Laboratory Animals of Tohoku University. The experimental protocol was approved by the Animal Care and Use Committee, Tohoku University Graduate School of Medicine.

Drugs

For locomotor testing, methamphetamine hydrochloride (Dainippon-Sumitomo Pharmaceuticals, Osaka, JAPAN) dissolved in 0.9% sodium chloride (saline) was administered subcutaneously in a volume of 10 ml/kg.

Measurement of locomotor activity

Mice were placed individually in clear plastic cages (40×30×26 cm), and locomotor activity was measured in 5-min bins using digital counters with passive infrared sensors (Supermex system, Muromachi Kikai, Tokyo, Japan). In MAP-induced locomotor activity tests, the subjects were first habituated to the apparatus for 180 min and then injected with the drug or saline control subcutaneously. Locomotor activity was then assessed for 60 min post-injection. In the locomotor sensitization paradigm, mice were habituated to the test environment for 180 min and then injected with MAP (1 or 2 mg/kg) or saline subcutaneously, and locomotor activity was assessed for 60 min post-injection. This procedure was conducted eight times; sessions 1–7 were performed at 2-day intervals, and session 8 occurred 7 days after the last injection.

Statistical analysis

All data are presented as the mean±SEM. Multipoint measurements of locomotor activity in a novel environment were submitted to two-way mixed design analysis of variance (ANOVA), with time as the within-subjects factor and genotype as the between-subjects factor. MAP-induced locomotor sensitization data were submitted to two-way mixed design ANOVA, with genotype as the between-subjects factor and session as the within-subjects factor. For post hoc comparisons, Dunnett's t test was applied. Contrasted to all the above data, data from the acute MAP injection were not normally distributed. By this reason, only the acute administration data were submitted to Kruskal-Wallis test followed by Wilcoxon test with Bonferroni correction for each genotype in each drug dose. All data analyses were performed using the Statistical Package for the Social Sciences (SPSS, Tokyo, Japan).

Results

DAT/VMAT2 double heterozygous mice (DAT+/–VMAT2+/–), as well as DAT heterozygous (DAT+/–), VMAT2 heterozygous (VMAT2+/–), and WT mice did not exhibit any gross behavioral or anatomical differences. There was no difference in the rate of weight gain between the littermates of the four genotypes (data not shown).

Baseline locomotion

All mice (WT, DAT+/–, VMAT2+/–, and DAT+/–VMAT2+/–) showed the normal pattern of locomotor activity in a novel environment, with initially elevated levels that habituated to near-zero levels by the end of the period of testing (Fig. 1). There was no significant genotype effect on baseline locomotion among WT, DAT+/–, VMAT2+/–, and DAT+/–VMAT2+/– mice ($F_{3,\ 76}=1.99$, $p>0.05$).

Locomotor-stimulating effects of acute methamphetamine treatment

Analysis of the locomotor response to acute MAP treatment revealed a significant effect of dose in WT, DAT+/–, VMAT2+/–, and DAT+/–VMAT2+/– mice (WT: $\chi^2=16.65$, $p<0.001$; DAT+/–: $\chi^2=13.37$, $p<0.01$; VMAT2+/–: $\chi^2=10.77$, $p<0.01$; DAT+/–VMAT2+/–: $\chi^2=7.45$, $p<0.05$; Fig. 2). In post hoc comparisons, administration of 1 mg/kg MAP caused significant locomotor increases in WT and VMAT2+/– mice ($p<0.01$, compared to saline, respectively), but it produced no significant locomotor increases in genotypes with heterozygous deletion of DAT (DAT+/– and DAT+/–VMAT2+/– mice). Administration of 2 mg/kg MAP caused significant locomotor increase in all four genotypes: WT, DAT+/–, VMAT2, and DAT+/–VMAT2+/– (WT: $p<0.001$; DAT+/–, VMAT2+/–, DAT+/–VMAT2+/–: $p<0.01$;

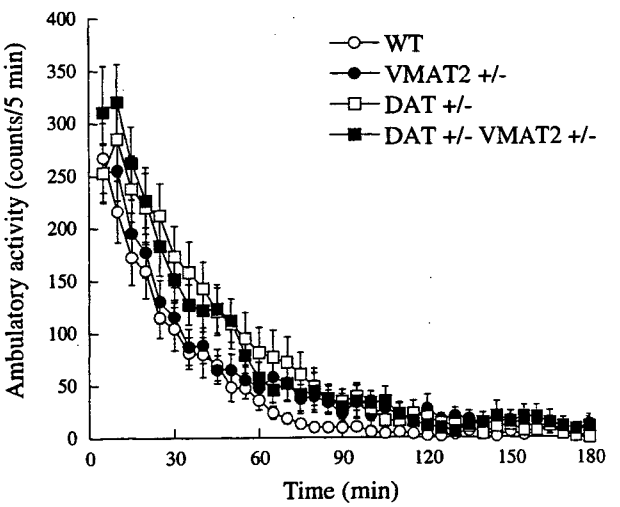

Fig. 1 Time course of locomotor activity to novelty environment in DAT and/or VMAT2 mutant mice. Time course of ambulatory activity (*beam breaks*) in WT, VMAT2+/–, DAT+/–, DAT+/–VMAT2+/– mice. Values represent mean±SEM. Number of mice (N)=19~20 per genotype

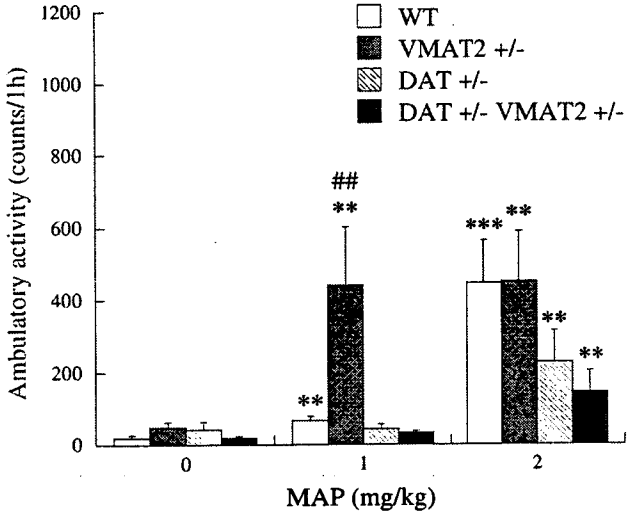

Fig. 2 Dose–response study of MAP-induced locomotor activity in mice with heterozygous deletion of the DAT and/or VMAT2 genes. Total ambulatory activity for 60 min after drug administration (saline or 1 or 2 mg/kg MAP s.c.) in WT, VMAT2+/–, DAT+/–, and DAT+/–VMAT2+/–mice. *Asterisk* Significant difference from saline treatment (**$p<0.01$, ***$p<0.001$, Wilcoxon test), *double number sign* significant difference from WT ($p<0.01$, Wilcoxon test). Values represent mean±SEM. $N=12\sim20$ per genotype

compared to saline, respectively). Treatment with 1 mg/kg MAP was significantly affected by genotype ($\chi^2=17.02$, $p<0.001$), and when analyzed separately in post hoc comparisons, VMAT2+/– showed significantly greater increases in locomotor activity ($p<0.01$) compared to all other genotypes. However, after treatment with 2 mg/kg MAP, there was no significant overall effect of genotype.

Locomotor sensitization by repeated methamphetamine injections

Repeated saline injection produced no significant change in locomotor activity in any of the four genotypes: WT, DAT+/–, VMAT2+/–, and DAT+/–VMAT2+/– (Fig. 3a). Repeated 1 mg/kg MAP injections produced a significant increase in locomotor response (session $F_{7,\ 476}=23.8$, $p<0.0001$) that differed among genotypes as demonstrated by a significant genotype × session interaction ($F_{21,\ 476}=3.36$, $p<0.001$), reflecting an increase in locomotor activity over test sessions in some groups but not others. VMAT2+/– and WT mice exhibited substantial sensitization of the locomotor stimulant effects of MAP, although VMAT2+/– mice showed significant higher locomotor activity compared to WT in sessions 1 and 2 ($p<0.01$). By contrast, DAT+/– and DAT+/–VMAT2+/– mice showed lower locomotor responses over test sessions compared to WT mice (genotype × session: $F_{7,\ 252}=8.09$, $p<0.001$ for DAT+/–; $F_{7,245}=4.27$, $p<0.05$ for DAT+/–VMAT2+/–). In comparing locomotor activity among genotypes for each session, DAT+/– and DAT+/–VMAT2+/– mice had significantly lower locomotor activity compared to WT mice from sessions 5 to 8 ($p<0.05$). Thus, although DAT+/– and DAT+/–VMAT2+/– mice did show some sensitization, this was greatly reduced compared to WT mice. In individual post hoc ANOVA of each genotype, MAP significantly increased locomotor activity in WT mice from sessions 5 to 8 ($p<0.05$ compared to session 1), but in DAT+/–, VMAT2+/–, and DAT+/–VMAT2+/– mice, MAP-induced locomotor activity was enhanced compared to values in session 1 only after sessions 7 and 8 ($p<0.05$ compared to session 1). These data demonstrate a delayed and attenuated development of sensitization in DAT+/– and DAT+/–VMAT2+/– mice compared to WT mice and a slight delay in VMAT2+/– mice (Fig. 3b).

Repeated MAP injections with a higher dose (2 mg/kg) produced a significant increase in the locomotor response overall (session $F_{7,\ 336}=11.02$, $p<0.0001$) that differed between groups as demonstrated by a significant genotype × session interaction ($F_{21,\ 336}=2.68$, $p<0.001$), again representing differences in sensitization dependent upon genotype. DAT+/– and DAT+/–VMAT2+/– mice had reduced locomotor responses initially compared to WT mice but eventually reached the same levels of the activity by session 8, whereas VMAT 2+/– mice showed greater locomotor responses from sessions 4 to 6, which also stabilized at near-WT levels by session 7 (genotype × session: $F_{7,\ 147}=3.84$, $p<0.01$). Individual post hoc tests for each genotype indicated that MAP significantly increased locomotor activity at sessions 4, 7, and 8 ($p<0.05$, compared to session 1) in WT mice, at sessions 4, 5, and 6 ($p<0.05$, compared to session 1) in VMAT2+/– mice, at sessions 6 and 8 ($p<0.05$, compared to session 1) in DAT+/– mice, and at sessions 6, 7, and 8 ($p<0.05$, compared to session 1) in DAT+/–VMAT2+/– mice. As was observed after sensitization with the lower dose of MAP, the development of sensitization was delayed in DAT+/– and DAT+/–VMAT2+/– mice compared to WT mice after repeated injection of 2 mg/kg MAP. VMAT2+/– mice showed significantly higher locomotor responses in sessions 5 and 6 compared to WT mice ($p<0.05$), but this difference was not observed in later sessions (Fig. 3c).

Discussion

Our results suggest that the reduced DAT and VMAT2 expression have different effects on MAP-induced locomotor responses and the development of sensitization and, furthermore, that the reduced expression of the DAT gene has a greater influence on MAP-induced sensitization than the reduced expression of the VMAT2 gene.

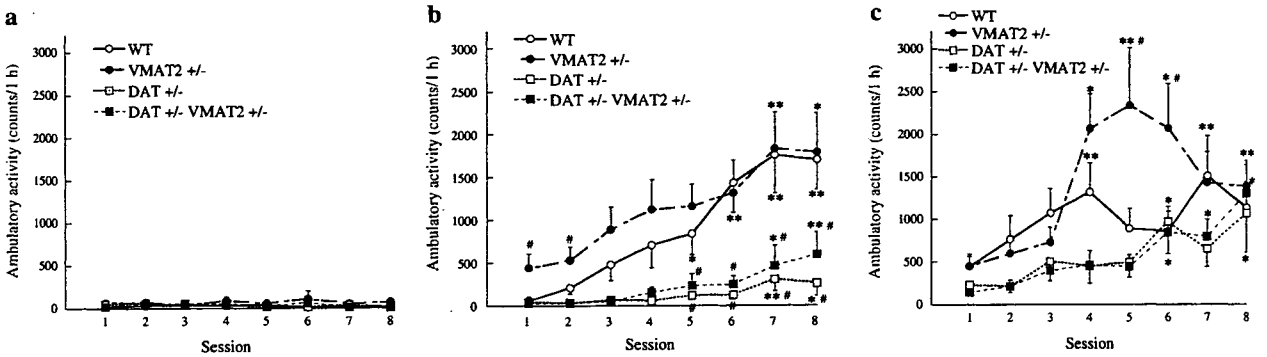

Fig. 3 **a–c** Sensitization of MAP-induced locomotor activity in mice with heterozygous deletion of the DAT and/or VMAT2 genes. Total ambulatory activity for 60 min of testing after saline (**a**), 1 mg/kg MAP (**b**), 2 mg/kg MAP (**c**) with 2-day intervals (sessions 1–7) and 1 week interval (session 8). Repeated administration of MAP in WT, VMAT2+/–, DAT+/–, and DAT+/–VMAT2+/–mice. *Asterisk* Significant difference from session 1 (*$p<0.05$, **$p<0.01$, Dunnett's *t* test), *number sign* significant difference from WT ($p<0.05$, Dunnett's *t* test). Values represent mean±SEM. $N=12\sim20$ per genotype

Acute administration of 1 mg/kg MAP did not increase locomotor activity in heterozygous DAT KO mice, while a higher dose of MAP (2 mg/kg) did induce a significant increase in locomotor activity in those mice compared to saline controls, but the magnitude of this increase was a half of that observed in WT mice. These data indicate a rightward shift in the dose–response curve for MAP in heterozygous DAT KO mice. Reports have suggested that DAT blockers suppress amphetamine-mediated dopamine release (Baumann et al. 2002; Sulzer et al. 1995; Villemagne et al. 1999) and that the dopamine-releasing action of amphetamines is dependent on DAT expression (Eshleman et al. 1994; Giros et al. 1996; Jones et al. 1998b). DAT expression in heterozygous DAT KO mice is reduced by half compared to WT mice (Sora et al. 1998, 2001). Thus, the decreased response of heterozygous DAT KO mice to acute MAP administration might reflect attenuation of MAP-induced dopamine release due to reduced DAT expression. Alternatively, changes in other aspects of dopamine neurotransmission in heterozygous DAT KO mice might underlie the decreased response to MAP administration in these mice. Of importance in considering these possibilities are previous in vivo microdialysis data demonstrating that heterozygous deletion of the DAT gene did not alter basal extracellular dopamine in the striatum or nucleus accumbens of mutant mice (Shen et al. 2004). While tissue dopamine concentrations and dopamine D2 receptor expression in the striatum were not reported to be altered in heterozygous DAT KO mice compared to WT mice (Sora et al. 2001), other studies have reported changes in dopaminergic function in heterozygous DAT KO mice. Using other methods in heterozygous DAT KO mice, in vivo quantitative microdialysis has demonstrated that heterozygous deletion of DAT induces a twofold elevation of basal extracellular dopamine level in the striatum (Jones et al. 1998a), while electrically stimulated dopamine release and the dopamine clearance rate constant are reduced by half compared to WT mice (Giros et al. 1996; Jones et al. 1998a), and tissue dopamine concentrations and tyrosine hydroxylase levels in the striatum were reported to be decreased (Jones et al. 1998a). In addition, dopamine D1 and D2 receptor mRNA levels are down-regulated in the basal ganglia of heterozygous DAT KO mice (Giros et al. 1996). Given the conflicting and apparently opposing changes in various indices of dopamine neurotransmission, the mechanism of the reduced behavioral responses of heterozygous DAT KO mice to MAP administration cannot be determined absolutely, although it appears likely that reduced DAT expression itself may be the main mechanism.

In contrast to the effects of heterozygous DAT KO, it is interesting to note that 1 mg/kg MAP administration had greater effects on locomotor activity in heterozygous VMAT2 KO mice than in WT mice. These effects with MAP are consistent with previous findings that heterozygous deletion of VMAT2 increased the locomotor-stimulating effects of *d*-amphetamine (Takahashi et al. 1997; Wang et al. 1997). Single 2 mg/kg MAP administration also increased locomotion of heterozygous VMAT2 KO mice, but the magnitude of the increase was equivalent to the increase observed in WT mice, indicating that VMAT2 KO induces a leftward shift in the dose–response curve. Previous in vivo microdialysis data have demonstrated that basal and *d*-amphetamine-induced extracellular dopamine in the striatum is reduced in heterozygous VMAT2 KO mice (Wang et al. 1997). In addition, amphetamine-induced dopamine release from midbrain cell cultures from heterozygous VMAT2 KO mice was reduced (Fon et al. 1997). In these mutant mice, striatal dopamine D1 and D2 receptor mRNA and overall receptor binding were not altered (Takahashi et al. 1997; Wang et al. 1997). Wang et al. (1997)

suggested that functional changes in dopamine receptors may underlie the hypersensitivity to the low dose of MAP in heterozygous VMAT2 KO mice. Alternatively, serotonin and norepinephrine neurotransmissions that are also affected by heterozygous VMAT2 gene deletion may have some role in the increased sensitivity to MAP in heterozygous VMAT2 KO mice.

In mice with combined heterozygous KO of DAT and VMAT2, 1 mg/kg MAP did not induce hyperlocomotion. The behavioral patterns of the DAT/VMAT2 double KO mice were the same as heterozygous DAT KO mice. Heterozygous VMAT2 KO mice have been reported to have normal levels (Gainetdinov et al. 1998) or slight (16%) reductions (Takahashi et al. 1997) in the number of striatal DAT binding sites, and the dopamine uptake function of DAT in heterozygous VMAT2 KO mice was not different from WT mice (Gainetdinov et al. 1998). Nonetheless, baseline and *d*-amphetamine-induced extracellular dopamine in the striatum is reduced in heterozygous VMAT2 KO mice (Wang et al. 1997). Thus, in mice with double heterozygous KO of DAT and VMAT2, reduction in released dopamine in the synaptic cleft due to half deletion of VMAT2, concurrent with the decrease in reuptake due to half deletion of DAT, might prevent some alterations in dopamine function that would otherwise occur from either KO alone. At the same time, the MAP-induced elevation of extracellular dopamine in the striatum may be more greatly affected by combined deletion of VMAT2 and DAT than that observed by VMAT2 alone. In transgenic mice with 95% reduced expression of the VMAT2 (KA1 VMAT2 mutant), amphetamine-induced dopamine release of striatal slices was reduced compared to WT mice, and the DAT inhibitor GBR12935 further attenuated the dopamine release (Patel et al. 2003), suggesting a synergy of effects on MAP-induced dopamine release by reduction in both DAT and VMAT2 expressions. However, the finding that single-MAP treatment in mice with double heterozygous deletion of DAT and VMAT2 produced almost the same effect as in heterozygous DAT KO mice indicates that half deletion of DAT is the dominant factor determining the locomotor-stimulating effect of single MAP treatment compared to the effect of half deletion of VMAT2.

Repeated administration of 1 mg/kg MAP sensitized locomotor responses in heterozygous DAT KO mice. However, the magnitude of increase in locomotor activity was lower, and the development of sensitization was delayed compared to WT mice. Lasting locomotor inhibitory responses and delayed sensitization in heterozygous DAT KO mice could be attributed to the initial poor locomotor response that was observed after acute MAP treatment of these mice. Interestingly, the dose of MAP that did not induce locomotor responses after the first treatment led to sensitization after repeated treatments. Spielewoy et al. (2001) reported that heterozygous DAT KO mice showed hypolocomotor activity after administration of 1 mg/kg *d*-amphetamine but not after higher doses up to 10 mg/kg and furthermore that the effect of *d*-amphetamine was enhanced after repeated exposures to the drug. Contrary to the effect of *d*-amphetamine on heterozygous DAT KO mice, the current study found that administration of MAP did not induce a hypolocomotor effect in heterozygous DAT KO mice. One possibility that might explain this discrepancy is that Spielewoy et al. (2001) used mice backcrossed for 12 generations on a C57BL/6 background, whereas we used mice on a mixed 129Sv/C57B6 background. Genetic background of mice is an important factor that may affect the consequences of gene deletion (Schlussman et al. 1998; Zhang et al. 2001). Another factor that may have an impact upon studies is the amount of habituation to the environment before drug injection. In the study of Spielewoy et al. (2001), mice were placed in the activity boxes immediately after injection, whereas mice were habituated in the test box for 3 h, and basal activity was largely, although not completely, equalized in our study. The degree of habituation has long been known to influence the effect of psychostimulants on locomotor activity (Dews and Wenger 1977; Robbins 1977). After repeated administration of higher doses (2 mg/kg) MAP, we observed that both the onset of the establishment of sensitization and the magnitude of sensitization were accelerated compared to 1 mg/kg MAP injections in heterozygous DAT KO mice as well as in WT mice. This result may reflect dose-dependent increase in MAP-induced extracellular dopamine levels in heterozygous DAT KO mice.

In the 1 mg/kg MAP sensitization experiment, development of sensitization in heterozygous VMAT2 KO mice was delayed. Although MAP-induced dopamine release was reduced in heterozygous VMAT2 mice (Fon et al. 1997; Wang et al. 1997), they had a greater initial locomotor response to 1 mg/kg MAP administration, suggesting that a greater behavioral response to first MAP administration does not necessarily accelerate the development of sensitization, and released dopamine levels may play more dominant role in the development of sensitization. Alternatively, hyperactivity after single MAP treatment may induce some tolerance in locomotor responses to initial MAP injections in heterozygous VMAT2 mice. In 2 mg/kg MAP sensitization experiments, there was no difference in heterozygous VMAT2 mice in the onset of sensitization compared to WT mice, indicating that half deletion of VMAT2 has some effect on the development of MAP sensitization but is not sufficient to completely eliminate it.

In double heterozygous DAT/VMAT2 KO mice, repeated administration of either 1 mg/kg or 2 mg/kg MAP

induced sensitization. The magnitude of the locomotor response and latency to the onset of sensitization were similar to what was observed in heterozygous DAT KO mice but not heterozygous VMAT2 KO mice, suggesting that the half deletion of DAT is more crucial in determining the magnitude of locomotor responses in chronic MAP administration and latency to the establishment of sensitization than the half deletion of VMAT2.

In conclusion, behavioral effects of both acute and chronic MAP administration were suppressed in heterozygous DAT KO mice, whether or not it was combined with heterozygous VMAT2 KO. Contrary to the effect observed in heterozygous DAT KO mice, acute MAP administration produced greater locomotor responses in heterozygous VMAT2 KO mice. While repeated 1 mg/kg MAP administration induced sensitization to an equivalent extent in heterozygous VMAT2 KO and WT mice, the latency until the establishment of behavioral sensitization was delayed in the mutant mice. In response to repeated 2 mg/kg MAP injections, there was no difference in the onset of sensitization between heterozygous VMAT2 KO and WT mice. These findings indicate that the half deletion of DAT plays a major role in both acute and chronic behavioral responses to MAP, while the effect of the half deletion of VMAT2 is less prominent. These findings lead us to hypothesize that DAT variants may have more profound effects than VMAT2 variants on the clinically important consequences of acute and chronic MAP abuse in humans.

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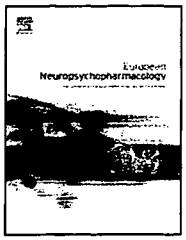

Phencyclidine-induced cognitive deficits in mice are improved by subsequent subchronic administration of the glycine transporter-1 inhibitor NFPS and D-serine

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Abstract

Accumulating evidence suggests that the glycine modulatory site on the NMDA receptor could be potential therapeutic target for cognitive deficits in schizophrenia. The present study was undertaken to examine the effects of the glycine transporter-1 (GlyT-1) inhibitor, (R)-(*N*-[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl])sarcosine (NFPS), on cognitive deficits in mice after repeated administration of the NMDA receptor antagonist phencyclidine (PCP). PCP (10 mg/kg/day for 10 days)-induced cognitive deficits were significantly improved by subsequent subchronic (2-week) administration of NFPS (1.0 and 3.0 mg/kg/day) or D-serine (600 mg/kg/day). However, PCP-induced cognitive deficits were not improved by a single administration of NFPS (3.0 mg/kg). Furthermore, Western blot analysis revealed that levels of GlyT-1 in the hippocampus, but not frontal cortex, of the PCP (10 mg/kg/day for 10 days)-treated mice were significantly higher than those of saline-treated mice. An *in vivo* microdialysis study revealed that repeated PCP administration significantly decreased the extracellular levels of glycine in the hippocampus, but not frontal cortex, of mice. These findings suggest that repeated PCP administration increased the density of GlyT-1 in the hippocampus of mouse brain, and that the GlyT-1 inhibitor NFPS could ameliorate cognitive deficits in mice after repeated administration of PCP.

1. Introduction

Cognitive deficits in patients with schizophrenia are a core feature of the illness and are predictive of vocational and social disabilities for patients (Coyle and Tsai, 2004; Freedman, 2003; Green et al., 2004; Kurtz, 2005). Several lines of evidence suggest that a dysfunction in the glutamatergic

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neurotransmission *via* *N*-methyl-D-aspartate (NMDA) receptor might be involved in the pathophysiology of schizophrenia (Javitt and Zukin, 1991; Olney and Farber, 1995; Krystal et al., 1999; Coyle and Tsai, 2004; Hashimoto et al., 2003, 2004, 2005c). NMDA receptor antagonists such as phencyclidine (PCP) and ketamine are known to induce schizophrenia-like symptoms including positive and negative symptoms, and cognitive deficits in healthy subjects (Javitt and Zukin, 1991; Krystal et al., 1999). Therefore, PCP has been used as an animal model of cognitive deficits in schizophrenia (Jentsch and Roth, 1999; Javitt et al., 2004; Hashimoto et al., 2005b, 2006, 2007). We reported that PCP-induced cognitive deficits in the novel object recognition test (NORT) could be significantly improved by subsequent subchronic (2-weeks) administration of clozapine, but not haloperidol (Hashimoto et al., 2005b). These findings suggest that reversal of PCP-induced cognitive deficits as measured by the NORT may be a potential animal model of atypical antipsychotic activity in relation to the amelioration of cognitive deficits in schizophrenia (Hashimoto et al., 2005b, 2006, 2007).

In the central nervous system (CNS), synaptic levels of glycine are regulated by specific sodium/chloride-dependent transporters. The actions of glycine are terminated by reuptake *via* two high-affinity glycine transporters referred to as GlyT-1 and GlyT-2. GlyT-1 and GlyT-2 possess 12 putative transmembrane spanning domains, and share approximately 50% amino acid sequence identity (Danysz and Parsons, 1998; Aragon and Lopez-Corcuera, 2005; Eulenberg et al., 2005; Smith et al., 1992). GlyT-1 is widely expressed in the CNS, where it exists predominantly on glial cells. GlyT-1 is likely responsible for glycine reuptake in forebrain areas, and in some regions it may be co-localized with strychnine-insensitive glycine sites on the NMDA receptors (Smith et al., 1992; Zafra et al., 1995; Cubelos et al., 2005). A recent study has demonstrated that glycine transport may keep local synaptic glycine levels very low, suggesting that GlyT-1 could play a role in regulating glutamatergic neurotransmission *via* NMDA receptors (Bergeron et al., 1998). Given the NMDA receptor hypofunction hypothesis in schizophrenia, it seems that the glycine modulatory site on the NMDA receptors is a potential therapeutic target for cognitive deficits and negative symptoms in patients with schizophrenia. The ability of glycine levels to modulate NMDA receptor-mediated neurotransmission suggests that pharmacological manipulation of synaptic glycine might be effective in the treatment of conditions involving a hypofunction of the NMDA receptors (Vandenberg and Aubrey, 2001; Javitt, 2002, 2006; Millan, 2002; Coyle and Tsai, 2004; Sur and Kinney, 2004; Kinney and Sur, 2005; Harsing et al., 2006; Hashimoto, 2006a,b, in press).

In the present study, using the NORT, we examined the effects of subsequent acute or subchronic (2-week) treatment with the glycine transporter-1 (GlyT-1) inhibitor, (R)-(*N*-[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl])sarcosine (NFPS)(Atkinson et al., 2001; Kinney et al., 2003), on cognitive deficits in mice after repeated administration of PCP. Furthermore, we studied whether repeated PCP treatment alters the levels of GlyT-1 protein using Western blot analysis or extracellular levels of glycine in mouse brain using *in vivo* microdialysis.

2. Materials and methods

2.1. Animals

Male ICR mice (6 weeks old) weighing 25–30 g were purchased from SLC Japan (Hamamatsu, Shizuoka, Japan). Mice were housed in the clear polycarbonate cages (22.5×33.8×14.0 cm) and in groups of 5 or 6 mice under a controlled 12/12-h light–dark cycle (light from 7:00 AM to 7:00 PM), with room temperature at 23±1 °C and humidity at 55±5%. The mice were given free access to water and food pellets for mice. The experimental procedure was approved by the Animal Care and Use Committee of Chiba University Graduate School of Medicine.

2.2. Drugs

PCP hydrochloride was synthesized in our laboratory. (R)-(*N*-[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl])sarcosine hydrochloride (NFPS) was purchased from Sigma-Aldrich Corporation (St. Louis, MI, USA). Other drugs were purchased from commercial sources.

2.3. Drug administration

The treatment schedule was performed by the methods as described previously (Hashimoto et al., 2005b, 2006, 2007). Briefly, saline (10 ml/kg) or PCP (10 mg/kg expressed as a hydrochloride salt) were administered subcutaneously (s.c.) for 10 days (once daily on days 1–5, 8–12) with no treatment on days 6, 7, 13 and 14. In the experiment of acute treatment, 3 days after a final administration of saline or PCP (day 15), vehicle (10 ml/kg; 1% Tween-80 solution), or NFPS (0.3, 1.0 or 3.0 mg/kg) was administered intraperitoneally (i.p.). In the experiment of subchronic (2-weeks) treatment, 3 days after a final administration of saline or PCP (day 15), vehicle (10 ml/kg/day; 1% Tween-80 solution), NFPS (0.3, 1.0 or 3.0 mg/kg/day), or D-serine (600 mg/kg/day) was administered i.p. for consecutive 2 weeks (once daily on days 15–28). The doses of NFPS and D-serine were selected because the doses were effective in the models of prepulse inhibition (PPI) and latent inhibition (LI) after administration of the NMDA receptor antagonist dizocilpine (Lipina et al., 2005).

2.4. Novel object recognition test (NORT)

In the experiment of acute treatment, 1 h after a final administration of vehicle or NFPS, NORT was performed as previously reported (Hashimoto et al., 2005b, 2006, 2007). In the experiment of subchronic (2-weeks) treatment, NORT was performed 1 day after the final administration of vehicle (1% Tween-80 solution), NFPS (0.3, 1.0 or 3.0 mg/kg), or D-serine (600 mg/kg). The apparatus for this task consisted of a black open field box (50.8×50.8×25.4 cm). Before the test, mice were habituated in the box for 3 days. During a training session, two objects (various objects differing in their shape and color but similar in size) were placed in the box 35.5 cm apart (symmetrically) and each animal was allowed to explore in the box for 10 min. The animals were considered to be exploring the object when the head of the animal was facing the object within an inch from the object or any part of the body, except for the tail, was touching the object. The time that mice spent exploring each object was recorded. After training, mice were immediately returned to their homecages, and the box and objects were cleaned with 75% ethanol to avoid any possible distinctive odorant cues. Retention tests were carried out at 1-day intervals following the respective training. During the retention test, each mouse was placed back into the same box, in which one of the objects used during training was replaced by a novel one. The mice were then allowed to freely explore for 5 min and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a

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counter-balanced manner in terms of their physical complexity. A preference index, a ratio of the amount of time spent exploring any one of the two objects [training session: the time exploring the object B / (the time exploring the two objects A and B)] or the novel one [retention session: the time exploring the object C / (the time exploring the two objects A and C)] over the total time spent exploring respective to both objects, was used to measure memory performance.

2.5. Western blot analysis

Three days (day 15) after the final administration of saline (10 ml/kg/day for 10 days; days 1–5 and days 8–12) or PCP (10 mg/kg/day for 10 days; days 1–5 and days 8–12), mice were sacrificed by decapitation. Then, frontal cortex and hippocampus were dissected on ice. Western blotting for GlyT-1 was performed according to the method of the previous report (Yee et al., 2006) with a slight modification. Briefly, brain tissue was homogenized in 10 vol of 5 mM Tris/HCl, pH 7.4, containing 0.32 M sucrose and centrifuged for 10 min at 10,000 ×*g*. The resulting supernatant was recentrifuged for 10 min at 40,000 ×*g* to obtain the crude membrane fraction. The pellet was washed twice in buffer and resuspended. Aliquots (25 μg protein) of the membranes were incubated for 5 min at 95 °C with an equal volume of 125 mM Tris/HCl, pH 6.8, 20% glycerol, 0.002% bromphenol blue, 10% β-mercaptoethanol, 4% SDS, and subjected to SDS-PAGE using 10% mini-gels (Mini Protean II; Bio-Rad, Hercules, CA). Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes using a Trans Blot Mini Cell (Bio-Rad, Hercules, CA). For immunodetection, the blots were blocked for 1–2 h in TBST (10 mM Tris/HCl, pH 8, 0.15 M NaCl, 0.05% Tween 20) containing 5% nonfat dry milk at room temperature (RT), followed by incubation with goat anti-GlyT-1 antibody (1:2,000, Cat. No: AB 1770, Chemicon International Inc., Temecula, CA) overnight at 4°C in TBST/5% blocker. Preabsorption of this antibody with the immunogen peptide completely abolishes the immunostaining, indicating the specificity of this antibody for GlyT-1 (Chemicon International Inc., Temecula, CA). The blots were washed once with 20 mM Tris, pH 7.5, 60 mM NaCl, 2 mM EDTA, 0.4% SDS, 0.4% Triton X-100, 0.4% deoxycholate, and three times with TBST. Incubation with the secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was performed for 1 h at RT. After extensive washing, immunoreactivity was detected by ECL plus Western Blotting Detection system (GE Healthcare Bioscience, UK). Images were captured using a Fuji LAS3000-mini imaging system (Fujifilm, Tokyo, Japan), and immunoreactive bands were quantified. β-Actin immunoreactivity was used to monitor equal sample loading.

2.6. Measurement of amino acids in mouse brain

Measurement of glycine, glutamate, and glutamine levels was carried out according to established methods (Hashimoto et al., 2005a) using a high performance liquid chromatography (HPLC) system with fluorescence detection. Measurement of D- and L-serine levels was carried out by a column switching HPLC as reported previously (Fukushima et al., 2004; Yamada et al., 2005). Three days (day 15) after the final administration of saline (10 ml/kg/day, 10 days) or PCP (10 mg/kg/day, 10 days), mice were sacrificed by decapitation. Then brains were dissected on ice, and frontal cortex and hippocampus were weighed. The brain tissues were homogenized in 20 volumes of methanol (HPLC grade) on ice. The homogenates were centrifuged at 4500 ×*g* for 10 min, and 20 μl of supernatant was evaporated to dryness at 40 °C. To the residue, 20 μl of H_2O (HPLC grade), 20 μl of 0.1 M borate buffer (pH 8.0) and 60 μl of 50 mM 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F; Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) in CH_3CN (HPLC grade) were added. The reaction mixture was then heated at 60 °C for 1 min, and immediately supplemented with 100 μl of H_2O/CH_3CN (90/10) containing 0.1% trifluoroacetic acid to stop the reaction. Ten μl of the resultant solution was injected into the HPLC system.

2.7. *In vivo* microdialysis study

Three days (day 15) after the final administration of saline (10 ml/kg/day for 10 days; days 1–5 and days 8–12) or PCP (10 mg/kg/day for 10 days; days 1–5 and days 8–12), mice were anaesthetized with sodium pentobarbital (40 mg/kg, i.p.) and were implanted with a probe, cemented in place in the frontal cortex (coordinates: from bregma (in mm), AP=+2.0, ML=−0.5, DV=−1.5) or hippocampus (coordinates: from bregma (in mm), AP=+2.2, ML=1.8, DV=1.6). Mice were allowed to recover from the surgery overnight. After recovery, the probes were continuously perfused with an artificial cerebrospinal fluid (147 mM NaCl, 4 mM KCl, and 2.3 mM $CaCl_2$) at a flow rate of 2.0 μl/min. Dialysate samples were collected every 30 min in the frontal cortex and hippocampus of mouse brain in a free moving state. Four fractions were collected to obtain extracellular levels of amino acids. Measurement of amino acids levels was carried out using HPLC as described above (Fukushima et al., 2004; Hashimoto et al., 2005a; Yamada et al., 2005).

2.8. Statistical analysis

Data are expressed as mean±S.E.M. Statistical analysis was performed by using Student *t*-test or one-way analysis of variance (ANOVA) and *post hoc* Bonferroni/Dun test. The *P* values less than 0.05 was considered statistically significant.

3. Results

3.1. Effects of NFPS and D-serine on the cognitive deficits in mice with repeated PCP treatment

In the NORT, repeated administration of PCP (10 mg/kg/day for 10 days; days 1–5 and days 8–12) caused significant cognitive deficits in mice, consistent with previous reports (Hashimoto et al., 2005b, 2006, 2007). During the training session, there were no significant differences between the six groups in the total amount of time spent exploring two objects and the exploratory preference (Fig. 1A).

In the retention session, the exploratory preference (approximately 40%) of the PCP-treated group was significantly lower than that (approximately 50%) of the saline-treated group (Fig. 1B), suggesting that our model of PCP-induced cognitive deficits using NORT may show negative symptoms such as social withdrawal, which are related to cognitive deficits (Hashimoto et al., 2005b). PCP-induced cognitive deficits were significantly improved after subsequent subchronic (2-weeks) administration of higher doses (1.0 and 3.0 mg/kg/day), but not lower dose (0.3 mg/kg/day), of NFPS. In the training session, the exploratory preference of six groups was not different (F (5,83)=1.822, P=0.117) (Fig. 1A). However, in the retention session, ANOVA analysis revealed that the exploratory preference of the six groups was significantly different (F (5,83)=12.39, P<0.001) (Fig. 1B). A *post hoc* Bonferroni test indicated that the exploratory preference of the PCP-treated group was significantly increased after subchronic (2-weeks) administration of NFPS (1.0 or 3.0 mg/kg/day), but not NFPS (0.3 mg/kg/day) (Fig. 1B). Furthermore, the exploratory preference of the PCP-treated group was significantly increased after subchronic (2-weeks) administration of D-serine (600 mg/kg/day) (Fig. 1B).

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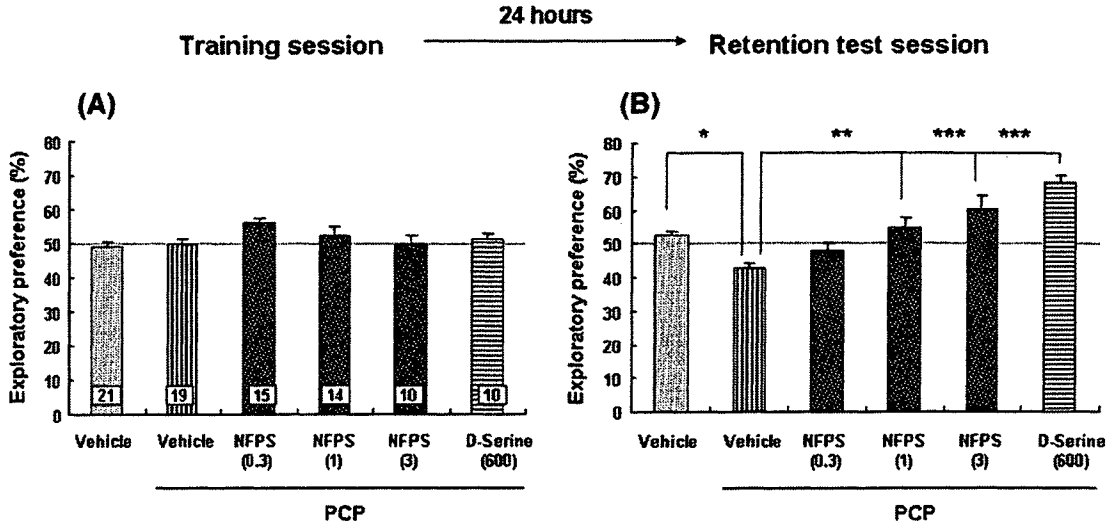

Figure 1 Effects of subchronic administration of NFPS and D-serine on PCP-induced cognitive deficits in mice Saline (10 ml/kg/day) or PCP (10 mg/kg/day) was administered s.c. for 10 days (once daily on days 1–5, 8–12). Three days after the last administration of saline or PCP (day 15), vehicle (10 ml/kg; 0.1% Tween-80), NFPS (0.3, 1.0 or 3.0 mg/kg) or D-serine (600 mg/kg) was administered i.p. into mice for consecutive 2 weeks (once daily on days 15–28). On day 29 and 30, the novel object recognition test (NORT) was performed as described in Materials and methods. Values are the mean ± S.E.M (n=10–21). The numbers on the columns indicate the numbers of animals used. *P<0.05, **P<0.01, ***P<0.001 as compared with PCP plus vehicle-treated group.

Next, we examined PCP-induced cognitive deficits in mice 1 hr after a single acute administration of NFPS (3.0 mg/kg). In the training session, ANOVA analysis revealed that the exploratory preference of six groups was not different (F (2,31) = 1.713, P=0.197) (Fig. 2A). In the retention session, ANOVA analysis revealed that the exploratory preference of the three groups was significantly different (F (2,31) = 6.07, P=0.006) (Fig. 2B). A *post hoc* Bonferroni test indicated that PCP treatment impaired cognitive deficits, evidence in PCP-treated mice that received subsequent vehicle injection. However, PCP-induced deficits were not improved by a single administration of NFPS (3.0 mg/kg) (Fig. 2B).

3.2. Effects of repeated PCP administration on the levels of GlyT-1 in mouse brain

Western blot analysis revealed that repeated administration of PCP (10 mg/kg/day for 10 days) significantly increased levels of GlyT-1 protein in the hippocampus (t=3.301, df=30, P=0.002),

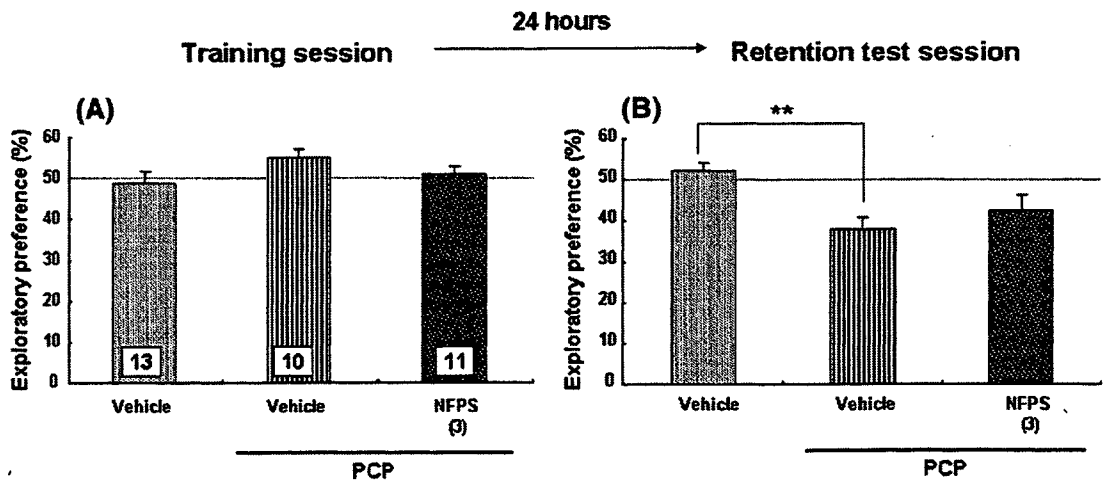

Figure 2 Effects of acute administration of NFPS on PCP-induced cognitive deficits in mice Saline (10 ml/kg/day) or PCP (10 mg/kg/day) was administered s.c. for 10 days (once daily on days 1–5, 8–12). Three days after the last administration of saline or PCP (day 15), vehicle (10 ml/kg; 1% Tween-80), or NFPS (3.0 mg/kg) was administered i.p. into mice. The novel object recognition test (NORT) was performed 1 hr after administration. Values are the mean ± S.E.M (n=10–13). The numbers on the columns indicate the numbers of animals used. **P<0.01 as compared with PCP plus vehicle-treated group.

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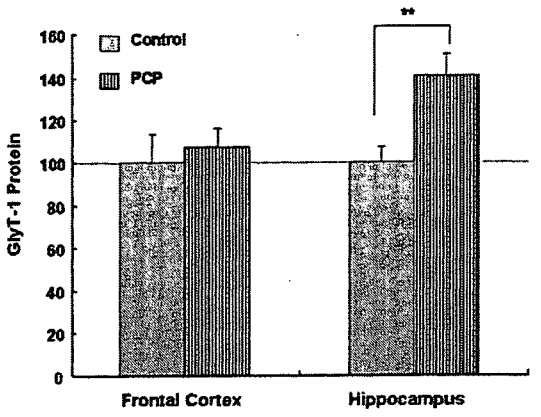

Figure 3 Effects of repeated administration of PCP on GlyT-1 protein in mouse brain Saline (10 ml/kg/day) or PCP (10 mg/kg/day) was administered s.c. for 10 days (once daily on days 1–5, 8–12). Three days after the last administration of saline or PCP (day 15), mice were sacrificed by decapitation. Western blot analysis using GlyT-1 antibody was performed as described in Materials and methods. Values are the mean ± S.E.M (n=16). **P<0.01 as compared with saline-treated group (Control).

but not frontal cortex (t=0.464, df=30, P=0.646), of mouse brain (Fig. 3).

3.3. Concentration of amino acids in mouse brain

We measured the tissue concentration of amino acids (glutamate, glutamine, glycine, L-serine, D-serine) in the frontal cortex and hippocampus 3 days after the last administration of saline (10 ml/kg/day for 10 days) or PCP (10 mg/kg/day for 10 days). As shown in the Table 1, repeated PCP administration did not alter the concentration of amino acids (glutamate, glutamine, glycine, L-serine, D-serine) in the frontal cortex. However, repeated PCP administration significantly decreased the concentration of glutamate, but not other amino acids (glutamine, glycine, L-serine, D-serine), in the hippocampus (Table 1).

3.4. *In vivo* microdialysis

In order to examine the effects of repeated PCP administration on extracellular levels of amino acids, we used an *in vivo* microdialysis technique to examine extracellular levels of amino acids in the frontal cortex and hippocampus of conscious mice. Repeated administration of PCP (10 mg/kg/day for 10 days) significantly (t=2.423, df=16, P=0.028) decreased the extracellular levels of glycine in the hippocampus, but not in the frontal cortex (Fig. 4). However, repeated administration of PCP (10 mg/kg/day for 10 days) did not alter the extracellular levels of D-serine in the frontal cortex and hippocampus (Fig. 4). In addition, repeated administration of PCP (10 mg/kg/day for 10 days) did not alter the extracellular levels of amino acids (L-serine, glutamine) in the frontal cortex and hippocampus (data not shown).

4. Discussion

The major findings of the present study are that PCP-induced cognitive deficits could be improved by subsequent subchronic (2-week) administration of the GlyT-1 inhibitor NFPS, and that repeated PCP administration increased the density of GlyT-1 in mouse hippocampus. We also found that D-serine, the endogenous agonist at the glycine modulatory site of NMDA receptor (Snyder and Ferris, 2000; Schell, 2004; Hashimoto et al., 2005c; Hashimoto and Hattori, 2007), also attenuated PCP-induced cognitive deficits in mice, consistent with the report demonstrating that D-serine has therapeutic effects on schizophrenic patients (Tsai et al., 1998). We reported that, in the NORT, PCP-induced cognitive deficits could be improved by subsequent subchronic (2-weeks) administration of the atypical antipsychotic drug clozapine, but not the typical antipsychotic drug haloperidol, suggesting that reversal of PCP-induced cognitive deficits using the NORT may be a potential animal model of atypical antipsychotic activity in relation to amelioration of cognitive deficits in schizophrenia (Hashimoto et al., 2005b). Therefore, it is likely that the GlyT-1 inhibitor NFPS could be potential therapeutic drugs for the treatment of cognitive deficits in schizophrenia.

In vivo NFPS significantly enhanced long-term potentiation in the hippocampal dentate gyrus induced by high-frequency electrical stimulation of the afferent perforant pathway, and NFPS enhanced prepulse inhibition (PPI) of the acoustic startle response in DBA/2J mice, a strain with low basal levels of PPI (Kinney et al., 2003). Furthermore, it has been reported that the PPI disruptive effects of the NMDA receptor antagonist dizocilpine (1 mg/kg) could be reversed by NFPS, and that NFPS could reverse abnormal persistent latent inhibition by dizocilpine (0.15 mg/kg)(Lipina et al., 2005). Moreover, the selective GlyT-1 inhibitor ORG24598 reversed neonatal ventral hippocampal lesions-induced PPI deficits in rats (Le Pen et al., 2003). Very recently, Depoortere et al. (2006) reported the neuropharmacological profiles of the novel and selective GlyT-1 inhibitor SSR504734. First, SSR504734 prevented the NMDA receptor antagonist ketamine-induced metabolic activation in the limbic areas of mouse brain, and reversed dizocilpine-induced

Table 1 Levels of amino acids in the frontal cortex and hippocampus after repeated administration of saline or PCP

Amino acids	Saline (n=10)	PCP (n=11)
Frontal cortex	nmol/mg tissue	
Glutamate	12.85±0.42	12.51±0.48
Glutamine	7.27±0.52	7.28±0.27
Glycine	1.34±0.04	1.30±0.06
L-serine	0.879±0.053	0.824±0.042
D-serine	0.416±0.014	0.400±0.060
Hippocampus	nmol/mg tissue	
Glutamate	15.12±0.44	13.72±0.45*
Glutamine	7.48±0.43	7.55±0.31
Glycine	1.62±0.06	1.64±0.12
L-serine	0.911±0.040	0.835±0.038
D-serine	0.337±0.010	0.321±0.011

Tissue concentration of amino acids in the frontal cortex and hippocampus 3 days after a final administration of repeated administration of saline (10 ml/kg/day for 10 days) or PCP (10 mg/kg/day for 10 days) was measured by HPLC. Values are the mean ± SEM of 10 mice (saline group) or 11 mice (PCP-treated group). *P<0.05 as compared to saline-treated group (control).

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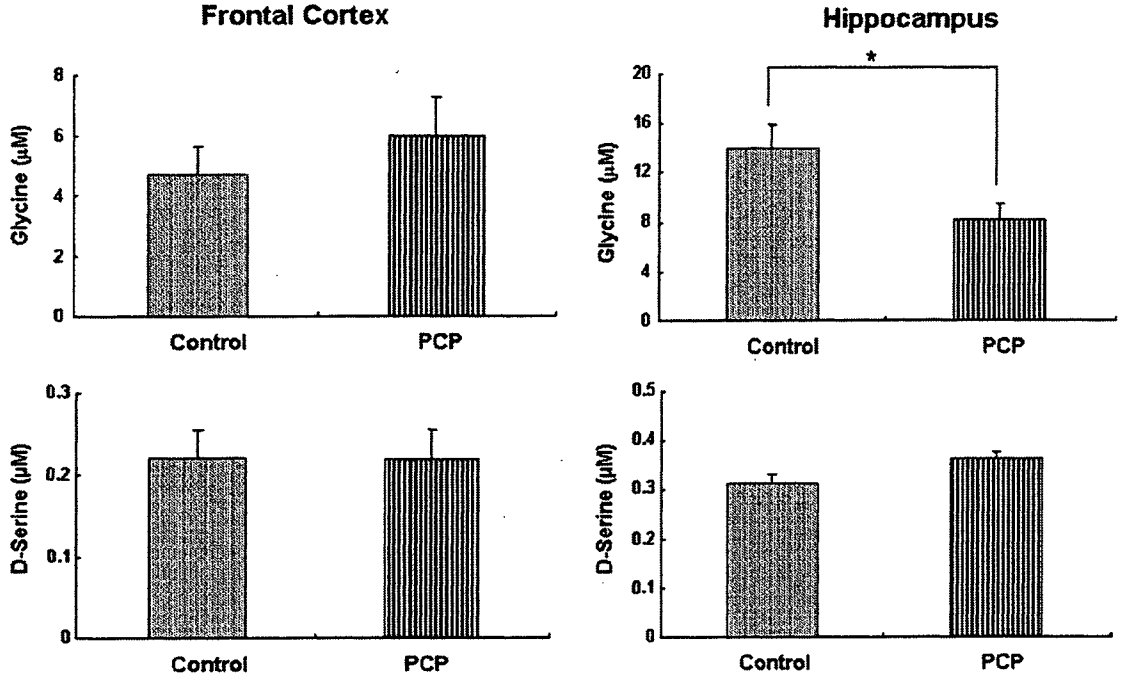

Figure 4 Effects of repeated administration of PCP on the extracellular levels of amino acids in mouse brain Saline (10 ml/kg/day) or PCP (10 mg/kg/day) was administered s.c. for 10 days (once daily on days 1–5, 8–12). Three days after the last administration of saline or PCP (day 15), *in vivo* microdialysis in the frontal cortex and hippocampus of conscious mice was performed as described in Materials and methods. Values are the mean±S.E.M ($n=10$). $^{**}P<0.05$ as compared with saline-treated group (control).

hyperactivity and increase in EEG spectral energy in mice and rats, respectively (Depoortere et al., 2006). Second, SSR504734 normalized spontaneous PPI deficits in DBA/2 mice, and reversed hyperactivity to locomotor effects of D-amphetamine and selective attention deficits in adult rats treated neonatally with PCP (Depoortere et al., 2006). Taken together, these findings suggest that GlyT-1 could offer promising targets for the development of novel therapies for schizophrenia (Vandenberg and Aubrey, 2001; Javitt, 2002, 2006; Millan, 2002; Coyle and Tsai, 2004; Sur and Kinney, 2004; Kinney and Sur, 2005; Harsing et al., 2006; Hashimoto, 2006a,b, 2007).

Among the brain regions that have been suspected to be linked to the etiology of schizophrenia, frontal cortex and hippocampus are the prominent regions. Hypofunction of frontal cortex is thought to play a role in the genesis of negative symptoms and cognitive deficits (Volk and Lewis, 2002), and hippocampus shows subtle neuroanatomical abnormality in schizophrenic patients (Harrison, 2004). In this study, we found that repeated PCP administration caused an increase of GlyT-1 protein as well as the reduction of extracellular glycine levels in the hippocampus, but not frontal cortex. To the best of our knowledge, this is the first report demonstrating that administration of PCP increased the density of GlyT-1 protein in the brain. The precise mechanism(s) underlying how repeated PCP administration could increase GlyT-1 in the hippocampus are currently unknown. Interestingly, we found that repeated PCP administration decreased the extracellular glycine levels in the hippocampus of mouse brain, resulting in hypofunction of the NMDA receptors in the hippocampus. It is possible that increased GlyT-1 protein may play a role in removing the extracellular glycine in synaptic cleft *via* GlyT-1, resulting in lower extracellular levels of glycine in the hippocampus. In this study, we found that extracellular glycine levels and GlyT-1 protein were not altered in the frontal cortex. It is likely that hippocampal GlyT-1 may play a role in the PCP-induced cognitive deficits although the reasons underlying this regional difference are currently unknown. Thus, it is likely that increased levels of GlyT-1 might be implicated in the pathophysiology of schizophrenia although it is currently unclear whether density of GlyT-1 is altered in the brain of schizophrenic patients. Taken together, GlyT-1 inhibitors may increase the extracellular levels of glycine, resulting in stimulation of the NMDA receptors on the postsynaptic neurons, suggesting that inhibition at GlyT-1 by pharmacological manipulation may potentiate the NMDA receptors (Hashimoto, 2006a,b, in press).

In an attempt to clarify the *in vivo* functional roles of GlyT-1 in the CNS, knockout mice deficient in GlyT-1 gene have been generated (Gomeza et al., 2003; Tsai et al., 2004b). Newborn mice deficient in GlyT-1 gene are anatomically normal but show severe motor and respiratory deficits and die during the first postnatal day (Gomeza et al., 2003). Furthermore, it has been demonstrated that hippocampal NMDA receptor function and memory retention are enhanced in heterozygous knockout mice (reduced expression of GlyT-1), and that these mice are protected against a disruption of sensory gating by amphetamine, suggesting that GlyT-1 inhibitors might have both cognitive-enhancing and antipsychotic effects (Tsai et al., 2004b). Very recently, Yee et al. (2006) reported the generation of a novel mouse line (CamKIIαCre; GlyT1tmi.2fl/fl) with a neuron and forebrain

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selective disruption of GlyT-1. The mutant mice were less responsive to PCP than controls. The mutation enhanced aversive Pavlovian conditioning without affecting spontaneous anxiety-like behavior in the elevated plus maze and augmented a form of attentional learning called latent inhibition in three different experimental paradigms: conditioned freezing, conditioned active avoidance, conditioned taste aversion (Yee et al., 2006). These findings suggest that augmentation of forebrain neuronal glycine transmission may also offer an effective therapeutic intervention against the cognitive and attentional impairments characteristic of schizophrenia. Taken together, the elevation of synaptic glycine levels, through blockade of the GlyT-1, can stimulate NMDA receptor function *in vivo*, and GlyT-1 inhibitors might have both cognitive-enhancing and antipsychotic effects.

In a recent 6-week double-blind, placebo-controlled trial of the GlyT-1 inhibitor *N*-methylglycine (sarcosine: 2 g/day), schizophrenic patients who received sarcosine treatment showed significant improvements in their positive, negative, cognitive, and general psychiatric symptoms (Tsai et al., 2004a). Sarcosine was also well-tolerated, and no significant side effects were noted (Tsai et al., 2004a). A recent double-blind, placebo-controlled study has demonstrated that sarcosine, superior to D-serine, can benefit not only patients with long-term stable disease but also acutely ill persons with schizophrenia, suggesting that a GlyT-1 inhibitor may be more efficacious than NMDA-glycine site agonists for adjunct treatment of schizophrenia, at least during the acute phase (Lane et al., 2005). The significant improvement with the sarcosine supports the hypothesis of the NMDA receptor hypofunction in schizophrenia. Therefore, GlyT-1 could be a novel target for the pharmacotherapy to enhance NMDA receptor function, and GlyT-1 is a good target for therapeutic intervention in schizophrenia.

In conclusion, the present findings suggest that repeated PCP administration caused the reduction of GlyT-1 in the hippocampus of mouse brain, and that the GlyT-1 inhibitor NFPS could improve cognitive deficits in mice after repeated administration of PCP. Taken together, it is likely that GlyT-1 inhibitors could be potential therapeutic drugs for schizophrenia.

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Contributors

K. Hashimoto designed the study and wrote the protocol. Y. Fujita and T. Ishima performed the experiments of the study. S. Chaki, M. Iyo, and K. Hashimoto undertook the statistical analysis of the data. Y. Fujita and K. Hashimoto wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

All authors have no conflict of interests.

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ORIGINAL ARTICLES

Protective Effects of Minocycline on the Reduction of Dopamine Transporters in the Striatum After Administration of Methamphetamine: A Positron Emission Tomography Study in Conscious Monkeys

Kenji Hashimoto, Hideo Tsukada, Shingo Nishiyama, Dai Fukumoto, Takeharu Kakiuchi, and Masaomi Iyo

Background: Positron emission tomography (PET) studies of methamphetamine (METH) abusers suggest that psychotic symptoms of METH abusers may be attributable to the reduction of dopamine transporters (DAT) in the human brain. However, there are currently no particular pharmacological treatments for the wide range of symptoms associated with METH abuse.

Methods: Using a PET study in conscious monkeys, we investigated whether the second generation antibiotic minocycline could protect against the reduction of DAT in monkeys treated with METH (2 mg/kg × 3, 3-hour intervals).

Results: Pretreatment and subsequent administration of minocycline significantly attenuated the reduction of DAT in the striatum of monkeys treated with METH. Furthermore, posttreatment and subsequent administration of minocycline also significantly attenuated the reduction of DAT. In contrast, repeated administration of minocycline alone did not alter the density of DAT in the striatum of monkeys treated with METH.

Conclusions: Our findings suggest that minocycline protects against METH-induced neurotoxicity in the monkey brain. Therefore, minocycline is likely to be a promising therapeutic agent for the treatment of several symptoms associated with METH use in humans.

Key Words: Dopamine transporter, methamphetamine, minocycline, monkey brain, neurotoxicity, positron emission tomography

Methamphetamine (METH) abuse has become a major public health problem worldwide, as demonstrated by increases in the number of emergency room visits, substance abuse treatment episodes, and arrests attributable to METH manufacture and abuse. However, there are currently no particular pharmacological treatments for the wide range of symptoms associated with METH abuse (National Institute on Drug Abuse 2002). Multiple lines of evidence indicate that dopamine (DA) plays a key role in a variety of motivated behaviors associated with abused drugs, including METH (Nestler 2001, 2002; Pierce and Kumaresan 2006). In addition, it is well known that METH elevates extraneuronal DA concentrations through its actions on the plasma membrane DA transporter (DAT) (Davidson et al 2001; Hanson et al 2004; Mortensen and Amara 2003).

Positron emission tomography (PET) studies of METH users have demonstrated that the reduction of DAT in the striatum is associated with motor and cognitive impairment (Volkow et al 2001) and that the reduction of DAT is also associated with the duration of METH use and the severity of psychiatric symptoms (Sekine et al 2001, 2003). These findings suggest that psychotic symptoms of METH users may be attributable to the reduction of DAT in the brain. Furthermore, it has been demonstrated that the densities of DAT in the striatum are significantly decreased in the postmortem brains of chronic METH users (Wilson et al 1996). Thus, although METH-induced neurotoxicity in the dopaminergic terminals is well documented, the precise mechanism underlying METH-induced neurotoxicity remains unknown (Cadet et al 2003). In addition, chronic METH users show severe structural and functional deficits in areas of the brain associated with emotion, especially depression and anxiety, as well as memory (London et al 2004). From the point of view of developing novel pharmacological interventions for the treatment or prevention of METH abuse, it is necessary to develop therapeutic drugs to protect against the reduction of DAT in the brain associated with METH use.

Minocycline is a second-generation tetracycline that has been in use for over 30 years. This drug easily crosses the blood-brain barrier and has powerful neuroprotective properties in several models of neurological diseases, including Parkinson disease, Huntington disease, amyotrophic lateral sclerosis (ALS), and ischemic stroke (Blum et al 2004; Domercq and Matute 2004; Stirling et al 2005; Yong et al 2004). For example, the impressive therapeutic effects of minocycline have been demonstrated in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) models of Parkinson's disease (Du et al 2001; Wu et al 2002). Minocycline mitigates both the demise of nigrostriatal dopaminergic neurons and the formation of nitrotyrosine produced by MPTP (Wu et al 2002). In addition, minocycline not only prevents MPTP-induced activation of microglia but also the formation of mature interleukin-1β and the activation of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase and induction of nitric oxide synthase (iNOS), three key microglial-derived cytotoxic mediators (Wu et al 2002). Thus, it is likely that a blockade of microglial activation by minocycline plays a role in the neuroprotective actions of this drug (Tikka et al 2001; Wu et al 2002; Zhu et al 2002). On the other hand, direct neuronal

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protection by minocycline has been documented and probably involves the preservation of mitochondrial integrity and cytochrome c, followed by the suppression of caspase-dependent as well as caspase-independent cell death (Chen et al 2000; Wang et al 2003). Taken together, these findings indicate that minocycline holds great promise as a therapeutic drug for the treatment of human neurodegenerative diseases (Blum et al 2004; Domercq and Matute 2004; Stirling et al 2005; Yong et al 2004).

The current study was conducted to investigate whether minocycline could protect against the reduction of DAT in monkeys treated with METH. For this purpose, we used PET imaging in conscious monkeys.

Methods and Materials

Subjects

Ten young-adult male rhesus monkeys (*Macaca mulatta*) weighing from 4 kg to 6 kg were used for PET measurements. Monkeys were maintained and handled in accordance with the recommendations of the US National Institutes of Health and also the guidelines of the Central Research Laboratory, Hamamatsu Photonics (Hamamatsu, Shizuoka, Japan). The monkeys were trained to sit on a chair by means of twice-weekly training sessions over the course of 3 months. The magnetic resonance images (MRI) of all monkeys were obtained with a Toshiba MRT-50A/II (.5T) (Toshiba Medical Systems Corporation, Tokyo, Japan) under anesthesia with pentobarbital. The stereotactic coordinates of PET and MRI were adjusted based on the orbitomeatal (OM) line with monkeys secured in a specially designed head holder (Takechi et al 1994). At least 1 month before the PET study, an acrylic plate, with which the monkey was fixed to the monkey chair, was attached to the head under pentobarbital anesthesia as described previously (Onoe et al 1994).

Drug Administration

Methamphetamine hydrochloride (Dainippon Pharmaceuticals Ltd., Osaka, Japan) was administered intramuscularly (2 mg/kg as a salt, three times at 3-hour intervals) into each monkey using the previously reported method (Hashimoto et al 2004) with slight modifications. This dose regimen of METH closely approximates the binge use of METH by some humans (20 to 40 mg every 2 to 3 hours) (Konuma 1994).

In the first trial, which consisted of minocycline pretreatment, METH administration, and subsequent minocycline administration, subjects (n = 3) received minocycline (Wako Pure Chemicals Ltd., Tokyo, Japan; 200 mg, subcutaneous [SC], 0800 hours) or vehicle (physiological saline 1 mL, SC, 0800 hours) as a control condition 30 minutes before administration of METH and a subsequent administration of minocycline (200 mg, SC, twice daily [b.i.d.], 0800 and 2000 hours) or vehicle according to the method reported previously with slight modifications (Diguet et al 2004) (Figure 1A). In the second trial, which consisted of METH administration, minocycline posttreatment, and subsequent minocycline administration, subjects (n = 2) received minocycline (200 mg, SC, 2000 hours) 30 minutes after the final administration of METH, followed by subsequent administration of minocycline (200 mg, SC, b.i.d., 0800 and 2000 hours) or vehicle. In both experiments, the subsequent administration of minocycline was performed for 6 consecutive days (Figure 1B; day 2 to day 7). In the third trial, to examine the effect of minocycline alone on the DAT in the monkey brain, subjects (n = 2) received minocycline (200 mg, SC, b.i.d, 0800 and 2000 hours) for 7 days (Figure 1C).

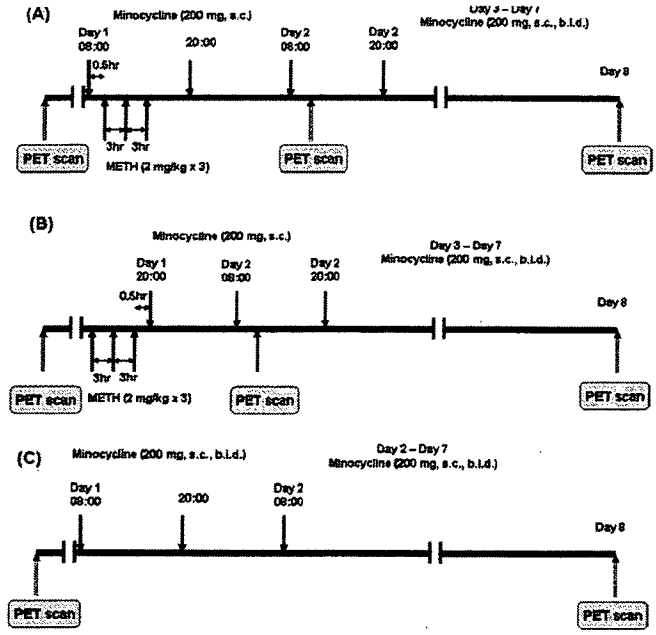

Figure 1. Treatment schedule of METH and/or minocycline in the monkeys. **(A)** Minocycline (200 mg, SC, 0800 hours) or vehicle (saline; 1 mL) was administered to monkeys (n = 3). Thirty minutes after injection, METH (2 mg/kg × 3, 3-hour intervals) was administered to the subjects (day 1). Then, minocycline (200 mg, SC, 2000 hours) or vehicle (saline; 1 mL) was administered to the monkeys (day 1). Minocycline (200 mg, b.i.d., 0800 and 2000 hours) was administered daily for 6 consecutive days (day 2 to day 7). **(B)** METH (2 mg/kg × 3, 3-hour intervals) was administered to the monkeys (n = 2). Thirty minutes after injection, minocycline (200 mg, SC, 2000 hours) was administered to the subjects (day 1). Then, minocycline (200 mg, b.i.d., SC, 0800 and 2000 hours) was administered daily for 6 consecutive days (day 2 to day 7). **(C)** Minocycline (200 mg, SC, 0800 and 2000 hours) was administered to the monkeys (n = 2) (day 1 to day 7). METH, methamphetamine; SC, subcutaneous; b.i.d., twice daily.

Synthesis of [^{11}C]-Labeled Compounds

Carbon-11 (^{11}C) was produced by ^{14}N (p,α)^{11}C nuclear reaction using a cyclotron (HM-18; Sumitomo Heavy Industries, Tokyo, Japan) at the Hamamatsu Photonics PET Center and obtained as [^{11}C]CO2, which was converted to [^{11}C]methyl iodide. [^{11}C]2β-carbomethoxy-3β-(4-fluorophenyl)tropane (β-CFT) (for DAT) and [^{11}C]SCH 23390 (for DA D_1 receptors) were synthesized as previously reported (Harada et al 2002; Tsukada et al 2001). The radiochemical and chemical purities of labeled compounds were greater than 98% and 99%, respectively. After analysis for identification, the solution was passed through a .22-μm pore size filter before intravenous administration to the monkeys.

PET Scans

Positron emission tomography data were collected before (control) and at 1 day (day 2) and 7 days (day 8) after the repeated administration of METH or METH/minocycline. In the trial in which minocycline was administered alone, PET data were collected before (control) and 7 days (day 8) after the repeated administration of minocycline. Data were collected on a high-resolution PET scanner (SHR-7700; Hamamatsu Photonics K. K., Hamamatsu, Japan) with transaxial resolution of 2.6-mm full-width at half-maximum (FWHM) and a center-to-center distance of 3.6 mm (Watanabe et al 1997). The PET camera allowed 31 imaging slices to be recorded simultaneously. After an overnight fast, animals were fixed to the monkey chair with

stereotactic coordinates aligned parallel to the OM line. A cannula was implanted in the posterior tibial vein of the monkey for administration of [^{11}C]-labeled compounds. The [^{11}C]β-CFT or [^{11}C]SCH 23390 was injected through the posterior tibial vein cannula. For [^{11}C]SCH 23390, a PET scan was performed for 64 minutes with 6 time frames at 10-second intervals, 6 time frames at 30-second intervals, 12 time frames at 1-minute intervals, and 16 time frames at 3-minute intervals. For [^{11}C]β-CFT, additional scans of nine time frames at 3-minute intervals were done to collect data for 91 minutes total. After completion of the first scan with [^{11}C]β-CFT, scans with [^{11}C]SCH 23390 were continuously performed at 3-hour intervals. Due to the very short half-life of ^{11}C (20.4 minutes), a time lag of at least 3 hours between scans provided a sufficient decay time of radioactivity in monkeys (approximately 1/400 of the injected dose). Therefore, the level of radioactivity associated with the previous injection of labeled compound did not interfere with the next scan.

Data Analysis and Statistical Analysis

For quantitative analysis, time-activity curves of radioactivity in the cerebellum, used as an input function because of the much lower density of dopamine receptors and DAT (Creese et al 1975; Kaufman and Madras 1993), and each region of interest (ROI) were fitted to a two-compartment model using the least-squares fitting method to estimate the kinetic parameters, and the binding potential in each ROI was calculated as described previously (Hashimoto et al 2004; Lammertsma and Hume 1996). The differences between the control (pre-METH) monkeys and METH-treated (post-METH) monkeys were determined using a paired two-tailed *t* test. The data on effects of minocycline were analyzed using an unpaired two-tailed *t* test. Significance was set at $p < .05$.

Results

Positron emission tomography studies using [^{11}C]β-CFT (for DAT) or [^{11}C]SCH 23390 (for DA D_1 receptor) were performed before (control) and at 1 day (day 2) and 7 days (day 8) after repeated administration of METH (2 mg/kg × 3, 3-hour intervals). High accumulation of radioactivity in the striatum after intravenous administration of [^{11}C]β-CFT or [^{11}C]SCH 23390 was detected in the control monkeys (Figure 2). Repeated administration of METH (2 mg/kg × 3, 3-hour intervals) significantly ($t = 10.27$, $p = .009$) decreased the binding of [^{11}C]SCH 23390 to DA D_1 receptors at 1 day (day 2) after administration of METH (Figure 2 and Table 1), although the difference (less than 10%) was small. However, the binding of [^{11}C]SCH 23390 in the striatum was recovered to control levels at 7 days (day 8) after administration of METH (Figure 2 and Table 1). In contrast, repeated administration of METH (2 mg/kg × 3, 3-hour intervals) markedly decreased the binding of [^{11}C]β-CFT (for DAT) in the striatum at 1 day (day 2; $t = 16.82$, $p = .004$) or 7 days (day 8; $t = 28.60$, $p = .001$) after administration of METH (Figure 2 and Table 1).

First, we examined the effect of pretreatment and subsequent administration of minocycline (200 mg b.i.d.) on the reduction of DAT in the striatum after repeated administration of METH (2 mg/kg × 3, 3-hour intervals) (Figure 1A). Pretreatment (30 minutes before administration of METH) and subsequent administration of minocycline (200 mg b.i.d.) (Figure 1A) significantly (day 2: $t = 12.18$, $p < .001$; day 8: $t = 22.97$, $p < .001$) attenuated the reduction of DAT in the striatum after administration of METH as compared with that of the striatum of monkeys treated with METH alone (Figure 2 and Table 1).

Second, we examined the effect of posttreatment and subsequent administration of minocycline (200 mg b.i.d.) on the reduction of DAT in the striatum after repeated administration of METH (2 mg/kg × 3, 3-hour intervals) (Figure 1B). Interestingly, posttreatment (30 minutes after the final administration of METH) and subsequent administration of minocycline (200 mg b.i.d.) significantly (day 2: $t = 4.33$, $p = .023$; day 8: $t = 13.69$, $p = .001$) attenuated the reduction of DAT in the striatum of monkeys treated with METH (Figure 2 and Table 1).

Finally, we examined the effects of minocycline alone on the binding potential of [^{11}C]β-CFT binding in the monkey striatum (Figure 1C). Repeated administration of minocycline (200 mg b.i.d. for 7 days) did not alter the binding potential of [^{11}C]β-CFT binding in the monkey striatum (Figure 2 and Table 1), suggesting that minocycline alone does not affect DAT in the monkey striatum.

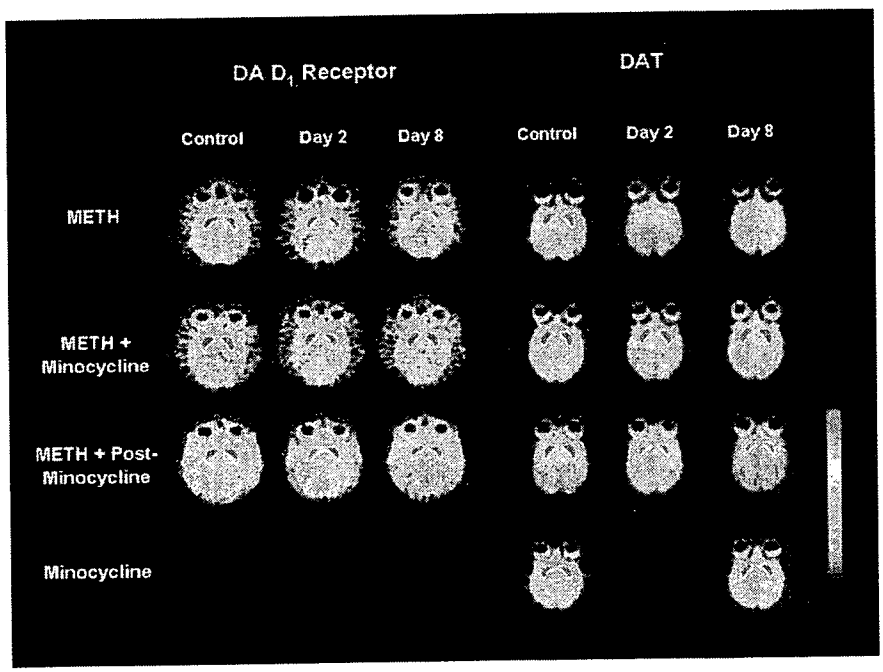

Figure 2. Representative PET images of [^{11}C]SCH 23390 binding (for DA D_1 receptor) and [^{11}C]β-CFT binding (for DAT) in the brains of monkeys. PET images of [^{11}C]SCH 23390 and [^{11}C]β-CFT in the brains of monkeys were obtained before (control) and at 1 day (day 2) and 7 days (day 8) after the repeated administration of METH (2 mg/kg × 3, 3-hour intervals). The PET image of [^{11}C]SCH 23390 was generated by the summation of data from 37 to 64 minutes after injection. PET images for [^{11}C]β-CFT were generated by the summation of data from 61 to 91 minutes after injection. PET, positron emission tomography; [^{11}C]SCH 23390, ; DA, dopamine; [^{11}C]β-CFT, [^{11}C]2β-carbomethoxy-3β-(4-fluorophenyl)tropane; DAT, dopamine transporter; METH, methamphetamine.

Table 1. Effects of Minocycline on the Binding Potential of DA D_1 Receptors and DAT in the Monkey Striatum After Administration of METH

Groups	Control Study	Day 2	Day 8
DA DI Receptors			
METH (n = 3)	100.0 ± 12.69	93.90 ± 1.00[b]	98.52 ± 16.91
METH + Preminocycline (n = 3)	100.0 ± 19.55	91.78 ± 3.00[a]	96.67 ± 5.25
METH + Postminocycline (n = 2)	100.0 ± 1.69	91.91 ± 2.39	108.5 ± 6.09
Minocycline (n = 2)	ND	ND	ND
DAT			
METH (n = 3)	100.0 ± 8.36	39.51 ± 6.20[b]	40.0 ± 3.54[b]
METH + Preminocycline (n = 3)	100.0 ± 11.28	88.66 ± 3.16[a,e]	89.99 ± 1.03[b,e]
METH + Postminocycline (n = 2)	100.0 ± 2.89	65.28 ± 7.20[c]	78.05 ± 1.10[a,d]
Minocycline (n = 2)	100.0 ± 3.96	ND	97.45 ± 5.30

PET studies were performed before (control) and at 1 day (day 2) and 7 days (day 8) after the repeated administration of METH (2 mg/kg × 3, 3-hour intervals). The binding potential of each monkey at control study was expressed as 100%. Values are the mean ± SD of three or two monkeys.

DA, dopamine; DAT, dopamine transporter; METH, methamphetamine; PET, positron emission tomography; ND, not determined.

[a] $p < .05$ as compared with control (paired t test).
[b] $p < .01$ as compared with control (paired t test).
[c] $p < .05$ as compared with METH group (unpaired t test).
[d] $p < .01$ as compared with METH group (unpaired t test).
[e] $p < .001$ as compared with METH group (unpaired t test).

Discussion

The major finding of the present study was that minocycline showed protective effects against METH-induced neurotoxicity in the monkey striatum. It is noteworthy that posttreatment with minocycline (30 minutes after the final administration of METH) followed by subsequent administration of minocycline conferred neuroprotection against METH-induced neurotoxicity in the monkey striatum without minocycline pretreatment. Recently, we found that posttreatment with minocycline (2 hours after the final administration of METH) and the subsequent administration of minocycline significantly attenuated the METH-induced neurotoxicity in the mouse striatum (Zhang et al, in press). In addition, we found that repeated administration of minocycline alone did not affect the density of DAT in the striatum of mice (Zhang et al, in press) and monkeys (this study). Taking these results together, minocycline is likely to be a useful therapeutic drug for treatment of METH-induced neurotoxicity in the human brain.

A recent study demonstrated that activation of microglia might contribute to METH-induced neurotoxicity in the striatum (Thomas et al 2004). It has also been reported that minocycline inhibits microglial activation and neurotoxicity in the striatum of MPTP-treated mice (Wu et al 2002), suggesting that microglia-related inflammatory events play a role in MPTP-induced neurotoxicity and that minocycline may be a valuable neuroprotective agent for the treatment of Parkinson disease (Wu et al 2002). Recently, we found that minocycline significantly attenuated the METH-induced neurotoxicity in the mouse striatum and that minocycline significantly attenuated microglial activation in the mouse striatum by repeated administration of METH (Zhang et al, in press). Therefore, it is likely that minocycline may, at least in part, attenuate METH-induced neurotoxicity in the monkey striatum via the inhibition of microglial activation.

Accumulating evidence suggests that METH induces neuronal apoptosis by activating the mitochondrial cell death pathway, and mitochondrial dysfunction may play a role in METH-induced neurotoxicity (Deng et al 2002; Jayanthi et al 2004). Administration of METH was shown to cause the gradual appearance of cytochrome c in the cytosol of the mouse striatum, and these changes were countered by marked decreases in cytochrome c in the mitochondrial fraction (Jayanthi et al 2004). It has been shown that minocycline blocks the release of the proapoptotic factors cytochrome c and apoptosis-inducing factor (Domercq and Matute 2004; Wang et al 2003; Zhu et al 2002). Furthermore, it has been demonstrated that minocycline delayed mortality and/or progression in mouse models of Huntington disease (Chen et al 2000) and ALS (Zhu et al 2002), presumably by inhibiting caspase-3 expression and cytochrome c release, suggesting that the primary target of minocycline is cytochrome c release (Chen et al 2000; Zhu et al 2002). Taken together, these results suggest that a blockade of the release of cytochrome c by minocycline might, at least in part, contribute to its neuroprotective activity in the context of METH-induced neurotoxicity in the striatum, although further studies will be needed to determine the precise mechanisms of minocycline on METH-induced neurotoxicity.

In clinical trials, minocycline was well tolerated, and no side effects or negative interactions with other simultaneously administered drugs were observed (Blum et al 2004; Domercq and Matute 2004; Smith and Leyden 2005; Yong et al 2004). Minocycline is an antibiotic that possesses superior penetration through the blood-brain barrier (Aronson 1980; Barza et al 1975; Zhang et al, in press), and the bioavailability of minocycline is very high in humans (Kelly and Kanegis 1967). Therefore, the present study suggests that minocycline might serve as a promising therapeutic drug for prevention of the long-term effects associated with METH abuse in humans.

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Phencyclidine-Induced Cognitive Deficits in Mice are Improved by Subsequent Subchronic Administration of Fluvoxamine: Role of Sigma-1 Receptors

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This study was undertaken to examine the effects of the selective serotonin reuptake inhibitors fluvoxamine and paroxetine on cognitive deficits in mice after repeated administration of the *N*-methyl-D-aspartate receptor antagonist phencyclidine (PCP). In the novel object recognition test, repeated administration of PCP (10 mg/kg/day, 10 days) significantly decreased the exploratory preference in the retention test session, but not in the training test session. PCP-induced cognitive deficits were significantly improved by subsequent subchronic (2-week) administration of fluvoxamine (20 mg/kg/day), but not paroxetine (10 mg/kg/day). Furthermore, the effect of fluvoxamine on PCP-induced cognitive deficits was antagonized by co-administration of the selective sigma-1 receptor antagonist NE-100 (1 mg/kg/day). Moreover, PCP-induced cognitive deficits were also significantly improved by subsequent subchronic (2-week) administration of the selective sigma-1 receptor agonist SA4503 (1 mg/kg/day) or neurosteroid dehydroepiandrosterone 3-sulfate (DHEA-S; 25 mg/kg/day). The effects of SA4503 or DHEA-S were also antagonized by co-administration of NE-100 (1 mg/kg/day), suggesting the role of sigma-1 receptors in the active mechanisms of these drugs. In contrast, acute single administration of these drugs (fluvoxamine, paroxetine, SA4503) alone or combination with NE-100 did not alter PCP-induced cognitive deficits. The present study suggests that agonistic activity of fluvoxamine at sigma-1 receptors plays a role in the active mechanisms of fluvoxamine on PCP-induced cognitive deficits in mice. Therefore, sigma-1 receptor agonists such as fluvoxamine would be potential therapeutic drugs for the treatment of the cognitive deficits of schizophrenia.

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INTRODUCTION

Cognitive deficits in patients with schizophrenia are core features of the illness, and predict vocational and social disabilities for patients (Freedman, 2003; Coyle and Tsai, 2004; Green *et al*, 2004; Kurtz, 2005). Multiple lines of evidence suggest that a dysfunction in glutamatergic neurotransmission via the *N*-methyl-D-aspartate (NMDA) receptors might be involved in the pathophysiology of schizophrenia (Javitt and Zukin, 1991; Olney and Farber, 1995; Coyle, 1996; Krystal *et al*, 1999; Moghaddam, 2003; Hashimoto *et al*, 2003, 2004, 2005b; Javitt, 2004). NMDA receptor antagonists such as phencyclidine (PCP) and ketamine are known to induce schizophrenia-like symptoms, including cognitive deficits and negative symptoms in healthy subjects (Javitt and Zukin, 1991; Krystal *et al*, 1999); consequently, NMDA receptor antagonists, including PCP and (+)-MK-801 (dizocilpine), have been used widely in animal models of schizophrenia (Hashimoto *et al*, 1997; Javitt *et al*, 2004; Jentsch and Roth, 1999; Mandillo *et al*, 2003; Morimoto *et al*, 2002; Okamura *et al*, 2004; Sams-Dodd, 1998).

We recently found that PCP-induced cognitive deficits in the novel object recognition test could be significantly improved by subsequent subchronic (2-week) administration of clozapine, but not haloperidol. Our observations suggested that reversal of PCP-induced cognitive deficits as measured by the novel object recognition test may be a potential animal model of atypical antipsychotic activity in relation to the amelioration of cognitive deficits in schizophrenia (Hashimoto *et al*, 2005a).

One approach used to improve cognitive deficits in schizophrenic patients is adjunctive medication to antipsychotic treatment. Some selective serotonin reuptake inhibitors (SSRIs) such as fluvoxamine have shown promising results in patients (Silver, 2001, 2003, 2004;

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