.

3.2. Synergistic effect of galantamine with risperidone on impairment of social interaction in PCP-treated mice

Treating animals with risperidone at 0.025 mg/kg and galantamine at 0.05 mg/kg simultaneously failed to reverse the impairment of social interaction (data not shown). Co-administration of risperidone and galantamine both at the none-effective doses of 0.05 mg/kg greatly reversed the impairment of social interaction in PCP-treated mice ($p < 0.01$) (Fig. 3).

3.3. Synergistic effect of galantamine with risperidone was abrogated by a nicotinic AChR antagonist, mecamylamine, but not by a muscarinic AChR antagonist, scopolamine

We investigated whether nAChR and mAChR are involved in the synergistic effect of the co-administration, by observing the effects of mecamylamine (3 mg/kg) and scopolamine (0.1 mg/kg) on the synergism of galantamine (0.05 mg/kg) with risperidone (0.05 mg/kg). Mecamylamine (3 mg/kg) was s.c. injected to the mice 20 min after the co-administration. Forty minutes after the injection, mecamylamine abrogated the effect of the co-administration on the impairment of social interaction induced by PCP treatment ($p < 0.01$) (Fig. 4a). The co-administration of risperidone (0.05 mg/kg) and galantamine (0.05 mg/kg) did not significantly affect the performance in saline-treated mice, and mecamylamine at the dose of 3 mg/kg also did not significantly block the effect of the co-administration (Fig. 4a).

Scopolamine (0.1 mg/kg) was s.c. injected to the mice 20 min after the co-administration. The performance of the normal mice in the test was impaired by scopolamine at

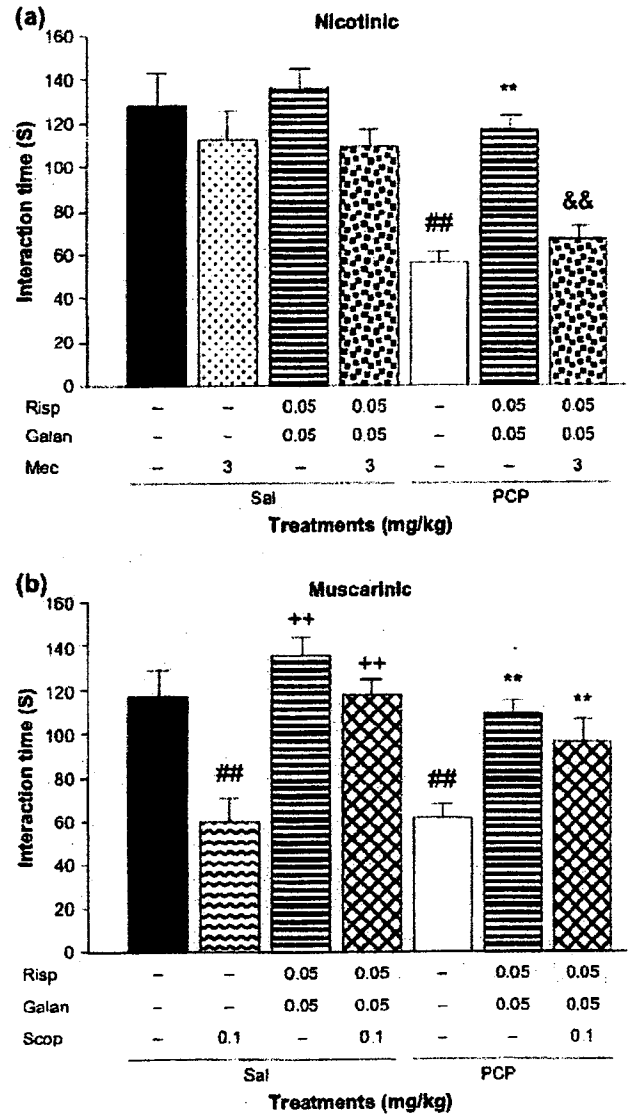

Fig. 4. The synergistic effect of galantamine with risperidone on PCP-induced impairment of social interaction was abrogated by the nAChR antagonist mecamylamine, but not the muscarinic AChR antagonist scopolamine. (a) The synergistic effect was abrogated by the nAChR antagonist mecamylamine (3 mg/kg), $F_{6,78} = 9.793$ ($p < 0.01$). (b) The synergistic effect was not abrogated by the mAChR antagonist scopolamine (0.1 mg/kg), $F_{6,77} = 8.748$ ($p < 0.01$). Results are expressed as means + S.E.M., $n = 10$–12, analyzed by a one-way ANOVA, followed by the modified Tukey's test for multiple comparisons. $^{\#\#}p < 0.01$, compared to Sal/Veh-treated group. $^{++}p < 0.01$, compared to Sal/Scop-treated group. $^{**}p < 0.01$, compared to PCP/Veh-treated group. $^{\&\&}p < 0.01$, compared to PCP/Risp/Galan-treated group. Sal: saline; PCP: phencyclidine; Risp: risperidone; Galan: galantamine; Mec: mecamylamine; Scop: scopolamine.

a relatively lower dose of 0.1 mg/kg (Fig. 4b), which is just enough to induce behavioral deficit in normal mice in our preliminary experiments. Scopolamine at the dose of 0.1 mg/kg did not abrogate the synergistic effect of galantamine (0.05 mg/kg) with risperidone (0.05 mg/kg) in PCP-treated mice (Fig. 4b). In order to understand it well, we also

investigated the effect of the co-administration followed by injection of scopolamine (0.1 mg/kg) in normal mice. The treatment with scopolamine at the dose of 0.1 mg/kg also failed to abrogate the effect of the co-administration in normal mice (Fig. 4b). In other words, the impairing effect of scopolamine at 0.1 mg/kg was compensated by the co-administration of risperidone (0.05 mg/kg) and galantamine (0.05 mg/kg).

3.4. Synergistic effect of galantamine with risperidone was mediated by D_1 receptors

Mesocortical dopaminergic neurotransmission through D_1 receptors is thought to be correlated with the negative symptoms of schizophrenia (Abi-Dargham, 2004; Albert et al., 2004; Kolb, 1990). We investigated whether the synergistic effect of galantamine with risperidone is mediated by dopaminergic D_1 receptors.

As shown in Fig. 5a, the effect of the co-administration of risperidone and galantamine on the impairment of social interaction in PCP-treated mice was abrogated by systemic administration of a D_1 receptor antagonist, SCH 23390 (0.02 mg/kg) ($p < 0.01$).

The mesocortical dopaminergic system projects to the PFC. In order to know whether the effects of the co-administration of risperidone and galantamine on the impairment of social interaction is mediated by D_1 receptors in the PFC, intra-mPFC microinfusion of SCH 23390 was performed in the present study. SCH 23390 (0.02 μg/0.5 μL/mouse) was infused into the mPFC of the PCP-treated mice 45 min after the co-administration of risperidone (0.05 mg/kg) and galantamine (0.05 mg/kg). The mPFC-local microinfusion of the D_1 receptor antagonist SCH 23390 abrogated the ameliorating effect of the co-administration on the impairment of social interaction in PCP-treated mice ($p < 0.01$) 15 min after the microinfusion (Fig. 5b).

3.5. Synergistic effect of galantamine with risperidone on extracellular concentration of dopamine in mPFC of PCP-treated mice and involvement of nAChR in the effect

As shown in Fig. 6a, basal extracellular concentration of dopamine decreased in the mPFC of PCP-treated mice compared with saline-treated mice, consistent with previous publications (Jentsch et al., 1998a,b; Jentsch and Roth, 1999). The change in the basal level was mirrored in the dopaminergic response to potassium stimulation at high concentration, which was observed in another preliminary study in our laboratory, in which the release of dopamine in the mPFC of PCP-treated (10 mg/kg, 14 days) mice was insensitive to the potassium stimulation.

The extracellular concentration of dopamine in the mPFC of PCP-treated mice was not increased significantly by treating animals with risperidone (0.05 mg/kg) or galantamine (0.05 mg/kg) alone. In contrast to the individual treatments at their non-effective doses, the co-administration of risperidone (0.05 mg/kg) and galantamine (0.05 mg/kg) significantly

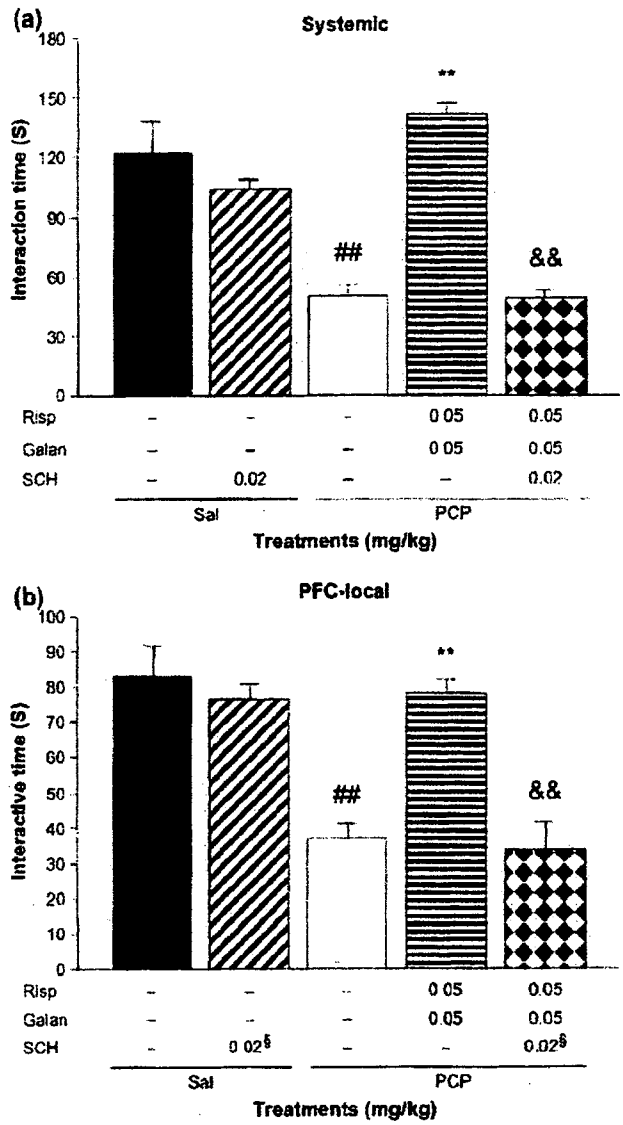

Fig. 5. The synergistic effect of galantamine with risperidone was mediated by D_1 receptors. (a) Systemic administration of SCH 23390 (0.02 mg/kg). $F_{3,19} = 19.595$ ($p < 0.01$). (b) mPFC-local microinjection of SCH (0.02 μg/0.5 μL/mouse). $F_{3,42} = 13.367$ ($p < 0.01$). Results are expressed as means ± S.E.M., $n = 9$–12, analyzed by a one-way ANOVA, followed by the modified Tukey's test for multiple comparisons. $^{\#\#}p < 0.01$, compared to Sal/Veh-treated group. $^{**}p < 0.01$, compared to PCP/Veh-treated group. $^{\&\&}p < 0.01$, compared to PCP/Risp/Galan-treated group. Sal: saline; PCP: phencyclidine; Risp: risperidone; Galan: galantamine; SCH: SCH 23390

increased extracellular concentration of dopamine 60 min after the co-administration (Fig. 6b).

Ichikawa et al. (2002) have reported that risperidone increases cortical acetylcholine release, which in turn may promote dopamine release. In the present study, the increasing effect of the co-administration on extracellular concentration of dopamine was abrogated by the nAChR antagonist mecamylamine (3 mg/kg, s.c. injected 20 min after the co-administration). The individual administration of mecamylamine itself at the dose of 3 mg/kg did not significantly change the

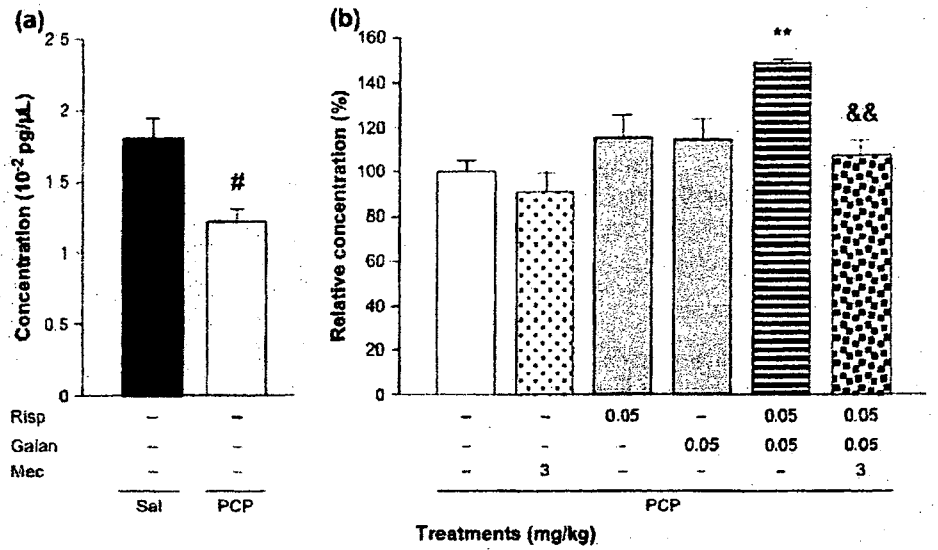

Fig. 6. Synergistic effect of galantamine with risperidone on extracellular concentration of dopamine in the mPFC of PCP-treated mice and the involvement of nAChR in the effect. (a) Basal extracellular concentration of dopamine in the mPFC of Sal- and PCP-treated mice. $^{\#}p < 0.05$, compared to Sal-treated group. Results are expressed as means ± S.E.M., $n = 3$–5, analyzed by Student's t-test. (b) Synergistic effect of galantamine with risperidone and the involvement of nAChR in the effect. Extracellular concentration of dopamine in the mPFC was tested 60 min after the co-administration. Results are expressed as means ± S.E.M., $n = 4$–5. $F_{5,23} = 6.294$ ($p < 0.01$), analyzed by a one-way ANOVA, followed by the modified Tukey's test for multiple comparisons. $^{**}p < 0.01$, compared to PCP/Vel-treated group. $^{\&\&}p < 0.01$, compared to PCP/Risp/Galan-treated group. Sal: saline; PCP: phencyclidine; Risp: risperidone; Galan: galantamine; Mec: mecamylamine.

extracellular concentration of dopamine in the mPFC of PCP-treated mice (Fig. 6b).

4. Discussion

PCP induces psychomimetic state that closely resembles schizophrenia and incorporates the positive, negative and cognitive symptoms of schizophrenia (Javitt and Zukin, 1991; Nabeshima et al., 1989; Noda et al., 1995). Acute treatment with PCP increases dopamine release in the striatum including nucleus accumbens (Balla et al., 2001; Greenslade and Mitchell, 2004). In contrast to the consequence of acute PCP exposure, repeated treatment with PCP reduces basal extracellular concentration of dopamine in the PFC as shown in the present study, which simulates the prominent characteristic of dopaminergic dysfunction in the PFC of schizophrenia patients that accounts for the negative symptoms (Abi-Dargham, 2004; Albert et al., 2004; Fink-Jensen, 2000; Kolb, 1990).

Social withdrawal is a key component of the negative symptoms, and social behavior plays an important role in the natural behavior of mice (Qiao et al., 2001). There are a number of studies describing PCP-induced impairment of social interaction in rats and monkeys that may serve as models of the negative symptoms of schizophrenia (Corbett et al., 1993; Qiao et al., 2001; Sams-Dodd, 1996; Schlemmer and Davis, 1983). Compared with the acute treatment, repeated PCP exposure-induced impairment of social interaction would be more relevant to social dysfunction in schizophrenia because this deficit in schizophrenia is enduring and not usually accompanied by transient confusion (Qiao et al., 2001). The PCP-induced impairment of social interaction is not due to motor dysfunction since the locomotor activity in the mice is not affected by the repeated PCP treatment without challenge (Noda et al., 1995; Qiao et al., 2001).

As noted above, dopaminergic dysfunction in the PFC constitutes a prominent characteristic of schizophrenia that accounts for the negative symptoms. In the present study, repeated PCP treatment induced dopaminergic hypofunction in the mPFC, and the co-administration of risperidone and galantamine improved the dopamine release in the mPFC. The striatal and limbic dopaminergic functions are under inhibitory control of the PFC, and lesions of the dopaminergic terminals in the mPFC lead to enhanced dopamine release in the nucleus accumbens (mesolimbic) and other nuclei of basal ganglia (Mitchell and Gratton, 1992; Pycock et al., 1980). These phenomena suggest that the PFC-dopaminergic hypofunction that causes the negative symptoms may exacerbate the positive symptoms, and vice versa.

Typical neuroleptics generally impair social behavior in human and animals whereas the atypical antipsychotic risperidone improved social behavior in PCP-treated mice at a dose of 0.1 mg/kg in the present study. Interestingly, a medicine for Alzheimer's disease, galantamine, also ameliorated the impairment of social interaction at the dose of 0.3 mg/kg. Further, the co-administration of risperidone and galantamine at their non-effective doses of 0.05 mg/kg showed significant effect on the impairment. These results indicate that galantamine may be effective in treating the negative symptom of schizophrenia and it may have synergistic effect with risperidone. The synergistic effect of galantamine with risperidone

was abolished by the systemic administration of a D_1 receptor antagonist, SCH 23390, at a dose that did not significantly affect the general behavior of normal mice, and also abolished by mPFC-local microinfusion of SCH 23390. Further, risperidone and galantamine synergistically increased dopamine release in the mPFC. These data confirmed that the synergistic effect of galantamine with risperidone on the impairment of social interaction is mediated by D_1 receptors in the mPFC through increased dopamine release.

It has been reported that risperidone increases cortical acetylcholine release without affecting the release in the nucleus accumbens and dorsal striatum (Ichikawa et al., 2002). The cortical acetylcholine release-increasing effect of risperidone is indispensable in elucidating the mechanism of the synergistic effects of galantamine with risperidone in the present study. Activation of nAChRs promotes the release of DA (Cao et al., 2005; Salminen et al., 2004; Wonnacott, 1997; Zhang et al., 2004), and this notion is consistent with the data of another study on dopamine release-increasing effect of galantamine, in which galantamine promotes DA release by allosterically potentiating nAChR (Wang et al., in press). Galantamine improves the efficiency of coupling between the binding of acetylcholine and the assumption of the open or activated channel configuration of nAChRs, and decreases the likelihood of the problematic rapid desensitization subsequent to the binding of nAChR agonists (Deutsch et al., 2005; Friedman, 2004). In the present study, the synergistic effect of galantamine with risperidone on the release of dopamine in the mPFC was abrogated by the nAChR antagonist mecamylamine. These findings clearly showed that the synergistic effect of the co-administration on the release of dopamine is mediated, at least partially, through the activation of nAChRs. As a potent allosteric potentiating ligand of nAChR, galantamine takes effect in the same window of concentrations (i.e., 0.1–1 μM), which correlates with the cerebrospinal fluid concentration of the drug at the recommended daily dosage (Samochocki et al., 2003). Galantamine is also a rapidly reversible and rather modest AChE inhibitor (IC_{50} in the frontal cortex and the hippocampus of mouse and human in the range from 2.8 to 3.9 μM) (Bickel et al., 1991b; Samochocki et al., 2003; Thomsen et al., 1991). At the doses used in the present study that are far below those required to reach its IC_{50} value for AChE inhibition, the effect of galantamine on the release of dopamine in the mPFC mainly arises from allosteric potentiation of the nAChR (Bickel et al., 1991a; Farlow, 2003; Scott and Goa, 2000; Samochocki et al., 2003), but not from the inhibition of AChE, which is supported by the publication that there is only 1–12% brain AChE inhibition 1 h after s.c. injection of galantamine at 3 mg/kg (Geerts et al., 2005), and in the present study the treatment with galantamine at 3 mg/kg was not more effective than that at 0.3 mg/kg. It is worth mentioning that in contrast to the PFC, the mesolimbic dopamine release related with positive symptoms is not affected by galantamine in the experiment of Sharp et al. (2004).

The deficit in the nAChR-dopaminergic systems is one of the multi-facets of neuronal degeneration induced by PCP treatment. As shown in the present study, the synergistic effect of galantamine with risperidone at the present doses quite depends on the function of nAChR-dopaminergic systems in PCP-treated mice, therefore the effect is 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, since they can somewhat be restored by compensating mechanisms that are not very clear until now.

Although the atypical antipsychotic risperidone exhibits relatively lower extrapyramidal side effects in clinical practice and catalepsy in animals compared to conventional antipsychotics, its extrapyramidal side effects increases dose-dependently, in which mAChR subtypes are closely involved (Corbett et al., 1993; Dagaev et al., 2004; Mesotten et al., 1989). Galantamine is an allosteric potentiating ligand of nAChRs, but not mAChRs (Samochocki et al., 2003). The activation of the nAChRs may ameliorate the extrapyramidal side effects (Kumari and Postma, 2005). Although galantamine is only a rather modest AChE inhibitor (IC_{50} value of 2.8–3.9 μM) compared to other AChE inhibitors presently used in clinical trials, such as rivastigmine (IC_{50} value of 4 nM) and donepezil (IC_{50} value of 15–24 nM), its inhibiting effect can be dose-dependently increased. In addition, as donepezil and rivastigmine, galantamine acts as a nAChR inhibitor at very high concentration (>10 μM) by direct blockade of ACh-activated channels (Samochocki et al., 2003; Sharp et al., 2004). Based on the above reasons and the fact that galantamine is effective to improve the social behavior synergistically at a relatively low dose that mainly allosterically potentiates nAChRs, we suppose that it is preferable to use galantamine at relatively lower doses, and it is most desirable to develop new derivatives of galantamine preserving the nAChR-allosteric potentiating effect and being devoid of AChE-inhibiting effect for treating the negative symptoms of schizophrenia.

5. Conclusion

Galantamine has synergistic effect with risperidone on the impairment of social interaction induced by repeated PCP treatment, and the co-administration of risperidone and galantamine may be used as a new strategy for treating the negative symptoms of schizophrenia. The synergistic effect may be mediated by D_1 receptors in the mPFC through nAChR activation-increased dopamine release.

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Synergistic effect of combined treatment with risperidone and galantamine on phencyclidine-induced impairment of latent visuospatial learning and memory: Role of nAChR activation-dependent increase of dopamine D_1 receptor-mediated neurotransmission

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Abstract

The clinically achievable efficacy of the atypical antipsychotics on cognitive symptoms of schizophrenia is practically limited by their dose-dependent side effects. Thus, there is the need for adjuvant treatments or strategies for the cognitive impairments. Further, human autopsy and genetic data in schizophrenia have indicated the existence of the abnormality of nicotinic acetylcholine receptors (nAChR). In the present study, we aimed to investigate the synergistic effect and mechanisms of a combined treatment with an atypical antipsychotic risperidone and galantamine, which is a nAChR-allosteric modulator and a modest cholinesterase inhibitor, on the impairment of latent visuospatial learning and memory in mice resembling the cognitive impairment of schizophrenia. Repeated treatment with phencyclidine (PCP, 10 mg/kg, 14 days)-induced cognitive impairment in mice in a one trial water-finding test was used as a model of the cognitive impairment of schizophrenia. In vivo microdialysis was used to investigate the extracellular concentration of dopamine in the medial prefrontal cortex (mPFC). Combined treatment with galantamine and risperidone, at low, ineffective doses (both at 0.05 mg/kg) showed a synergistic effect to reverse cognitive impairment and increase extracellular concentration of dopamine in the mPFC. The synergistic behavioral effect was abolished by a dopamine-D_1 receptor antagonist, SCH 23390, and a nAChR antagonist, mecamylamine, but not a muscarinic AChR (mAChR) antagonist, scopolamine. Mecamylamine also blocked the synergistic effect on dopamine release in the mPFC of PCP-treated mice. The study indicates that galantamine and risperidone may have synergistic effect on the cognitive impairments in schizophrenia patients by synergistically promoting the nAChR activation-dependent increase of dopamine D_1 receptor-mediated neurotransmission.

Keywords: Phencyclidine; Cognitive impairment; Schizophrenia; Risperidone; Galantamine; Synergistic effect; nAChR; Dopamine

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1. Introduction

The symptoms of schizophrenia are classified as positive (e.g., hallucination, delusion), negative (e.g., anhedonia, social withdrawal or poor social interaction) and cognitive symptoms (e.g., deficits in attention, working memory, mental flexibility), most of which are related with dopaminergic aberration (Abi-Dargham, 2004). Among the dopaminergic projections, the mesolimbic and mesocortical pathways are tightly involved in the pathophysiology of schizophrenia. The positive symptoms are thought to arise from a subcortical hyperstimulation of dopamine D_2 receptors, especially in striatal areas, whereas the negative and cognitive symptoms arise from a cortical dopaminergic neurotransmission mediated by dopamine-D_1 receptors in the dorsolateral prefrontal cortex (PFC) in schizophrenia patients that corresponds to the medial PFC (mPFC) in rodents (Abi-Dargham, 2004; Albert et al., 2002; Fink-Jensen, 2000; Kolb, 1990).

Although the aberration of dopaminergic system is critical in the pathophysiology of schizophrenia, other neurotransmitter systems are more or less involved in the pathophysiology of schizophrenia and interact with the dopaminergic system (Fink-Jensen, 2000; Javitt and Zukin, 1991; Noda et al., 2000, 2001). PCP, a non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, induces psychotomimetic states in humans and rodents, incorporating not only the positive symptoms (e.g. hallucinations, paranoia) but also the negative symptoms (e.g. social withdrawal, motor retardation) and cognitive deficits (e.g., impairment of attention and working memory), thus, PCP-treated animals have been proposed as a preclinical model of schizophrenia (Castner et al., 2004; Javitt and Zukin, 1991; Morris et al., 2005; Noda et al., 1995, 2000, 2001).

Deficits in attention and information-processing mechanisms have been suggested to play a critical role in schizophrenia, therefore, the study of cognitive function related to sensory information or attention has been of central importance in the attempt to understand this disorder (Noda et al., 2001). The water-finding test is thought to be a latent visuospatial learning and memory paradigm related to the ability to sort visuospatial information and to attention process (Mackintosh, 1975; Ichihara et al., 1993). This test does not need any motivation to train animals, and animals are deprived of water only before the testing trial (Ichihara et al., 1993). The end of the water nozzle is set further above the floor in the testing trial than in training to decrease the probability of being found by chance. The PCP-induced behavioral deficit in mice in this paradigm best resembles the facts found in a clinical task made by Daniel et al. (2006) that schizophrenia patients have deficits in localizing the objects in the space that they previously explored, and in remembering the spatial relations among landmarks in the environment. Alternatively, the deficit in PCP-treated mice in the water-finding test resembles the poor performance in schizophrenia patients in the typical object-relocation task, independent of overall intellectual ability (Gillett, 2002; Van't Wout et al., 2006). There are also many other reports that indicate the visuospatial deficits in schizophrenia patients (Bilder et al., 2000; Brewer et al., 2005; Gabrovska et al., 1997; Glahn et al., 1997), although the precise nature of the deficit still remains unclear. Among those reports, Maruff et al. (1995) have reported the asymmetries in the covert orienting of visual spatial attention in schizophrenia, and this attentional deficit is dynamic and may reflect disruption to the neurocognitive network controlling attention at the level of the anterior cingulate cortex in the PFC. Poor performance in figure-ground segregation has also been found in schizophrenic patients in several visuospatial tests like the hidden figures test or the embedded figures test, which require the observers to identify which one of several simple figures (perceptually present) is hidden in a complex visual configuration (Loas, 2004).

Risperidone is an atypical antipsychotic drug with antagonistic properties at D_2, 5-HT_{2A} and α_1 receptors (Shayegan and Stahl, 2004). It has much better efficacy on the positive symptoms of schizophrenia than conventional neuroleptics (Khan, 1997). Risperidone also has some effects on the cognitive symptoms, however these effects are practically limited by various side effects. Therefore, there is still the need for adjuvant drugs for the cognitive symptom. A number of studies have indicated that there is a deficit with the nicotinic acetylcholine receptors (nAChRs) in the PFC of schizophrenia patients (Arnold et al., 2004; Deutsch et al., 2005), which has been postulated to be related with the cognitive symptoms (Kumari and Postma, 2005). It has been found in clinical surveys that nicotine-containing cigarettes improve cognitive function in schizophrenia patients compared with nicotine-free cigarettes, which is supposed to reflect nicotine's ability to raise dopamine levels in the PFC (Kumari and Postma, 2005). Galantamine, a medicine for Alzheimer's disease, is an allosteric modulator of nAChRs, and the weakest acetylcholinesterase (AChE) inhibitor among the three cholinesterase (ChE) inhibitors presently used in clinical trials (Samochocki et al., 2003; Sharp et al., 2004). In a clinical case report, galantamine enhanced cognition in 5 schizophrenia patients treated with clozapine (Bora et al., 2005), which is an atypical antipsychotic.

The present study was designed to test the hypothesis that co-administration of galantamine and risperidone synergistically attenuates cognitive deficit in a PCP-treated animal model of schizophrenia, and to analyze the mechanism underlying the effect.

2. Methods and materials

2.1. Animals

Male mice of the ICR strain (Japan SLC Inc., Shizuoka, Japan), 6 weeks old at the beginning of experiments, were used. They were housed in plastic cages, five mice per cage through out the research, received food (CE2; Clea Japan Inc., Tokyo, Japan) and water ad libitum, and were maintained on a 12/12-h light/dark cycle (lights on from 8:00 AM to 8:00 PM). Behavioral experiments were carried out in a sound-attenuated and air-regulated experimental room, to which mice were habituated for at least 1 h. All experiments were performed following the Guidelines for Animal Experiments of Nagoya University, which conformed to the international guidelines set out in the "Guide for the Care and Use of laboratory Animals" (ILAR-NRC publication, revised in 1996).