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Minocycline Attenuates Hyperlocomotion and Prepulse Inhibition Deficits in Mice after Administration of the NMDA Receptor Antagonist Dizocilpine

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The present study was undertaken to examine whether the second generation antibiotic drug minocycline attenuates behavioral changes (eg, acute hyperlocomotion and prepulse inhibition (PPI) deficits) in mice after the administration of the *N*-methyl-D-aspartate (NMDA) receptor antagonist (+)-MK-801 (dizocilpine). Dizocilpine (0.1 mg/kg)-induced hyperlocomotion was significantly attenuated by pretreatment with minocycline (40 mg/kg). Furthermore, the PPI deficits after a single administration of dizocilpine (0.1 mg/kg) were attenuated by pretreatment with minocycline (10, 20, or 40 mg/kg), in a dose-dependent manner. Moreover, *in vivo* microdialysis study in the free-moving mice revealed that pretreatment with minocycline (40 mg/kg, i.p.) significantly attenuated the increase of extracellular dopamine (DA) levels in the frontal cortex and striatum after administration of dizocilpine (0.1 mg/kg), suggesting that the inhibition of dizocilpine-induced DA release by minocycline may, at least in part, be implicated in the mechanism of action of minocycline with respect to dizocilpine-induced behavioral changes in mice. These findings suggest that minocycline could attenuate behavioral changes in mice after the administration of the NMDA receptor antagonist dizocilpine. Therefore, it is possible that minocycline would be a potential therapeutic drug for schizophrenia.

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INTRODUCTION

Multiple lines of evidence suggest that a dysfunction in the 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; Tamminga, 1998; Hashimoto *et al*, 2003, 2004, 2005). Therefore, the NMDA receptor antagonists such as (+)-MK-801 (dizocilpine) have been used widely in animal models for schizophrenia (Al-Amin and Schwarzkopf, 1996; Hashimoto *et al*, 1997; Bakshi and Geyer, 1998; Varty *et al*, 1999; Morimoto *et al*, 2002; Okamura *et al*, 2004).

Prepulse inhibition (PPI) of the acoustic startle response is a form of sensorimotor gating, defined as an inhibition of the startle response when a low-intensity stimulus, the prepulse, precedes the startling stimulus (Braff and Geyer, 1990; Braff and Freedman, 2002; Geyer *et al*, 2001). Deficits in PPI have been reported in several psychiatric disorders including schizophrenia, suggesting that deficient PPI *per se* or abnormalities in neural circuits regulating PPI may cause some symptoms (eg, cognitive deficits) of schizophrenia (Braff and Geyer, 1990; Perry *et al*, 1999; Swerdlow and Geyer, 1998; Braff and Freedman, 2002). In experimental animals, PPI deficits can be induced by the administration of the NMDA receptor antagonist dizocilpine (Al-Amin and Schwarzkopf, 1996; Bakshi and Geyer, 1998; Varty *et al*, 1999; Yee *et al*, 2004; Long *et al*, 2006). Therefore, pharmacological models of PPI deficits by NMDA receptor antagonism are excellent predictors of antipsychotic activity (Swerdlow and Geyer, 1998; Geyer *et al*, 2001; Levin *et al*, 2005).

Accumulating evidence suggest that the second-generation tetracycline minocycline produces neuroprotective effects in several animal models of neurological diseases, including Parkinson's disease (Du *et al*, 2001; Wu *et al*, 2002), amyotrophic lateral sclerosis (Zhu *et al*, 2002), Huntington's disease (Chen *et al*, 2000; Wang *et al*, 2003), and ischemia (Yrjanheikki *et al*, 1998, 1999). The neuroprotective effects of minocycline can occur indirectly by microglial activation and proliferation (Yrjanheikki *et al*, 1998; Tikka *et al*, 2001; Wu *et al*, 2002; Domercq and Matute, 2004; Yong *et al*, 2004; Blum *et al*, 2004; Thomas

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and Le, 2004; Stirling *et al*, 2005). Recently, we reported that minocycline could ameliorate the behavioral changes (eg, acute hyperlocomotion and the development of behavioral sensitization) and neurotoxicity in mice or monkey by the administration of methamphetamine or 3,4-methylenedioxymethamphetamine (Zhang *et al*, 2006a, b; Hashimoto *et al*, 2007), suggesting that minocycline may be a potential therapeutic drug for neuropsychiatric disorders including schizophrenia.

In this study, we investigated the effects of minocycline on behavioral changes (acute hyperlocomotion and PPI deficits) in mice induced by the administration of dizocilpine. Furthermore, using *in vivo* microdialysis technique, we examined the effects of minocycline on the dopamine (DA) release in prefrontal cortex and striatum after the administration of dizocilpine as DA in these brain regions has been implicated in the behavioral changes by the NMDA receptor antagonism.

METHODS

Animals

Male Std:ddy mice (8 weeks old, 32–39 g body weight at the beginning of the experiment) were housed under a 12-h light/12-h dark cycle (lights on from 0700 to 1900 h; room temperature, 22±2°C; humidity, 55±5%) with free access to food and water. All experiments were performed in accordance with the Guide for Animal Experimentation, Chiba University Graduate School of Medicine.

Drugs Administration

(+)-MK-801 hydrogen maleate (dizocilpine) (0.1 mg/kg. as a hydrogen maleate salt; Sigma-Aldrich Corporation, St Louis, MO), dissolved in physiological saline, was injected subcutaneously (s.c.) in a volume of 10 ml/kg. The dose (0.1 mg/kg) of dizocilpine was selected because this dose caused PPI deficits in mice. Minocycline hydrochloride (10, 20, or 40 mg/kg as a hydrochloride salt; Wako Pure Chemical Industries, Ltd, Osaka, Japan), dissolved in physiological saline, was injected intraperitoneally (i.p.) in a volume of 10 ml/kg. The other chemicals used were purchased from commercial sources.

Effects of Minocycline on Hyperlocomotion after a Single Administration of Dizocilpine

Thirty minutes after a single i.p. injection of minocycline (10, 20, or 40 mg/kg, $n=6$) or vehicle (10 ml/kg, $n=6$), dizocilpine (0.1 mg/kg, $n=6$) or vehicle (10 ml/kg, $n=6$) was administered s.c. into the mice. Locomotor activity was measured using an animal movement analysis system (SCANET SV-10, Melquest, Toyama, Japan), as reported previously (Zhang *et al*, 2006a). The system consisted of a rectangular enclosure (480 × 300 mm). The side walls (height, 60 mm) of the enclosure were equipped with 144 pairs of photosensors located at 5-mm intervals at a height of 30 mm from the bottom edge. An animal was placed in the observation cage 60 min from injection of vehicle or dizocilpine. A pair of photosensors was scanned every 0.1 s to detect the animal's movements. The intersection of paired photosensors (10 mm apart) in the enclosure was counted as one unit of locomotor activity. Data collected for 180 min were used in this study.

Measurement of Acoustic Startle Reactivity and Prepulse Inhibition of Startle

The mice were tested for their acoustic startle reactivity (ASR) in a startle chamber (SR-LAB, San Diego Instruments, CA) using standard methods described by Swerdlow and Geyer (1998). After an initial 10-min acclimation period in the chamber, the test sessions began. They consisted of six trial types: (1) pulse alone, 40 ms broadband burst; pulse preceded 100 ms by a 20 ms prepulse that was (2) 4 dB, (3) 8 dB, (4) 12 dB, or (5) 16 dB over background (65 dB); and (6) background only (no stimulus). The amount of PPI is expressed as the percentage decrease in the amplitude of the startle reactivity caused by presentation of the prepulse (% PPI).

For the effect of minocycline on PPI, minocycline (10, 20, or 40 mg/kg) or vehicle (10 ml/kg) were administered 40 min (including 10-min acclimation period) before the machine records, and dizocilpine (0.1 mg/kg) or vehicle (10 ml/kg) was administered s.c. 10 min (including 10-min acclimation period) before. The PPI test lasted 20 min in total.

In Vivo Microdialysis

Mice were anesthetized with sodium pentobarbital before the stereotaxic implantation of a probe into the left frontal cortex (+2.1 mm anteroposterior, +1.0 mm mediolateral from the bregma, and −1.2 mm dorsoventral with respect to dura) or striatum (+0.0 mm anteroposterior, +2.5 mm mediolateral from the bregma, and −4.4 mm dorsoventral with respect to dura). Probes were secured onto the skull using stainless-steel screws and dental acrylic. Twenty-four hours after surgery, *in vivo* microdialysis was performed on conscious mice. Probes were perfused continuously with artificial CSF (147 mM NaCl, 4 mM KCl, and 2.3 mM $CaCl_2$) at a rate of 2 μl/min. The dialysate was collected in 30-min fractions. Levels of DA were measured by high-performance liquid chromatography (HPLC) using a reversed phase column (Eicompak CA-5ODS 2.1 mm × 150 mm; Eicom, Kyoto, Japan), as reported previously (Zhang *et al*, 2006a). Four samples were obtained in order to establish the baseline levels of extracellular DA before the administration of dizocilpine.

Statistical Analysis

The data are presented as the mean±standard error of the mean (SEM). The computation was carried out using the SPSS 12.0J software (SPSS 12.0J, Tokyo, Japan). The results of the acute behavioral study and *in vivo* microdialysis were analyzed by two-way analysis of variance (ANOVA) for repeated measures, with treatment as the between-subjects factor and time as the within-subjects factor. When appropriate, group means at individual time points were compared by one-way ANOVA, followed by Bonferroni/Dunn *a posteriori* analysis.

PPI was calculated as the percent inhibition of the startle amplitude evoked by the pulse alone: % PPI = 100 × (magnitude on pulse alone trial−magnitude on prepulse + pulse trial/magnitude on pulse alone trial). The PPI data were analyzed using a with treatment drug as a between-subjects factor and prepulse intensity as a within-subjects factor. There were significant effects of prepulse intensity (which were always significant), which will not be discussed, and drug treatment data were collapsed across prepulse intensity for presentation purposes. The PPI data were analyzed by multivariate analysis of variance (MANOVA). When appropriate, group means at individual dB levels were compared by one-way ANOVA, followed by Bonferroni/Dunn *a posteriori* analysis. The dose-dependent relationship was evaluated by MANOVA, followed by one-way ANOVA with contrast (polynomial). Significance for the results was set at $p<0.05$.

RESULTS

Effects of Minocycline on Hyperlocomotion after a Single Administration of Dizocilpine

A single administration of dizocilpine (0.1 mg/kg, s.c.) markedly increased locomotion in mice. Two-way ANOVA analysis revealed significant differences among the five groups studied ($F(44, 275)=2.599$, $p<0.0001$). Pretreatment with minocycline (40 mg/kg, i.p., 30 min before the administration of dizocilpine) significantly attenuated dizocilpine-induced hyperlocomotion in mice (Figure 1). In contrast, administration of minocycline (40 mg/kg) alone did not alter locomotion in mice.

Effects of Minocycline on PPI Deficits after a Single Administration of Dizocilpine

Figure 2 shows the effects of minocycline (10, 20, or 40 mg/kg) on dizocilpine (0.1 mg/kg)-induced PPI deficits in mice. The MANOVA analysis of all PPI data revealed that there was a significant effect (Wilks lambda = 0.395, $p<0.001$). Subsequent ANOVA analysis revealed significant differences at all dB groups (4, 8, 12, and 16 dB). A *posteriori* analysis indicated a significant ($p<0.01$) difference between vehicle + vehicle group and vehicle + dizocilpine (0.1 mg/kg) group (Figure 2). Furthermore, *a posteriori* analysis demonstrated that minocycline (40 mg/kg) significantly ($p<0.05$) attenuated PPI deficits in mice induced by dizocilpine (0.1 mg/kg) (Figure 2). Next, we analyzed whether the effects of minocycline on dizocilpine-induced PPI deficits were dose-dependent. The MANOVA analysis of four groups (0, 10, 20, and 40 mg/kg of minocycline) revealed a significance (Wilks lambda = 0.621, $p=0.029$). Moreover, the subsequent analysis using contrast (polynomial) showed that minocycline significantly attenuated dizocilpine-induced PPI deficits at 8 dB ($p=0.003$), 12 dB ($p<0.001$), and 16 dB ($p<0.001$), in a dose-dependent manner (Figure 2). In contrast, minocycline (40 mg/kg) alone did not alter PPI in mice (Figure 2).

Effects of Minocycline on Dizocilpine-Induced DA Release in the Frontal Cortex and Striatum

In order to explore the mechanisms by which minocycline inhibits the psychopharmacological effects of dizocilpine, we used an *in vivo* microdialysis technique to examine the *in vivo* effects of minocycline on the dizocilpine-induced increase in extracellular DA levels in the frontal cortex and striatum of conscious mice. A single administration of dizocilpine (0.1 mg/kg, s.c.) caused a marked increase in extracellular DA levels in the frontal cortex and striatum. Peak levels of extracellular DA were increased to approximately five-fold the baseline level. Two-way ANOVA analysis revealed significant differences among the three

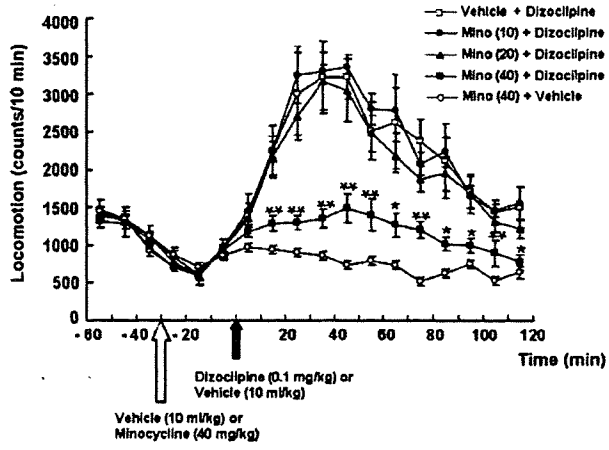

Figure 1 Effects of minocycline on dizocilpine-induced hyperlocomotion in mice. Thirty minutes after a single i.p. injection of minocycline (10, 20, or 40 mg/kg) or vehicle (10 ml/kg), dizocilpine (0.1 mg/kg) or vehicle (10 ml/kg) was administered s.c. into the mice. Behavior (locomotion) in the mice was evaluated. Each value (counts per 10 min) is the mean ± SEM ($n=6$ per group). *$p<0.05$, **$p<0.01$ as compared with the vehicle + dizocilpine group.

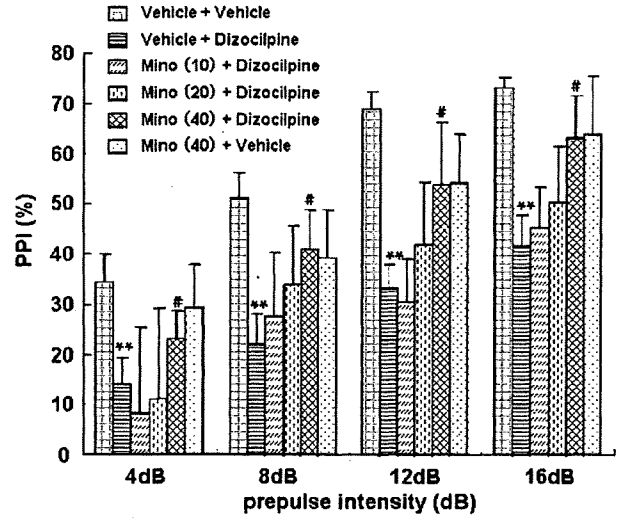

Figure 2 The effect of minocycline on dizocilpine-induced PPI deficits in mice. Thirty minutes after i.p. injection of vehicle (10 ml/kg) or minocycline (10, 20, or 40 mg/kg), dizocilpine (0.1 mg/kg) or vehicle (10 ml/kg) was administered s.c. to the mice. Each value is the mean ± SEM ($n=12$–14 per group). **$p<0.01$ as compared with vehicle + vehicle group, $^{\#}p<0.05$ as compared with vehicle + dizocilpine group.

groups studied (frontal cortex: $F(10, 110) = 58.47$, $p < 0.001$; striatum: $F(10, 100) = 60.07$, $p < 0.001$). Subsequent analysis revealed that pretreatment with minocycline (40 mg/kg, i.p., 30 min before dizocilpine treatment) significantly attenuated dizocilpine-induced increases in extracellular DA levels in the frontal cortex (Figure 3a) and in the striatum (Figure 3b). Effects of minocycline on dizocilpine-induced DA release in the frontal cortex were greater than those of minocycline in the striatum. In contrast, we found that minocycline alone did not alter the extracellular

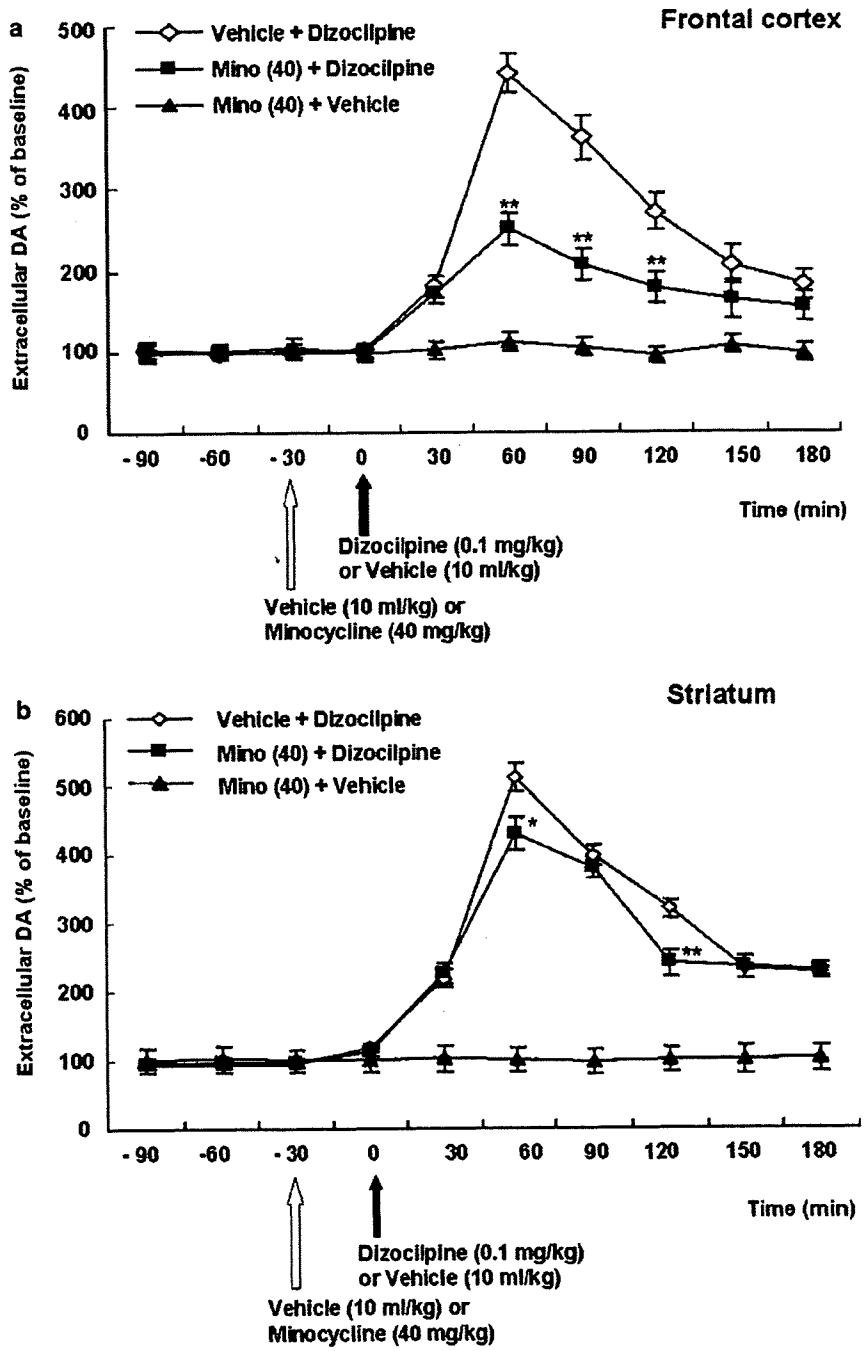

Figure 3 Effects of minocycline on extracellular DA levels in the frontal cortex and striatum after the administration of dizocilpine. Thirty minutes after i.p. injection of minocycline (40 mg/kg) or vehicle (10 ml/kg), MK-801 (0.1 mg/kg, s.c.) or vehicle (10 ml/kg, s.c.) was administered to mice. Extracellular levels of DA in the mouse frontal cortex (a) and striatum (b) were measured by *in vivo* microdialysis in conscious mice. The basal extracellular DA levels were 0.424 ± 0.019 pg/20 μl in the frontal cortex (mean ± SEM of 8–9 mice) and 2.697 ± 0.269 pg/20 μl in the striatum (mean ± SEM of 8–9 mice). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with dizocilpine-treated group.

DA levels in the frontal cortex (Figure 3a) and striatum (Figure 3b).

DISCUSSION

The major findings of the present study are that minocycline significantly attenuated behavioral changes (hyperlocomotion and PPI deficits) in mice after the administration of dizocilpine, and that minocycline significantly attenuated increase of extracellular DA levels in the frontal cortex and striatum after the administration of dizocilpine. To our knowledge, this is the first report demonstrating that minocycline can restore behavioral changes (eg, hyperlocomotion and sensorimotor gating deficits) induced by the NMDA receptor antagonist dizocilpine. Several studies demonstrated that atypical antipsychotic drugs including clozapine can ameliorate hyperlocomotion and PPI deficits in mice after the administration of dizocilpine (Leriche *et al*, 2003; Levin *et al*, 2005; Lipina *et al*, 2005; Long *et al*, 2006). Therefore, our findings indicate that minocycline has a potential antipsychotic activity in animal models of schizophrenia.

Schizophrenia is associated with a dysregulation of DA function in both the prefrontal cortex and striatum (reviewed by Goldman-Rakic, 1999; Goldman-Rakic *et al*, 2004; Weinberger *et al*, 2001; Abi-Dargham and Moore, 2003), and the role of prefrontal cortex in working memory had received a great deal of attention because most patients with schizophrenia exhibit deficits in working memory-related tasks (reviewed by Goldman-Rakic, 1999; Goldman-Rakic *et al*, 2004). It has been reported that the NMDA receptor antagonists such as dizocilpine and ketamine dose-dependently impaired the spatial delayed alteration performance, and that these drugs preferentially increased the release of DA in the prefrontal cortex compared with the striatum of rats (Verma and Moghaddam, 1996). Interestingly, it has been reported that repeated administration of dizocilpine significantly increased the density of DA D1 receptors in the prefrontal cortex and decreased working memory performance in monkeys (Tsukada *et al*, 2005), indicating the dizocilpine-induced impairment of DA neuronal system in prefrontal cortex. A recent report showed that DA D1 receptor agonists rather than D2 receptor agonists disrupt PPI in mice, suggesting that DA D1 receptors may play a more prominent role in the modulation of PPI in mice (Ralph-Williams *et al*, 2003). Taken together, it is likely that the inhibition of dizocilpine-induced DA release by minocycline in the prefrontal cortex may be implicated in the mechanism of action of minocycline with respect to dizocilpine-induced PPI deficits in mice although the mechanism(s) underlying the modulation of dizocilpine-induced DA release by minocycline are currently unclear. Therefore, it is likely that minocycline may have potential therapeutic activity for schizophrenia.

Some studies demonstrated that the medial prefrontal cortex (mPFC) might be involved in the PPI deficits after the administration of dizocilpine (Bakshi and Geyer, 1998; Schwabe and Koch, 2004). First, it has been reported that dizocilpine significantly decreased PPI after infusion into the amygdala or dorsal hippocampus, but not nucleus accumbens, ventral hippocampus, or dorsomedial thalamus, and that a trend toward PPI deficits was also observed with administration into mPFC (Bakshi and Geyer, 1998). These findings suggest that multiple limbic forebrain regions including mPFC might mediate dizocilpine-induced PPI deficits in rats (Bakshi and Geyer, 1998). Second, Schwabe and Koch (2004) reported that dizocilpine failed to disrupt PPI in rats with ibotenic acid lesions of the mPFC, suggesting that mPFC is an important brain region within the neuronal circuit responsible for dizocilpine-induced PPI deficits. In this study, we found that the increase in extracellular DA levels in prefrontal cortex after the administration of dizocilpine was significantly attenuated by pretreatment with minocycline (40 mg/kg). Based on the key role of DA in the behavioral changes by the NMDA receptor antagonists, it is also likely that the inhibition of dizocilpine-induced DA release by minocycline in the prefrontal cortex may, in part, be implicated in the mechanism of action of minocycline with respect to dizocilpine-induced behavioral changes in mice.

Minocycline can readily cross the blood–brain barrier, regardless of the dose and route of administration (Barza *et al*, 1975; Aronson, 1980; Zhang *et al*, 2006a). Recent clinical trials have been aimed primarily at assessing the safety and tolerability of minocycline in several neurodegenerative diseases (reviewed by Blum *et al*, 2004; Domercq and Matute, 2004; Thomas and Le, 2004; Yong *et al*, 2004; Stirling *et al*, 2005; Smith and Leyden, 2005). In these clinical trials, minocycline was well tolerated at 200 mg/day over 6 months, and no side effects or negative interactions with other simultaneously administered drugs were observed (Domercq and Matute, 2004). Taken together, it might be of great interest to study the effects of minocycline on several symptoms in schizophrenic patients.

In conclusion, the present findings suggest that minocycline ameliorated behavioral changes (hyperlocomotion and PPI deficits) in mice after the administration of the NMDA receptor antagonist dizocilpine, and minocycline significantly attenuated the release of DA in the frontal cortex after the administration of dizocilpine. Therefore, minocycline would be a potential therapeutic drug for schizophrenia.

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Phencyclidine-induced cognitive deficits in mice are improved by subsequent subchronic administration of the antibiotic drug minocycline

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Abstract

Background: The *N*-methyl-D-aspartate (NMDA) receptor antagonist phencyclidine (PCP)-induced cognitive deficits have been used as an animal model for schizophrenia. This study was undertaken to determine whether the antibiotic drug minocycline could improve PCP-induced cognitive deficits in mice.
Methods: Saline (10 ml/kg/day, s.c., once daily on day 1–5, 8–12) or PCP (10 mg/kg/day, s.c., once daily on day 1–5, 8–12) were administered to mice for 10 days. Subsequently, vehicle (10 ml/kg/day, i.p.) or minocycline (4.0 or 40 mg/kg/day, i.p.) was injected for 14 consecutive days. One day after the final injection, a novel object recognition test was performed.
Results: PCP-induced cognitive deficits in mice were significantly improved by subsequent subchronic (14 days) administration of minocycline (40 mg/kg), but not minocycline (4.0 mg/kg).
Conclusions: This study suggests that minocycline could be a potential therapeutic drug for cognitive deficits in schizophrenic patients.

Keywords: Cognition; Minocycline; NMDA receptor; Novel object recognition; Schizophrenia

1. Introduction

Cognitive deficits in patients with schizophrenia are a core feature of the illness, which predicts vocational and social disabilities for patients (Green, 1996). Accumulating evidence suggests that *N*-methyl-D-aspartate (NMDA) receptor plays a role in the pathophysiology of schizophrenia (Javitt and Zukin, 1991; Hashimoto et al., 2004, 2005b). The NMDA receptor antagonists such as phencyclidine (PCP) are known to induce schizophrenia-like symptoms including cognitive deficits in healthy subjects (Javitt and Zukin, 1991). In the novel object recognition test (NORT), we found that PCP-induced cognitive deficits in mice could be significantly improved by subsequent subchronic (14 days) administration of clozapine, but not haloperidol (Hashimoto et al., 2005a). These findings suggest that the 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., 2005a, 2006, 2007a).

Minocycline is a semisynthetic second-generation tetracycline which has anti-inflammatory effects that appear to be completely separate and distinct from its anti-microbial activity. Accumulating evidence suggests that minocycline has potential therapeutic effects in several animal models of neurological diseases (Domercq and Matute, 2004; Yong et al., 2004; Thomas and Le, 2004; Stirling et al., 2005). In addition, we reported that minocycline could ameliorate the behavioral changes (e.g., acute hyperlocomotion and the development of

Abbreviations: ANOVA, One-way analysis of variance; NMDA, *N*-methyl-D-aspartate; NORT, Novel object recognition test; PCP, Phencyclidine.

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behavioral sensitization) and neurotoxicity that occur in mice and monkeys due to the administration of methamphetamine or 3,4-methylenedioxymethamphetamine (Zhang et al., 2006a, 2006b; Hashimoto et al., 2007b). Furthermore, we found that the hyperlocomotion and prepulse inhibition deficits in mice that occur after the administration of the NMDA receptor antagonist dizocilpine were significantly attenuated by the administration of minocycline (Zhang et al., 2007). These findings suggest that minocycline may be a potential therapeutic drug for neuropsychiatric disorders including schizophrenia. In the present study, using the NORT, we examined the effects of subsequent subchronic (14 days) treatment with minocycline on cognitive deficits in mice after repeated administration of PCP.

2. Methods

2.1. Animals

Male ICR mice (6 weeks old) weighing 25–30 g were purchased from SLC Japan (Hamamatsu, Shizuoka, Japan). The mice were housed in 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) at a room temperature of 23±1 °C and humidity of 55±5%. The mice were given free access to water and to food pellets designed for mice. The experimental procedure was approved by the Animal Care and Use Committee of Chiba University Graduate School of Medicine.

2.2. Drug administration

PCP hydrochloride was synthesized by K.H. in our laboratory. Saline (10 ml/kg/day) or PCP (10 mg/kg/day expressed as a hydrochloride salt) were administered subcutaneously (s.c.) for 10 days (once daily on day 1–5, 8–12), and no treatment was given on days 6, 7, 13 and 14. In the experiment involving subchronic treatment, 3 days (day 15) after the final administration of saline or PCP, vehicle (10 ml/kg/day; physiological saline) or minocycline (4.0 or 40 mg/kg/day, Wako Pure Chemical Ltd., Tokyo, Japan) was administered intraperitoneally (i.p.) for 14 consecutive days (once daily on days 15–28). The dose (40 mg/kg) of minocycline was selected based on the fact that this dose was effective in mitigating the methamphetamine-induced hyperlocomotion (Zhang et al., 2006a) as well as prepulse inhibition deficits in mice after the administration of the NMDA receptor antagonist dizocilpine (Zhang et al., 2007). The dose (4.0 mg/kg) of minocycline was used as a low-dose, negative control dose. The experiments were conducted separately, and the individual dose groups were distributed across the duration of the experiments.

2.3. Novel object recognition test (NORT)

NORT was performed 1 day after a final administration of vehicle (10 ml/kg/day for 14 days) or minocycline (4.0 or 40 mg/kg/day for 14 days). 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 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 (5 min×2). The animals were considered to be exploring the object when the head of the animal was facing the object within an inch of the object or when any part of the body, except for the tail, was touching the object. The time that the mice spent exploring each object was recorded. After the training, the mice were immediately returned to their home-cages, and the box and objects were cleaned with 75% ethanol to avoid any possible instinctive odorant cues. Retention tests were carried out at 1-day intervals following the training. During the retention test, each mouse was placed back into the same box, with one of the objects used during training replaced by a novel object. 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 counter-balanced manner in terms of their physical complexity. A preference index, the ratio of the amount of time spent exploring any one of the two objects (training session) or the novel object (retention test session) over the total time spent exploring both objects, was used to measure the memory performance.

2.4. Statistical analysis

Data were expressed as means±S.E.M. Statistical analysis was performed using one-way analysis of variance (ANOVA) and the *post hoc* Bonferroni/Dunn test. *P*-values less than 0.05 were considered statistically significant.

3. Results

During the training session, there were no significant differences (F (4,80)=1.92, p=0.115) among the five groups in the total amount of time spent exploring two objects (Table 1). 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

Table 1
Total amount of time spent exploring both objects during object recognition in the training and retention sessions

Group	Training session (10 min)	Retention session (5 min)
	Time exploring objects (seconds)	
Vehicle+Vehicle	63.97±3.37	48.12±3.81
PCP+Vehicle	65.24±4.64	43.93±3.30
PCP+Minocycline (4.0 mg/kg)	63.47±6.92	42.65±5.99
PCP+Minocycline (40 mg/kg)	82.65±6.74	48.81±3.90
Vehicle+Minocycline (40 mg/kg)	71.08±13.14	32.44±6.67

Data are expressed as the mean±S.E.M (n=9–24). There were no significant differences among five groups.

group, suggesting that the behavior of the PCP-treated mice may not have been due to memory impairment (Hashimoto et al., 2005a). Therefore, it is likely that our model of PCP-induced cognitive deficits using NORT may show behavioral deficits such as reduction in motivation for a novel object, concentration, and withdrawal symptoms, which might be related to cognitive deficits.

We examined the effects of subsequent subchronic (14 day) administration of minocycline on PCP-induced cognitive deficits in mice. As shown in Fig. 1, PCP-induced deficits were significantly improved after subsequent subchronic (14 days) administration of minocycline (40 mg/kg/day), but not minocycline (4.0 mg/kg/day). In the training session, the exploratory preference was not different among the five groups (F (4,80)=1.011, p=0.407) (Fig. 1). However, in the retention test session, ANOVA analysis revealed that the exploratory preferences of mice in the five groups were significantly different (F (4,80)=14.30, p<0.001) (Fig. 1). A *post hoc* Bonferroni test indicated that the exploratory preference of the PCP-treated group was significantly (p<0.001) increased after subchronic (14 days) administration of minocycline (40 mg/kg/day) (Fig. 1). Furthermore, subchronic (14 days) administration of minocycline (40mg/kg/day) alone did not alter the exploratory preference in either the training session or the retention session (Fig. 1).

4. Discussion

In this study, we found that PCP-induced cognitive deficits could be improved by subsequent subchronic (14 days) administration of minocycline. In the NORT, no significant differences in the total amount of time spent exploring two objects or in exploratory preference were found among all of the groups during the training session, suggesting that the levels of motivation, curiosity, and interest in exploring novel objects were the same in all groups (Hashimoto et al., 2005a, 2006, 2007a). In the NORT, we reported that PCP-induced cognitive deficits in mice could be improved by subsequent subchronic (14 days) administration of the atypical antipsychotic drug clozapine, but not the typical antipsychotic drug haloperidol (Hashimoto et al., 2005a). Therefore, the reversal of PCP-induced cognitive deficits using 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., 2005a).

It has been reported that minocycline enhanced the discriminative stimulus effects of PCP in rats, suggesting that minocycline may interact either directly or indirectly with the NMDA receptors (Munzar et al., 2002). These data seem to be in conflict with the present data. The precise mechanisms underlying this discrepancy are currently unclear. The discrepancy may be due to the difference in the treatment schedule. In the paper by

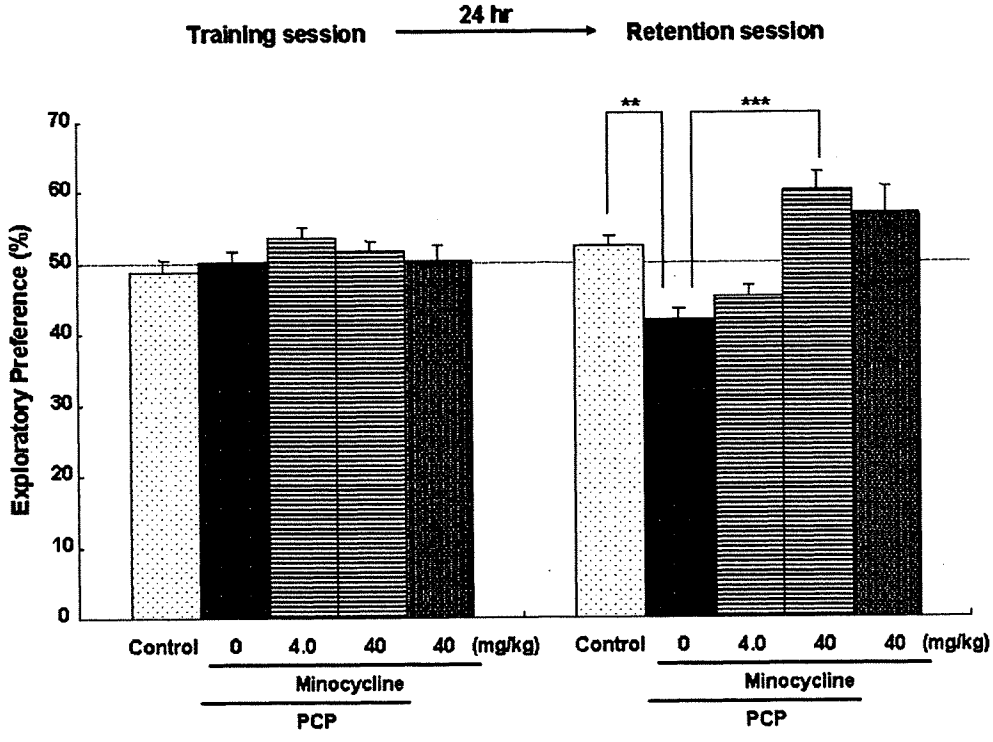

Fig. 1. Effects of minocycline on PCP-induced cognitive deficits in mice Saline (10 ml/kg/day) or PCP (10 mg/kg/day) were administered s.c. for 10 days (once daily on day 1–5, 8–12). (A) Three days (day 15) after the last administration of saline or PCP, vehicle (10 ml/kg/day; saline), or minocycline (4.0 or 40 mg/kg/day) were administered i.p. into mice for consecutive 14 days (once daily on day 15–28). Twenty four hours (day 29) after the last administration of vehicle or minocycline, the training session of NORT was performed. Then the retention test session was performed 24 h after the training session. Values are the mean±S.E.M. (n=9–24). **p<0.01, ***p<0.001 as compared with PCP plus saline-treated group.

Munzar et al. (2002), minocycline was administered 30 min before PCP administration. It is, therefore, possible that minocycline may increase PCP levels in the rat brain by pharmacokinetic interaction. In contrast, in the present study, minocycline was administered 24 h after the final administration of PCP.

Recently, we reported that minocycline significantly attenuated hyperlocomotion in mice after the administration of methamphetamine (Zhang et al., 2006a), and that hyperlocomotion and prepulse inhibition deficits in mice after the administration of the NMDA receptor antagonist dizocilpine were significantly attenuated by the administration of minocycline (Zhang et al., 2007). Taken together, these results suggest that minocycline is likely to have antipsychotic activity in animal models of schizophrenia. Although the molecular and cellular mechanisms underlying the efficacy of minocycline on PCP-induced cognitive deficits are currently unclear, it seems that at least two mechanisms (the attenuation of innate and adaptive immunity and the blockade of apoptotic cascades) (Domercq and Matute, 2004) may be implicated in the mechanism of minocycline. Further studies of the mechanism of minocycline are necessary.

Recently, Miyaoka et al. (2007) reported two cases of schizophrenic patients treated with minocycline. In the two patients, minocycline was effective in the treatment of acute schizophrenia with predominantly catatonic symptoms (Miyaoka et al., 2007). Ahuja and Carroll (2007) have hypothesized that minocycline acts as a functional NMDA receptor antagonist and helps improve the catatonic symptoms when used as an adjunct to antipsychotic medication. However, the precise mechanisms underlying the improvement induced by minocycline are currently unknown. Further double-blind placebo control studies of minocycline in schizophrenic patients as well as molecular and cellular studies of the mechanisms of minocycline are necessary.

5. Conclusion

This study shows that PCP-induced cognitive deficits in mice could be attenuated by subsequent subchronic administration of minocycline, although the precise mechanisms underlying the mechanism of action for minocycline remain unresolved. Therefore, minocycline may be a potential therapeutic drug for schizophrenia.

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Research Report

Mithramycin protects against dopaminergic neurotoxicity in the mouse brain after administration of methamphetamine

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ABSTRACT

The present study was undertaken to examine the effects of mithramycin, an inhibitor of transcription factor Specificity protein (Sp)-1, on the behavioral changes and dopaminergic neurotoxicity in the mouse striatum after administration of methamphetamine (METH). Pretreatment with mithramycin (75, 150 or 300 μg/kg) did not alter acute hyperlocomotion in mice after a single administration of METH (3 mg/kg). However, the development of behavioral sensitization in mice after repeated administration of METH (3 mg/kg/day, once daily for 5 days) was significantly blocked by pretreatment with mithramycin (300 μg/kg). Furthermore, pretreatment with mithramycin (300 μg/kg) significantly attenuated the hyperthermia in mice after repeated administration of METH (3 mg/kg×3, 3-h intervals). Moreover, the combination of pretreatment and subsequent administration of mithramycin (75, 150 or 300 μg/kg) significantly attenuated the reductions of dopamine (DA), its major metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) and DA transporter (DAT) in the striatum after repeated administration of METH (3 mg/kg×3, 3-h intervals), and these attenuations were dose dependent. These findings suggest that mithramycin attenuates the development of behavioral sensitization and dopaminergic neurotoxicity in mice after repeated administration of METH. Therefore, mithramycin could have potential for the treatment of METH abusers, particularly since this drug has been approved by the Food and Drug Administration in the United States. In the future, however, another Sp1 inhibitors with fewer side effects might be more appropriate.

1. Introduction

Abuse of methamphetamine (METH) is an extremely serious and growing worldwide problem. METH is a powerfully addictive stimulant associated with serious health conditions, including memory loss, aggression, psychotic symptoms and behavior and potential heart and brain damage (Ujike and Sato, 2004; Hashimoto, 2007). Repeated administration of METH induces dopaminergic neurotoxicity in rodents and nonhuman primates by producing long-term depletion of dopamine (DA) and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) and of the density of DA transporter (DAT) binding in the striatum (Davidson et al., 2001; Cadet et al., 2003; Zhang et al., 2006; Hashimoto et al., 2004; 2007). In addition, the levels of DA and the density of the DAT have been shown to be reduced in the postmortem

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striatum of chronic METH users (Wilson et al., 1996). Brain imaging studies using positron emission tomography (PET) have demonstrated that the density of the DAT in the caudate/putamen and nucleus accumbens of METH users was significantly lower than that of normal controls (Sekine et al., 2001; Volkow et al., 2001). Thus, although METH-induced neurotoxicity in the dopaminergic terminals is well documented, the precise mechanism of METH-induced neurotoxicity remains unknown (Cadet et al., 2003; Hashimoto, 2007).

Mithramycin is an aureolic acid polyketide antibiotic traditionally used to treat hypercalcemia/hypercalciuria and certain forms of cancers (Jones et al., 1995). Mithramycin binds to G–C-rich DNA sequences to inhibit the binding of transcription factor Specificity protein (Sp1) (Ray et al., 1989), which is known to affect neuronal survival/death pathways (Jones et al., 1995; Chatterjee et al., 2001; Ferrante et al., 2004; Lee et al., 2006; Citron et al., 2008). In addition, mithramycin may also indirectly regulate gene transcription by altering histone methylation (Ferrante et al., 2004), which is involved in the pathology of neuropsychiatric diseases (Jiang et al., 2008; Akbarian and Huang, 2009). In a mouse (R6/2) model of Huntington's disease, treatment with mithramycin prolonged survival and prevented the increase in histone H3 methylation observed in these mice, suggesting that the enhanced survival and neuroprotection might be attributable to alleviation of the repressed expression of genes vital to neuronal function and survival (Ferrante et al., 2004). Taken together, these findings suggest that mithramycin is likely to have neuroprotective effects in animal models of neurodegenerative diseases.

It is therefore of interest to study whether mithramycin can attenuate behavioral and pathological changes after administration of METH. The present study was undertaken to examine the effects of mithramycin on the behavioral changes and dopaminergic neurotoxicity in mice after administration of METH.

2. Results

2.1. *Effects of mithramycin on hyperlocomotion and development of behavioral sensitization after administration of METH*

A single administration of METH (3 mg/kg, s.c.) markedly increased locomotion in mice. Pretreatment with mithramycin (75, 150 or 300 μg/kg, i.p.) did not alter hyperlocomotion in mice after a single administration of METH (3 mg/kg, s.c.; Fig. 1). In addition, mithramycin (300 μg/kg, i.p.) alone did not alter locomotion in the normal mice (Fig. 1).

Repeated administration of METH (3 mg/kg, once daily for five consecutive days) significantly increased METH (1 mg/kg)-induced hyperlocomotion in mice as compared with the results obtained with the vehicle+vehicle group, indicating the development of behavioral sensitization by repeated treatment with METH (Fig. 2). One-way ANOVA revealed significant differences among the four groups [$F(3,31)=13.845$, $p<0.001$], and *post hoc* analysis indicated that pretreatment with mithramycin (300 μg/kg, i.p.) significantly ($p<0.01$) attenuated the development of sensitization after the administration of METH. In contrast, the locomotion in the mithramycin (300 μg/kg, i.p.)+vehicle group did not differ from that of the control (vehicle+vehicle) group (Fig. 2).

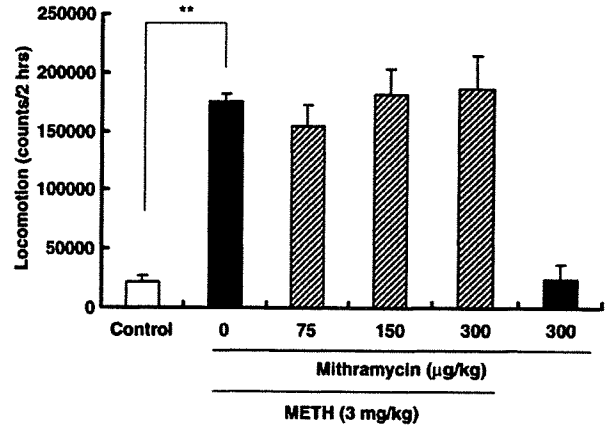

Fig. 1 – Effect of mithramycin on hyperlocomotion in mice after a single administration of METH. Thirty minutes after i.p. injection of vehicle (10 ml/kg) or mithramycin (75, 150 or 300 mg/kg), METH (3 mg/kg) or vehicle (10 ml/kg) was administrated s.c. to the mice. Behavior (locomotion) in the mice was evaluated for 3 h. Data were shown as total locomotion for 2 h after administration of METH or vehicle. Each value is mean±SEM ($n=8$–10, per group).

2.2. *Effects of mithramycin on hyperthermia induced by the repeated administration of METH*

Two-way ANOVA analysis revealed significant differences among the five groups [$F(4,60)=5.596$, $p<0.01$] (Fig. 3). Repeated injection of METH (3 mg/kg×3, 3-h intervals) produced hyperthermia in mice within 1 h after the first injection of METH. Pretreatment with the high doses of mithramycin (150 or 300 μg/kg, i.p.), but not pretreatment with the low dose (75 μg/kg, i.p.), significantly attenuated METH-induced hyperthermia in mice. Mithramycin (300 μg/kg, i.p.) alone did not alter rectal temperature in mice (Fig. 3).

2.3. *Effects of mithramycin on the reduction of DA and DOPAC in the mouse striatum by repeated administration of METH*

One-way ANOVA analysis revealed that striatal DA ($F(5,71)=7.298$, $p<0.001$) and DOPAC ($F(5,71)=9.556$, $p<0.001$) levels were significantly different among the six groups studied. Pretreatment and subsequent administration of mithramycin at the intermediate and high doses (150 and 300 μg/kg), but not at the low dose (75 μg/kg), significantly attenuated the reduction of DA and DOPAC in the striatum after repeated METH administration (3 mg/kg×3, 3-h intervals) (Fig. 4). Treatment with mithramycin (300 μg/kg) alone exerted no effect on the levels of DA and DOPAC in the mouse striatum (Fig. 4).

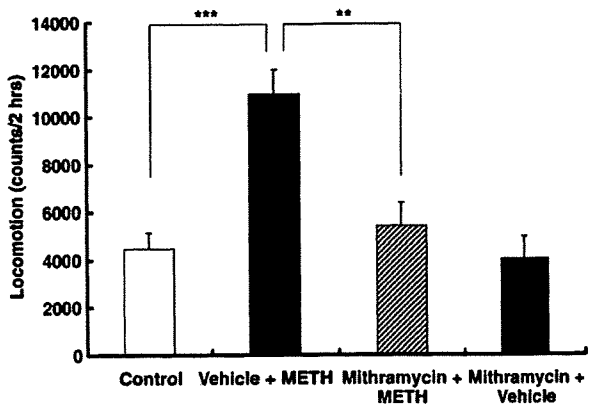

Fig. 2 – Effect of mithramycin on the development of behavioral sensitization in mice after repeated administration of METH. Vehicle (10 ml/kg) + vehicle (10 ml/kg) group, vehicle (10 ml/kg) + METH (3 mg/kg) group, mithramycin (300 μg/kg) + METH (3 mg/kg) group or mithramycin (300 μg/kg) + vehicle (10 ml/kg) group were treated daily for five consecutive days. Seven days after the final administration of METH, a lower dose of METH (1 mg/kg, s.c.) was administered to all mice. Behavioral (locomotion) in the mice was evaluated. Data were shown as total locomotion for 2 h after administration of METH. Each value is mean ± SEM (n = 8–10, per group). **$p < 0.01$, ***$p < 0.001$ as compared to the vehicle + METH group (Bonferroni/Dunn method).

2.4. *DAT immunohistochemistry*

Repeated administration of METH (3 mg/kg × 3, 3-h intervals) markedly decreased the density of the DAT in the mouse striatum (Fig. 5). One-way ANOVA analysis revealed significant differences in DAT immunoreactivity in the striatum [$F(3,18) = 11.262$, $p < 0.001$] among the four groups studied. Post hoc analysis indicated that pretreatment and subsequent administration of mithramycin (300 μg/kg) significantly ($p < 0.01$) attenuated the reduction of DAT immunoreactivity in the mouse striatum after repeated METH administration (Fig. 5). The administration of mithramycin (300 μg/kg) + vehicle did not alter the density of DAT immunoreactivity in the mouse striatum (Fig. 5).

3. Discussion

The major findings of present study are as follows: First, we found that mithramycin could ameliorate the development of behavioral sensitization after repeated METH administration, whereas this drug did not attenuate acute hyperlocomotion in mice after a single administration of METH. Therefore, it is possible that treatment with mithramycin may prevent METH-induced psychosis in abstinent METH users who relapse. Second, we found that pretreatment and subsequent administration of mithramycin significantly attenuated METH-induced dopaminergic neurotoxicity (e.g., reduced the levels of DA, DOPAC and DAT immunoreactivity) in the mouse

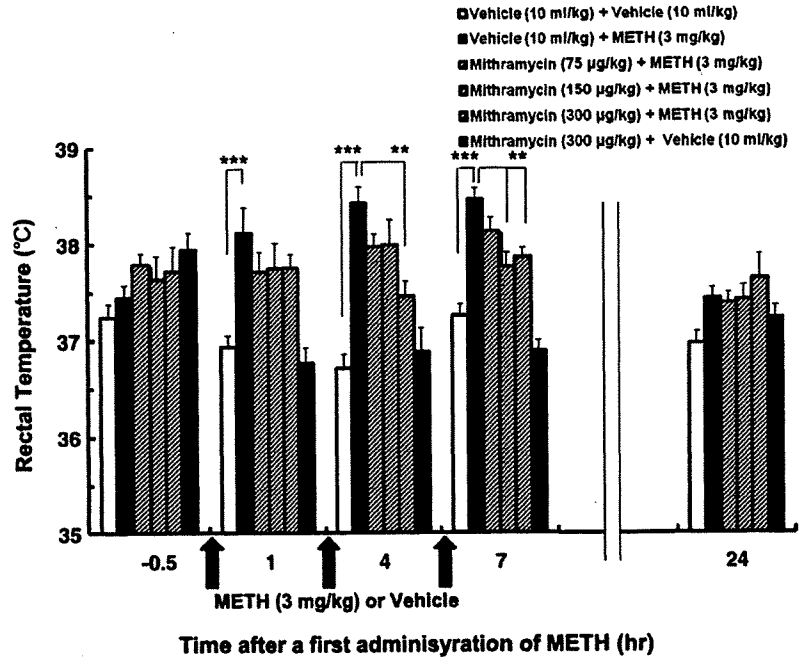

Fig. 3 – Effect of mithramycin on METH-induced hyperthermia in mice. Mice received three injections of vehicle (10 ml/kg, 3-h intervals, s.c) or METH (3 mg/kg, 3-h intervals, s.c). Vehicle (10 ml/kg, i.p.) or mithramycin (75, 150 or 300 μg/kg, i.p.) was injected into the mice 30 min prior the first injection of METH. Rectal temperature was recorded 30 min before the first injection of METH or vehicle and 1, 4, 7 or 24 h after the first METH (or vehicle) injection. Each value is mean ± SEM (n = 8–10 per group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as compared to the vehicle + METH group (Bonferroni/Dunn method).

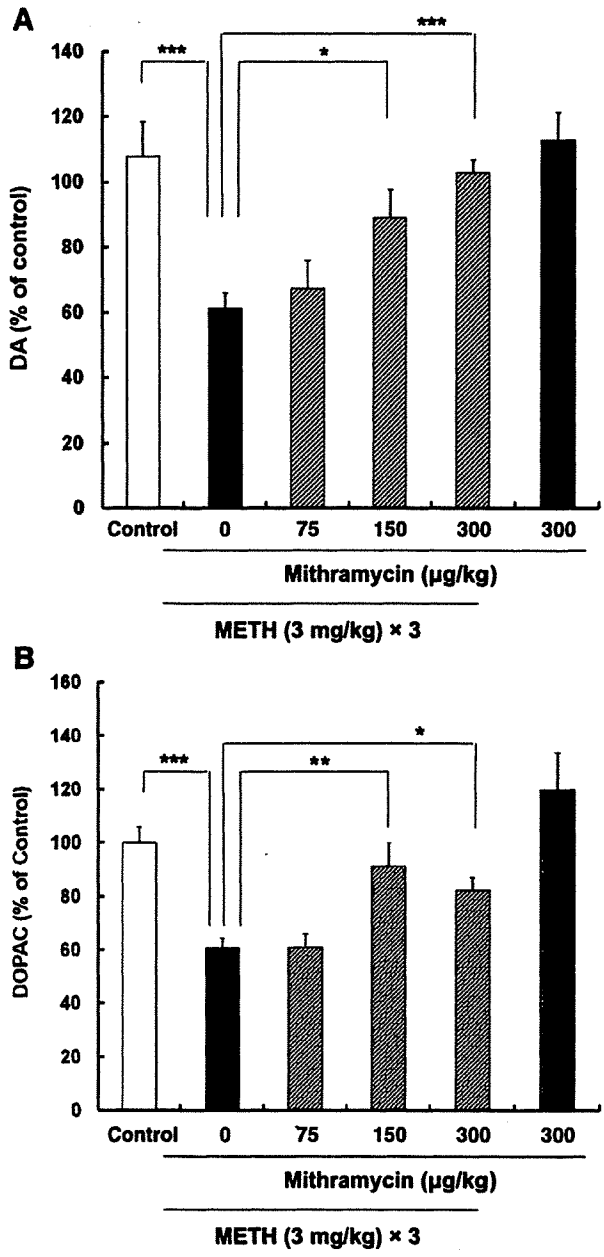

Fig. 4 – Effects of mithramycin on the reduction of DA and DOPAC levels in the mouse striatum after the repeated administration of METH. Thirty minutes after i.p. injection of mithramycin (300 mg/kg) or vehicle (10 ml/kg), mice received three injections of METH (3 mg/kg, s.c) or vehicle (saline, 10 ml/kg, s.c.) at 3-h intervals (day 1). Then mice received once a day injection of mithramycin (300 μg/kg) or vehicle (10 ml/kg) for two consecutive days (days 2 and 3). Mice were sacrificed 3 days after the administration of METH (day 4), and levels of DA and DOPAC in the mouse striatum were measured by HPLC. Values are the mean ± SEM (n = 8–10 per group). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ as compared to the vehicle+METH group (Bonferroni/Dunn method).

striatum. As mentioned in the Introduction, recent PET studies have shown a reduction of DAT in abstinent METH users, suggesting dopaminergic neurotoxicity in the human brain (Sekine et al., 2001; Volkow et al., 2001). Taken together, these findings suggest that mithramycin may have potential as a therapeutic drug for METH abusers, particularly since it has already been approved by the U.S. FDA. On the other hand, due to the documented low efficacy and toxicity of mithramycin for the treatment of hypercalcemia (Pecherstorfer et al., 2003), another Sp1 inhibitor might later be found to be more effective for these patients.

The transcription factor Sp1 is a zinc-finger domain transcriptional activator (Bouwman and Philipsen, 2002) that has been implicated in the expression of many genes in concert with other transcription factors (Suske, 1999; Chu and Ferro, 2005; Safe and Abdelrahim, 2005; Solomon et al., 2008; Tan and Khachigian, 2009). Sp1-mediated processes have been suggested to include aberrant transcriptional modulation of DA receptor genes and neurogeneration (Yajima et al., 1998; Dunah et al., 2002; Li et al., 2002; Ryu et al., 2003; Wang and Bannon, 2005; Qiu et al., 2006). In the present study, we could not determine the role of Sp1 in the mechanism of action of mithramycin because the Sp1 mRNA levels and DNA-binding activities of Sp1 were not altered by repeated METH administration (data not shown). In addition, Lee et al. (2002) reported that administration of METH induced AP-1 and CREB DNA-binding activities in the mouse brain, whereas METH did not affect the DNA-binding activities of Sp1. Nonetheless, we found that treatment with METH markedly increased the DNA-binding activity of Sp1 in neuroblastoma SK-N-SH cells and that mithramycin blocked the increase of DNA-binding activities of Sp1 by METH, suggesting that Sp1 plays a role in the mechanisms of action of mithramycin (data not shown). Further detailed studies will be needed to clarify the role of Sp1 in the mechanisms of action of mithramycin *in vivo*.

Recently, it was reported that Sp1 mRNA levels were altered in the postmortem brains of patients with schizophrenia and in lymphocytes of schizophrenic patients (Ben-Shachar and Karry, 2007). This finding suggested that abnormality in Sp1, which could be the main activator/repressor or could act in combination with additional transcription factors and is subject to environmental stimuli, might contribute to the pathology of schizophrenia (Ben-Shachar and Karry, 2007). It is well known that psychosis associated with repeated METH use might be similar to positive symptoms in schizophrenia (Sato et al., 1983; 1992; Ujike and Sato, 2004). It is therefore likely that mithramycin would attenuate METH-induced psychosis in humans since the severity of psychosis in METH users is associated with a reduction of DAT in the human striatum (Sekine et al., 2001). However, a randomized double-blind placebo-controlled study employing a large number of patients will be needed before it can be concluded that mithramycin is effective in the treatment of METH users.

In conclusion, the present study demonstrated that mithramycin attenuated the dopaminergic neurotoxicity and the development of behavioral sensitization after repeated METH administration. In this context, it is important to note that mithramycin has been used as a treatment for hypercalcemia. In such cases, mithramycin has been shown to have

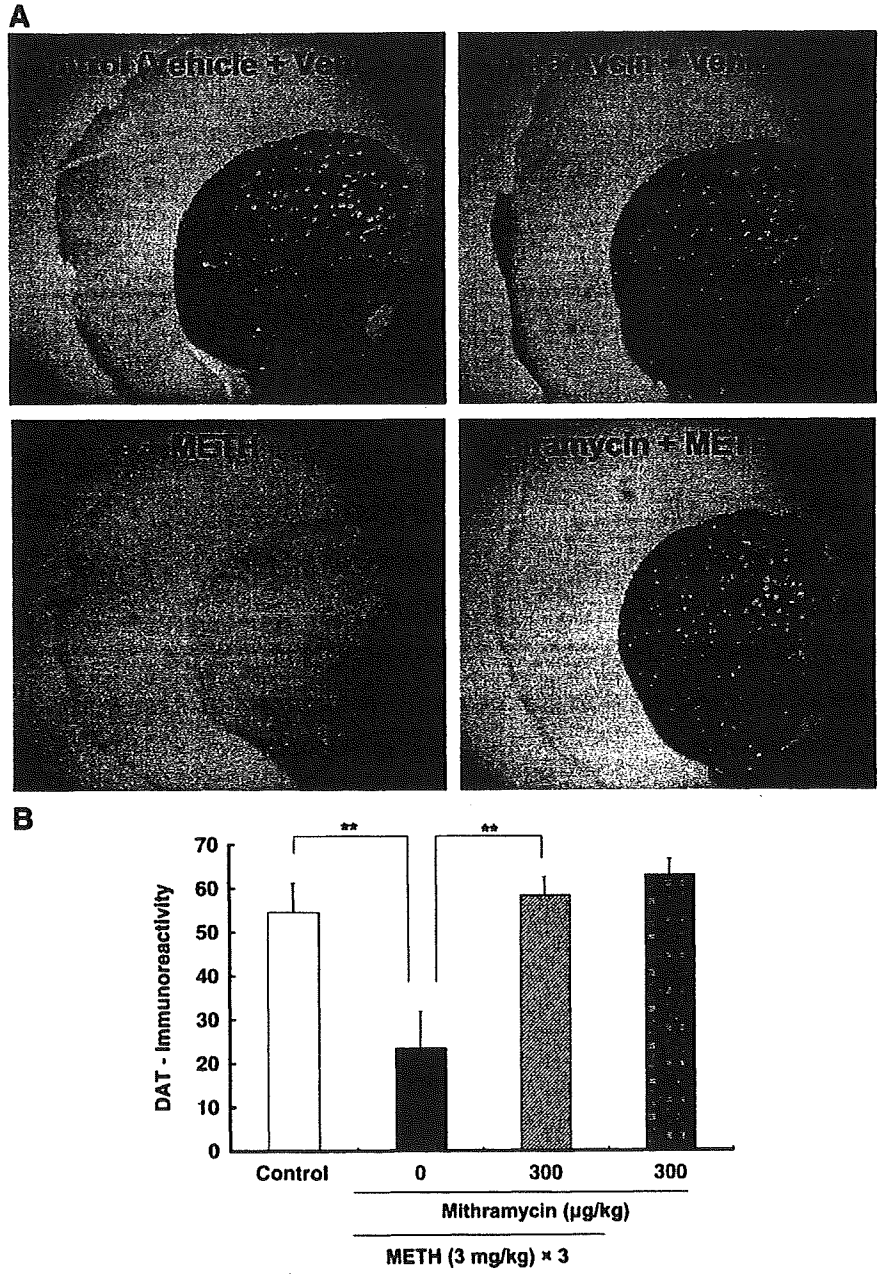

Fig. 5 – Effects of mithramycin on the reduction in DAT density in the mouse striatum after the repeated administration of METH. Vehicle+vehicle, vehicle+METH, mithramycin+METH or mithramycin+vehicle. Thirty minutes after i.p. injection of mithramycin (300 μg/kg) or vehicle (10 ml/kg), the mice received three injections of METH (3 mg/kg, s.c.) or vehicle (saline, 10 ml/kg, s.c.) at 3-h intervals (day 1). Then mice received once a day injection of mithramycin or vehicle for two consecutive days (days 2 and 3). Mice were perfused 3days after the administration of METH (day 4), and then DAT immunohistochemistry was performed. (A) Representative photomicrographs of DAT immunoreactivity in the striatum of the mice. (B) The quantification for DAT immunoreactivity staining was determined for each group and was expressed as a percentage of that of matched control mice. Each value is the mean±SEM (n= 8–10 per group). $^{**}p < 0.01$ as compared to the vehicle+METH group (Bonferroni/Dunn method).

toxicity and relatively low efficacy, and thus it has been recommended that its use be restricted to patients with hypercalcemia of malignancy who fail to respond to intravenous bisphosphonates (Pecherstorfer et al., 2003). Therefore, it is likely that other Sp1 inhibitors with fewer side effects would ultimately have better potential as therapeutic drugs for METH abusers, although mithramycin might be considered in the short term since it has already been approved by the U.S. FDA.

4. Experimental procedures

4.1. Animals

Male Balb/c AnNCrlCrlj mice (8 weeks old, 24–30 g body weight at the beginning of the experiment; Charles River Japan Inc., Tokyo, Japan) were housed under a 12-h light/12-h dark cycle (lights on from 0700 to 1900 h) at room temperature (22±2 °C; humidity, 55±5%) with free access to food and water. In this study, Balb/c mice were used since this strain has been shown to be sensitive to METH-induced dopaminergic neurotoxicity (Kita et al., 1998; Koike et al., 2005; Zhang et al., 2006). All procedures were performed in accordance with the Guidelines for Animal Experimentation of Chiba University.

4.2. Drugs

METH hydrochloride (*d*-methamphetamine; Dainippon Pharmaceutical Ltd., Osaka, Japan) and mithramycin A (Sigma-Aldrich Inc., St. Louis, MO) were dissolved in physiological saline. Other chemicals were purchased from commercial sources. METH was expressed as a hydrochloride salt.

4.3. Locomotor activity

4.3.1. *Effects of mithramycin on hyperlocomotion after a single administration of METH*

In the acute behavioral experiments, vehicle (10 ml/kg, i.p.) or mithramycin (75, 150 or 300 μg/kg) was administered intraperitoneally (i.p.) into mice. Thirty minutes after injection, METH (3.0 mg/kg) or vehicle (10 ml/kg) was injected subcutaneously (s.c.). Locomotor activity was measured over 3 h using an animal movement analysis system (SCANET SV-10; Melquest, Toyama, Japan) as reported previously (Zhang et al., 2006).

4.3.2. *Effects of mithramycin on the development of behavioral sensitization after repeated administration of METH*

Forty mice were divided into the following four groups: a vehicle (10 ml/kg, s.c.)+vehicle (10 ml/kg) group; a vehicle (10 ml/kg)+METH (3 mg/kg) group; a mithramycin (300 μg/kg)+METH (3 mg/kg) group; and a mithramycin (300 μg/kg)+vehicle (10 ml/kg) group. The interval between the first and second injections was 30 min. In this study, we used a 300-μg/kg dose of mithramycin, since this dose was effective in the METH-induced neurotoxicity. After the administration of METH, the mice were returned to their home cages. The same treatment was continued for each animal for five consecutive days. One week after the final treatment, each mouse was given a low dose of METH (1 mg/kg, s.c.), and the animal's behavioral changes (locomotion) were measured using an animal movement analysis system (SCANET SV-10; Melquest) as described above (Zhang et al., 2006).

4.4. Measurement of DA and DOPAC by HPLC

We examined the effects of pretreatment with and subsequent treatment of mithramycin on METH-induced neurotoxicity in mice. Thirty minutes after i.p. injection of mithramycin (75, 150 or 300 μg/kg) or vehicle (10 ml/kg), mice received three injections of METH (3 mg/kg, s.c.) or vehicle (10 ml/kg, s.c.) at 3-h intervals. Rectal temperature was measured using a TD-320 thermometer coupled to a rectal probe (Shibaura Electronics Co., Ltd., Saitama, Japan), and rectal temperatures were recorded 30 min before the first injection, and at 1, 4, 7 and 24 h after the first injection of METH. Mice received daily (24-h intervals) injections of mithramycin (75, 150 or 300 μg/kg) or vehicle (10 ml/kg) for two consecutive days. Mice were sacrificed 3 days after the administration of METH for measurement of DA and DOPAC. The brains were quickly removed and dissected on an ice-cold glass plate. The striatum was dissected, and the samples were stored at −80°C until used for the assay.

4.5. DAT immunohistochemistry

DAT immunohistochemistry in the mouse brain was performed as reported previously (Koike et al., 2005; Zhang et al., 2006). The drug treatment was performed using the same method as used to measure catecholamine. Three days after the administration of METH (3 mg/kg×3, 3-h intervals), the mice were deeply anesthetized with sodium pentobarbital and perfused transcardially with 10 ml of isotonic saline, followed by 40 ml of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed from the skulls and were postfixed overnight at 4 °C in the same fixative. For the immunohistochemical analysis, 50-μm-thick serial coronal sections of brain tissue were cut in ice-cold 0.01 M phosphate-buffered saline (PBS; pH 7.5) using a vibrating blade microtome (VT1000S; Leica Microsystems AG, Wetzlar, Germany). Free-floating sections were treated with 0.3% H_2O_2 in 0.05 M Tris–HCl saline (TBS) for 30 min and were blocked in TBS containing 0.2% Triton X-100 (TBST) and 1.5% normal serum for 1 h at room temperature. The samples were then incubated for 36 h at 4 °C with rat anti-DAT antibody (1:10,000; Chemicon International Inc., Temecula, CA). The sections were washed twice in TBST and were processed according to the avidin–biotin–peroxidase method (Vectastain Elite ABC; Vector Laboratories, Inc., Burlingame, CA). The processed sections were then reacted for 5 min in a solution of 0.15 mg/ml diaminobenzidine containing 0.01% H_2O_2 and were mounted on gelatinized slides, dehydrated, cleared and coverslipped under Permount® (Fisher Scientific, Fair Lawn, NJ). The samples were imaged, and the staining intensity of DAT immunoreactivity in the anterior regions of the striatum was analyzed by using a light microscope equipped with a CCD camera (LAS-3000Uvmini; Fujifilm, Tokyo, Japan) and the Multi Gauge ver. 3.0 software package.

4.6. Statistical analysis

The data are presented as the mean±standard error of the mean (SEM). The results of the behavioral study were analyzed using Student's t-test. The results of rectal temperature were analyzed by two-way analysis of variance (ANOVA) for repeated measures, with treatment as the between-subjects factor and time as the within-subjects factor. When appropriate, group means at individual time points were compared by one-way ANOVA, and *post hoc* comparisons were performed using the Bonferroni/Dunn test. Levels of DA and DOPAC, the densities of DAT-immunoreactive staining intensity and the

behavioral study were analyzed by one-way ANOVA, followed by the Bonferroni/Dunn test for multiple comparisons. For all analyses, p values of less than 0.05 were considered statistically significant.

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Selective Serotonin Reuptake Inhibitors, Fluoxetine and Paroxetine, Attenuate the Expression of the Established Behavioral Sensitization Induced by Methamphetamine

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To obtain an insight into the development of a new pharmacotherapy that prevents the treatment-resistant relapse of psychostimulant-induced psychosis and schizophrenia, we have investigated in the mouse the effects of selective serotonin reuptake inhibitors (SSRI), fluoxetine (FLX) and paroxetine (PRX), on the established sensitization induced by methamphetamine (MAP), a model of the relapse of these psychoses, because the modifications of the brain serotonergic transmission have been reported to antagonize the sensitization phenomenon. In agreement with previous reports, repeated MAP treatment (1.0 mg/kg a day, subcutaneously (s.c.)) for 10 days induced a long-lasting enhancement of the increasing effects of a challenge dose of MAP (0.24 mg/kg, s.c.) on motor activity on day 12 or 29 of withdrawal. The daily injection of FLX (10 mg/kg, s.c.) or PRX (8 mg/kg, s.c.) from 12 to 16 days of withdrawal of repeated MAP administration markedly attenuated the ability of the MAP pretreatment to augment the motor responses to the challenge dose of the stimulant 13 days after the SSRI injection. The repeated treatment with FLX or PRX alone failed to affect the motor stimulation following the challenge of saline and MAP 13 days later. These results suggest that the intermittent and repetitive elevation of serotonergic tone may inhibit the expression of the motor sensitization induced by pretreatment with MAP. It is proposed that clinically available serotonin reuptake inhibitors could be useful for preventing the recurrence of hallucinatory-paranoid state in drug-induced psychosis and schizophrenia.

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Keywords: behavioral sensitization; methamphetamine; fluoxetine; paroxetine; methamphetamine psychosis; schizophrenia

INTRODUCTION

The addiction of amphetamine, methamphetamine (MAP), cocaine, and other psychostimulants with dopamine agonist properties has been a serious worldwide health and social concern, and has been estimated to affect more than 60 million patients based on recent reports from the World Health Organization. The abuse of these drugs causes a growing intensification of craving of psychotomimetic substances, and stimulant-induced psychiatric symptoms exhibit progressive quantitative alterations from a non-psychotic to a prepsychotic and finally to a hallucinatory-paranoid state indistinguishable from that of schizophrenia (Ujike and Sato, 2004). The robust drug craving and psychotic state have been observed to easily reoccur even after long period of abstinence by reuse of a small amount of a stimulant or an unspecific stressor (Ujike and Sato, 2004). These observations indicate that the severe vulnerability to relapse of the above psychotomimetic effects may be established during stimulant abuse (Ujike and Sato, 2004). The difficult clinical problems of stimulant craving and psychosis, and their unpredictable relapses often lead to antisocial behavior and require the development of a novel treatment that can eliminate the enduring vulnerability.

One of the rational approaches to develop this type of treatment appears to explore the substances that reverse an animal model of the drug-induced craving and recurrent psychosis, psychostimulant-induced reverse tolerance, or behavioral sensitization. The behavioral sensitization is a characteristic phenomenon in that the single or repeated exposure to amphetamines and other psychostimulants results in a progressive enhancement of the psychotomimetic responses to these drugs or stress, including hyperactivity and stereotypy, in the rodents (Nishikawa *et al*, 1983; Robinson and Becker, 1986; Vanderschuren and Kalivas, 2000). The augmented behavioral responses have been shown to persist even long after drug discontinuation. Because the progressively intensifying, cross-reactive (to stimulants and stress), easily relapsing, long-lasting, and dopamine agonist-inducible nature of the behavioral sensitization of rodents seems to mimic that of stimulant-

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