研究成果の刊行に関する一覧表

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Ⅳ．　研究成果の刊行物・別刷

Pharmacology, Biochemistry and Behavior 100 (2012) 601–606

Contents lists available at SciVerse ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Minocycline produced antidepressant-like effects on the learned helplessness rats with alterations in levels of monoamine in the amygdala and no changes in BDNF levels in the hippocampus at baseline

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ARTICLE INFO

Article history:
Received 19 March 2011
Received in revised form 12 September 2011
Accepted 16 September 2011
Available online 24 September 2011

Keywords:
Learned helplessness (LH)
Minocycline
Depression
Monoamines
BDNF

ABSTRACT

Previous studies have indicated that minocycline might function as an antidepressant drug. The aim of this study was to evaluate the antidepressant-like effects of minocycline, which is known to suppress activated microglia, using learned helplessness (LH) rats (an animal model of depression). Infusion of minocycline into the cerebral ventricle of LH rats induced antidepressant-like effects. However, infusion of minocycline into the cerebral ventricle of naïve rats did not produce locomotor activation in the open field tests, suggesting that the antidepressant-like effects of minocycline were not attributed to the enhanced locomotion. LH rats showed significantly higher serotonin turnover in the orbitofrontal cortex and lower levels of brain-derived neurotrophic factor (BDNF) in the hippocampus than control rats. However, these alterations in serotonin turnover and BDNF expression remained unchanged after treatment with minocycline. On the contrary, minocycline treatment of LH rats induced significant increases in the levels of dopamine and its metabolites in the amygdala when compared with untreated LH rats. Taken together, minocycline may be a therapeutic drug for the treatment of depression.

1. Introduction

Minocycline, the second-generation tetracycline antibiotic drug, has powerful anti-inflammatory and neuroprotective effects. The action of minocycline is assumed to be exerted through the inhibition of cytochrome c release from the mitochondria, the inhibition of caspase expression, and the suppression of microglial activation (Domercq and Matute, 2004; Kim and Suh, 2009). Minocycline thereby reduces transcription of the downstream pro-inflammatory nitric oxide synthase and cyclooxygenase-2 and the subsequent release of interleukin1β (IL-1β), nitric oxide (NO), and prostaglandin E2.

Minocycline is currently receiving attention as a potential new agent for the treatment of major depression (Hashimoto, 2009; Pae et al., 2008). A previous case report documented the antidepressant effects of minocycline in a patient with bipolar disorder (Levine et al., 1996). In animal studies, minocycline reduced immobility by increasing climbing and enhanced the anti-immobility effect of subthreshold doses of desipramine in the forced swimming test (an antidepressant-screening model) (Molina-Hernandez et al., 2008). Furthermore, minocycline attenuated lipopolysaccharide (LPS)-induced expression of pro-inflammatory cytokines, and prevented LPS-induced development of depressive-like behaviors in mice (O'Connor et al., 2009). These lines of evidence suggest that minocycline is a potential antidepressant drug.

The prefrontal cortex, nucleus accumbens, hippocampus, and amygdala are candidates for the locus of depression, and their involvement in the pathophysiology of depression is well documented. Dysfunctional changes within these interconnected limbic regions have been implicated in depression and the actions of antidepressants (Berton and Nestler, 2006; Krishnan and Nestler, 2008). Postmortem and neuroimaging studies of depressed patients have revealed reductions in gray-matter volume and glial density in the prefrontal cortex and hippocampus (Drevets, 2001; Harrison, 2002; Sheline et al., 2003). Activity in the amygdala and anterior cingulate cortex is strongly correlated with dysphoric emotions: indices of neuronal activity within these regions are chronically increased in depressed individuals, but revert to normal levels after successful treatment (Drevets, 2001; Ressler and Mayberg, 2007).

The networks described above are significantly modulated by monoamine projections from the midbrain and brainstem nuclei. Abnormal monoamine metabolism is also observed in animal models of depression such as olfactory bulbectomized rats (Zhou et al., 1998), Wistar–Kyoto rats (De La Garza and Mahoney, 2004) and Flinders

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doi:10.1016/j.pbb.2011.09.008

Sensitive Line rats (Zangen et al., 1997, 1999), and treatment with antidepressants improves these monoaminergic dysfunctions (Zangen et al., 1997, 1999). Olfactory bulbectomized rats showed increased serotonin (5-HT) turnover in the frontal cortex (Zhou et al., 1998). Flinders Sensitive Line rats, a genetic model of depression, also showed increased dopamine turnover in the prefrontal cortex and decreased serotonin turnover in the nucleus accumbens (Zangen et al., 1997, 1999). Serotonergic neurons are known to be associated with depression-related neuropsychological functions including stress responsiveness, motivation, working memory, and anxiety (Jans et al., 2007). In support of this, a previous clinical study demonstrated that depressed patients exhibited significantly higher 5-HT turnover in plasma levels than normal controls (Mitani et al., 2006).

The monoamine hypothesis of depression posits that depression is caused by decreased monoamine function in the brain (Berton and Nestler, 2006). It is assumed that initial increases in the levels of synaptic monoamines (5-HT and norepinephrine (NE)) induced by antidepressant drugs produce secondary neuroplastic changes that involve transcriptional and translational changes, mediating molecular and cellular plasticity (Nestler et al., 2002; Pittenger and Duman, 2008). Although monoamine-based antidepressants remain the first line of therapy for depression, therapeutic delays and low remission rates have encouraged the search for more effective agents (Berton and Nestler, 2006; Mathew et al., 2008; Trivedi et al., 2006).

Brain-derived neurotrophic factor (BDNF) is implicated in neuronal plasticity and plays an important role in learning and memory. It has been reported that stress reduced the expression of BDNF in the hippocampus of rats, and that treatment with antidepressants or electroconvulsive therapy restored the reduced hippocampal BDNF levels in stressed rats. It is well known that subchronic treatments with antidepressants increase the BDNF expression in the hippocampus of animals (Duman and Monteggia, 2006; Nibuya et al., 1995). Direct infusion of BDNF into the hippocampus induces an anti-depressive effect in learned helplessness (LH) rats (Shirayama et al., 2002). Furthermore, treatments with antidepressants did not improve the depressive-like behavior in the forced swim test in mice whose expression of BDNF in the dentate gyrus of hippocampus was selectively attenuated (Adachi et al., 2008). Clinical studies including a recent meta-analysis study have reported that the concentration of serum BDNF was decreased in depressed patients, and that subsequent treatment with antidepressants increased the concentration of serum BDNF (Brunoni et al., 2008; Sen et al., 2008; Shimizu et al., 2003). Furthermore, external stressors activate cyclooxygenase enzymes that enable the production of prostaglandins, increasing the secretion and synthesis of BDNF (Toyomoto et al., 2004). Moreover, pro-inflammatory cytokines such as IL-1β, which are increased in clinical depression, impaired BDNF signal transduction (Tong et al., 2008).

LH is a widely used animal model of depression. In this model, application of an uncontrollable and unpredictable stressor such as inescapable shock leads to a helpless state in a variety of animals and humans (Overmier and Seligman, 1967; Maier and Seligman, 1976; Breier et al., 1987). Helpless animals lose weight, appear agitated, and have sleep disturbances, libido reduction, and associative-cognitive deficits (Henn and Vollmayr, 2005). LH animals are responsive to tricyclic antidepressants, selective serotonin reuptake inhibitors, monoamine oxidase inhibitors, and electroconvulsive treatment (Sherman et al., 1982; Shirayama et al., 2002). LH rats show changes in the NE and 5-HT systems. Thus, the NE-β receptor and 5-HT-1B receptor were up-regulated in the hippocampus of LH rats, and the neurochemical and behavioral changes were reversed with subchronic treatment with antidepressants (Henn and Vollmayr, 2005).

We examined whether minocycline could recover the behavioral deficits observed in LH rats. The focus of this investigation was to determine the mechanism of the antidepressant-like effects of minocycline on LH rats. Therefore, we examined the effects of minocycline on levels of monoamine and their metabolites after LH paradigm and after subsequent treatment with minocycline in the medial prefrontal cortex, orbitofrontal cortex, nucleus accumbens, striatum, hippocampus, and amygdala. These regions are possibly involved in the pathophysiology of depression (Pittenger and Duman, 2008). Moreover, we examined the BDNF level in the LH paradigm and after subsequent treatment with minocycline in the hippocampus.

2. Materials and methods

2.1. Animals and treatments

The animal procedures were in accordance with the Chiba University Graduate School of Medicine Guide for the Care and Use of Laboratory Animals and were approved by the Chiba University Graduate School of Medicine Animal Care and Use Committee. Male Sprague–Dawley rats (190–220 g) were housed under a 12-h light/12-h dark cycle at room temperature (22 ± 2 °C) with free access to food and water.

Surgery was performed using a stereotaxic apparatus (Kopf, Tujunga, CA) under anesthesia with pentobarbital sodium solution (50 mg/kg, intraperitoneal injection; Abbott Laboratories, Abbott Park, IL) 1 day after the acquisition of LH. The coordinates for the cerebral ventricle relative to the bregma according to the atlas of Paxinos and Watson (Paxinos and Watson, 1997) were as follows: −0.3 anteroposterior (AP), ±1.2 lateral, −3.4 dorsoventral (DV) from the dura. Minocycline hydrochloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was dissolved in physiological saline. Rats received bilateral microinjection of different amounts of minocycline (160 or 20 μg/side) or saline (control) into the cerebral ventricle. A total volume of 4.0 μl was infused into each side over 10 min, and the injection syringe was left in place for an additional 5 min to allow for diffusion.

2.2. LH paradigm

LH behavioral tests were performed using the Gemini Avoidance System (San Diego, CA, USA). This apparatus was divided into two compartments by a retractable door. On days 1 and 2, rats were subjected to 30 inescapable electric footshocks [0.65 mA, 30 s duration, at random intervals (averaging 18–42 s)]. On day 3, a two-way conditioned avoidance test was performed as a post-shock test to determine if the rats would show the predicted escape deficits. This screening session consisted of 30 trials in which the electric footshocks [0.65 mA, 6 s duration, at random intervals (mean of 30 s)] were preceded by a 3 s conditioned stimulus tone that remained on until the shock was terminated. Rats with more than 25 escape failures in the 30 trials were regarded as having reached the criterion. Approximately 65% of the rats reached this criterion.

On day 4, rats received bilateral microinjections of minocycline into the ventricle.

On day 8, a two-way conditioned avoidance test was performed. This test session consisted of 30 trials in which electric footshock [0.65 mA, 30 s duration, at random intervals (mean of 30 s, averaging 18–42 s)] was preceded by a 3 s conditioned stimulus tone that remained on until the shock was terminated. The numbers of escape failures and latency to escape in each of 30 trials were recorded by the Gemini Avoidance System.

2.3. Open field test

Four days after the surgery, locomotor activity was measured in the open field test in a square area (76.5 × 76.5 × 49 cm) using a standard procedure (Lacroix et al., 1998). This experiment was performed separately from the two-way conditioned avoidance test using different animals. The open field was divided into two areas, a peripheral area and a square center (40 × 40 cm). The test room was dimly

illuminated (60 W lights, indirect). Rats were allowed to explore for 45 min. The computer software (BeTrace: Behavioral and Medical Sciences Research Consortium, Hyogo, Japan) calculated the velocity of movement, the distance traveled, and time spent in the center of the open field. These parameters are assumed to reflect locomotor activity and fear or anxiety, respectively.

2.4. Measurement of monoamines

On day 8, animals were decapitated and the brains were immediately removed. These animals had not been subjected to the two-way conditioned avoidance test or open field test. The prefrontal cortex, nucleus accumbens, striatum, amygdala, and hippocampus were dissected and stored at −80 °C until used for the assay. Tissue samples were homogenized in 0.2 M perchloric acid ($HClO_4$) containing 100 μM disodium EDTA and 100 ng/ml isoproterenol (internal standard), and were then centrifuged at 20,000×*g* for 15 min at 4 °C. The supernatants were filtered through a 0.45 μm pore membrane (Millex-LH, 4 mm; Millipore, Tokyo, Japan) and were analyzed for dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), norepinephrine (NE) and 3-methoxy-4-hydroxyphenylglycol (MHPG) by high-performance liquid chromatography (HPLC) coupled with electrochemical detection. The HPLC system consisted of a liquid chromatograph pump (EP-300, Eicom, Kyoto, Japan), degasser (DG-300, Eicom), reversed phase column (Eicompak SC-5ODS 3.0×150 mm; Eicom), ECD-300 electrochemical detector (Eicom), and data processor (EPC-300, Eicom). The mobile phase consisted of 0.1 M acetate–citric acid buffer (pH 3.5) containing 13% methanol, 5 mg/l disodium EDTA, and 190 mg/l sodium octyl sulfate.

2.5. Measurements of BDNF protein levels

On day 8, animals were decapitated and the hippocampus was dissected out. These rats had not been subjected to the two-way conditioned avoidance test or open field test. The samples were homogenized by a Polytron in 3 ml of buffer containing 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 4 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin. The homogenized samples were spun at 15,000 rpm for 30 min, and the supernatants were analyzed for BDNF using a two-site enzyme-linked immunosorbent assay (ELISA). BDNF proteins were quantified by using the BDNF Emax immunoassay system (Promega Co., Madison, WI, USA). Data were expressed as percent of control and are the means with S.E.M.

2.6. Statistical analysis

Statistical differences among three groups were determined by one-way ANOVA, followed by post hoc analysis (Tukey's test). For comparison of the mean values between the two groups, statistical evaluation was done using the two-tailed Student's *t*-test. Differences were considered to be significant when the P values were less than 0.05.

3. Results

3.1. LH and conditioned avoidance test

LH rats that received bilateral microinjections of minocycline into the cerebral ventricle demonstrated a significant improvement on the conditioned avoidance test relative to saline-treated controls (Fig. 1).

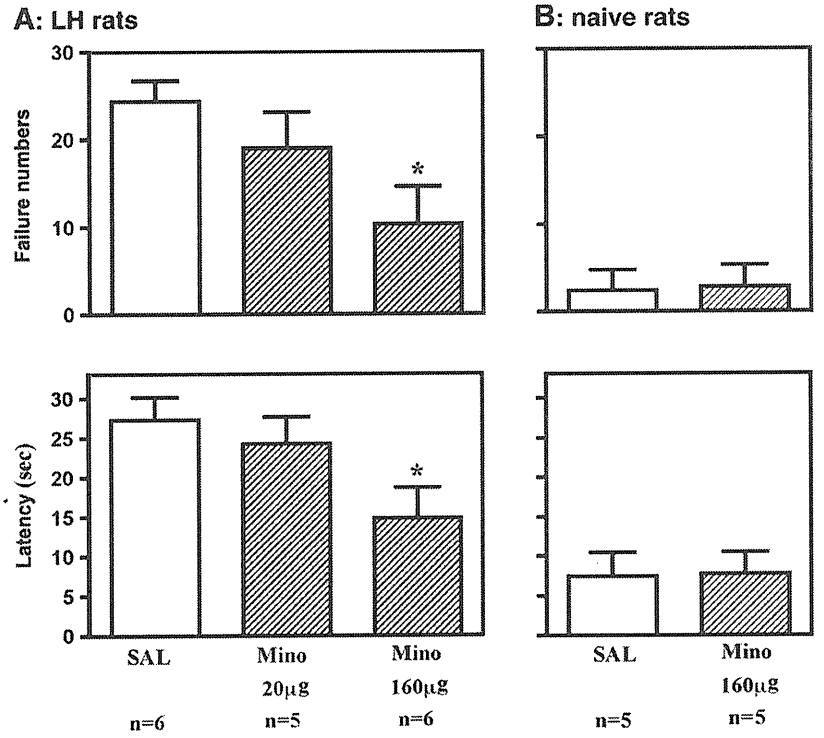

Fig. 1. Minocycline decreased escape failure in the LH paradigm. Minocycline (Mino) or saline (SAL) was administered via bilateral infusion into the cerebral ventricle, and animals were subjected to a conditioned avoidance test 4 days later. Escape failure and latency to escape were determined. The results were expressed as mean ± S.E.M. The number of animals is listed under each column. Shown on the right are the results of minocycline-injection into naïve rats for comparison. Left top, $F(2, 14) = 4.052$, $p = 0.0409$; left bottom, $F(2, 14) = 3.861$, $p = 0.0462$; right top, $t = 0.114$, $p = 0.9120$; right bottom, $t = 0.072$, $p = 0.9442$. *$p < 0.05$ when compared with saline-treated controls (ANOVA followed by Tukey's test).

Meanwhile, injection of minocycline into the cerebral ventricle of naïve rats failed to induce the antidepressant-like effects in the conditioned avoidance test (Fig. 1).

3.2. Locomotor activity

Infusions of minocycline into the cerebral ventricle of naïve rats failed to affect the time spent in the center and distance traveled, but decreased velocity in the open field test (Fig. 2). This is not the result expected if a general increase in locomotor activity contributed to the effect of minocycline on conditioned avoidance in the LH models of depression.

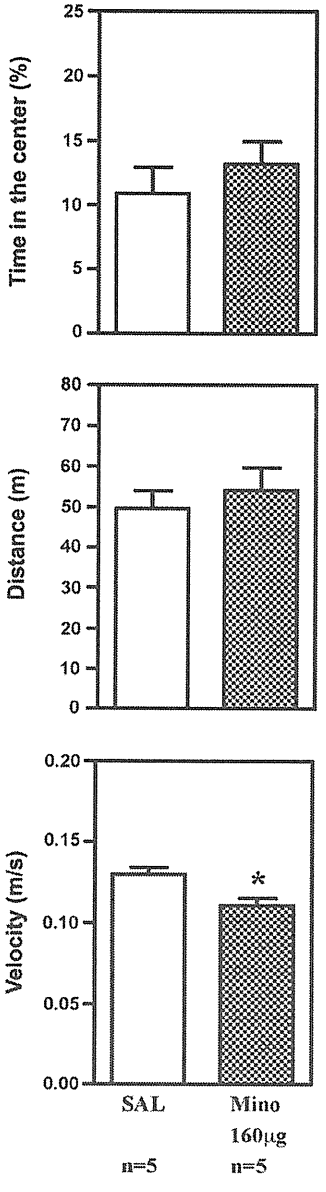

Fig. 2. Effects of minocycline infusion into the cerebral ventricle of naïve rats on locomotor activity. Minocycline (Mino) or saline (SAL) was administered via bilateral infusion into the cerebral ventricle, and 4 days later, the time spent in the center, distance traveled, and velocity in an open field were determined. The results were expressed as mean ± S.E.M. The number of animals is listed under each column. Top, $t = 0.873$, $p = 0.4079$; middle, $t = 0.642$, $p = 0.5389$; bottom, $t = 3.058$, $p = 0.0156$. *$p < 0.05$ when compared to saline-injected controls (Student's *t*-test).

3.3. Monoamines and their metabolites

LH rats showed a significant increase in 5-HT turnover in the orbitofrontal cortex, and the alteration remained unchanged after treatment with minocycline ($F(2,26) = 5.542$, $P = 0.0099$; Table 1). No alterations were found in 5-HT levels, 5-HIAA levels, and 5-HIAA/5-HT ratio in the medial prefrontal cortex, nucleus accumbens, striatum, hippocampus, or amygdala (Table 1).

No changes in the levels of DA, DOPAC, or HVA, or in the (DOPAC + HVA)/DA ratio were seen in the medial prefrontal cortex, orbitofrontal cortex, nucleus accumbens or striatum (Table 2). On the contrary, subsequent treatment with minocycline significantly increased levels of DA and DOPAC in the amygdala when compared with LH rats (DA, $F(2,25) = 4.189$, $P = 0.0270$; DOPAC, $F(2,25) = 5.290$, $P = 0.0121$; Table 2).

LH rats did not show any alterations in the NE levels, MHPG levels or MHPG/NE ratios in the medial prefrontal cortex, orbitofrontal cortex or nucleus accumbens (Table 3).

3.4. BDNF levels

LH rats showed a significantly decreased level of BDNF in the hippocampus compared with control rats (Fig. 3). However, subsequent treatment with minocycline did not result in any improvement in the decreased expression of BDNF (Fig. 3).

4. Discussion

The primary finding of the present study is that infusion of minocycline into the cerebral ventricle produced antidepressant-like effects in LH rats, an animal model of depression. The open field test showed a decrease in velocity and no alterations in distance traveled

Table 1
Levels of serotonin metabolism and its turnover in brain regions.

		5-HT	5-HIAA	5-HIAA/5-HT
<*Medial prefrontal cortex*>				
Control	n = 11	0.330 ± 0.021	0.455 ± 0.021	1.414 ± 0.080
LH	n = 10	0.315 ± 0.024	0.436 ± 0.015	1.449 ± 0.114
LH + Mino	n = 9	0.340 ± 0.021	0.496 ± 0.023	1.478 ± 0.057
<*Orbitofrontal cortex*>				
Control	n = 11	0.463 ± 0.021	0.371 ± 0.013	0.762 ± 0.030
LH	n = 10	0.430 ± 0.027	0.418 ± 0.020	0.920 ± 0.042*
LH + Mino	n = 10	0.455 ± 0.015	0.402 ± 0.016	0.886 ± 0.033*
<*Nucleus accumbens*>				
Control	n = 11	0.395 ± 0.025	0.700 ± 0.024	1.825 ± 0.101
LH	n = 10	0.439 ± 0.047	0.756 ± 0.056	1.793 ± 0.092
LH + Mino	n = 10	0.391 ± 0.037	0.738 ± 0.020	1.900 ± 0.180
<*Striatum*>				
Control	n = 11	0.342 ± 0.019	0.628 ± 0.028	1.857 ± 0.067
LH	n = 10	0.358 ± 0.033	0.644 ± 0.041	1.843 ± 0.076
LH + Mino	n = 10	0.333 ± 0.028	0.667 ± 0.025	2.082 ± 0.116
<*Hippocampus*>				
Control	n = 11	0.289 ± 0.023	0.509 ± 0.031	1.891 ± 0.086
LH	n = 10	0.311 ± 0.013	0.512 ± 0.019	1.667 ± 0.083
LH + Mino	n = 10	0.267 ± 0.018	0.485 ± 0.017	1.964 ± 0.149
<*Amygdala*>				
Control	n = 10	0.665 ± 0.058	0.776 ± 0.024	1.242 ± 0.099
LH	n = 9	0.629 ± 0.042	0.700 ± 0.019	1.146 ± 0.068
LH + Mino	n = 10	0.617 ± 0.041	0.782 ± 0.046	1.291 ± 0.068

Monoamine level (ng/mg tissue) and turnover are indicated as mean ± SEM.
Sample numbers are indicated in each row.
5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid.
* $P < 0.05$ when compared to control animals (ANOVA followed by Tukey's test).

Table 2
Levels of dopamine metabolism in brain regions.

		DA	DOPAC	HVA	(DOPAC + HVA)/DA
<Medial prefrontal cortex>					
Control	n = 11	0.168 ± 0.011	0.072 ± 0.007	0.106 ± 0.007	1.066 ± 0.054
LH	n = 10	0.140 ± 0.017	0.062 ± 0.005	0.092 ± 0.002	1.110 ± 0.080
LH ± Mino	n = 9	0.134 ± 0.012	0.056 ± 0.006	0.110 ± 0.011	1.248 ± 0.084
<Orbitofrontal cortex>					
Control	n = 11	0.331 ± 0.088	0.101 ± 0.025	0.139 ± 0.020	1.063 ± 0.142
LH	n = 10	0.252 ± 0.079	0.098 ± 0.022	0.132 ± 0.014	1.420 ± 0.271
LH + Mino	n = 10	0.274 ± 0.076	0.081 ± 0.016	0.129 ± 0.015	1.135 ± 0.183
<Nucleus accumbens>					
Control	n = 11	7.239 ± 0.245	2.664 ± 0.188	0.953 ± 0.064	0.503 ± 0.034
LH	n = 10	6.908 ± 0.452	2.780 ± 0.223	0.908 ± 0.063	0.537 ± 0.021
LH + Mino	n = 10	7.492 ± 0.442	3.013 ± 0.202	1.108 ± 0.117	0.553 ± 0.033
<Striatum>					
Control	n = 11	11.176 ± 0.384	2.799 ± 0.168	1.127 ± 0.037	0.351 ± 0.012
LH	n = 10	9.901 ± 0.619	2.424 ± 0.188	1.038 ± 0.073	0.348 ± 0.010
LH + Mino	n = 10	10.235 ± 0.456	2.548 ± 0.141	1.169 ± 0.065	0.363 ± 0.011
<Amygdala>					
Control	n = 10	1.001 ± 0.102	0.293 ± 0.031	0.157 ± 0.012	0.494 ± 0.059
LH	n = 9	0.679 ± 0.131	0.176 ± 0.029	0.116 ± 0.015	0.501 ± 0.101
LH + Mino	n = 9	1.385 ± 0.250#	0.346 ± 0.048#	0.186 ± 0.035	0.411 ± 0.025

Monoamine level (ng/mg tissue) and turnover are indicated as mean ± SEM.
Sample numbers are indicated in each row.
DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid.
# $P<0.05$ when compared to LH rats (ANOVA followed by Tukey's test).

or time spent in the center, suggesting that the antidepressant-like effects of minocycline may not be attributed to enhanced locomotion.

Second, LH rats showed decreased levels of DA and DOPAC in the amygdala, and minocycline significantly increased the levels of DA and DOPAC in the amygdala when compared with untreated LH rats. Previous studies showed that manipulation of the amygdala exerted antidepressant-like effects (Wallace et al., 2004; Shirayama et al., 2011). Therefore, the mechanism of minocycline could be attributable to a significant alteration in DA and DOPAC in the amygdala.

Third, serotonin turnover (5-HIAA/5-HT ratios) was statistically increased in the orbitofrontal cortex of LH rats when compared with control rats, but the increases in 5-HT turnover remained unchanged after treatment with minocycline. This is in partial agreement with the recent study in which depressed patients exhibited higher 5-HT turnover levels in plasma than normal controls (Mitani et al., 2006). It demonstrates that LH contributed to alteration of the 5-HT systems in the orbitofrontal cortex. The orbitofrontal cortex is involved in motivation, which is lowered in depression. This is compatible with a working hypothesis that antidepressant drugs, especially selective serotonin uptake inhibitors, exert their beneficial effects through activating serotonergic neural transmission (Jans et al., 2007). Further study will be needed to elucidate the role of 5-HT in the antidepressant effects of minocycline.

We did not find statistically significant results for NE. However, a recent study showed that minocycline administration reduced immobility in the forced swim test (an antidepressant-screening model) by increasing climbing (Molina-Hernandez et al., 2008), indicating that minocycline exerts an antidepressant-like effect through the NE system because a previous study on antidepressants indicated that increased climbing reflects the NE system whereas increased swimming reflects the 5-HT system in the forced swim test (Lucki, 1997). Further studies will be needed to elucidate the involvement of NE systems in LH rats during stressful conditions.

A previous study showed that Wistar–Kyoto rats, which are prone to develop stress-induced anhedonia, exhibited increased DA and 5-

Table 3
Levels of norepinephrine in brain regions.

		NE	MHPG	MHPG/NE
<Medial prefrontal cortex>				
Control	n = 11	0.334 ± 0.009	0.199 ± 0.012	0.599 ± 0.038
LH	n = 10	0.323 ± 0.008	0.192 ± 0.013	0.603 ± 0.050
LH + Mino	n = 9	0.310 ± 0.019	0.233 ± 0.025	0.691 ± 0.067
<Orbitofrontal cortex>				
Control	n = 11	0.263 ± 0.008	0.177 ± 0.011	0.682 ± 0.053
LH	n = 10	0.274 ± 0.005	0.176 ± 0.016	0.635 ± 0.051
LH + Mino	n = 10	0.253 ± 0.011	0.209 ± 0.022	0.751 ± 0.061
<Nucleus accumbens>				
Control	n = 10	0.335 ± 0.029	0.181 ± 0.019	0.565 ± 0.081
LH	n = 9	0.354 ± 0.038	0.194 ± 0.028	0.595 ± 0.130
LH + Mino	n = 9	0.388 ± 0.069	0.199 ± 0.028	0.549 ± 0.135

Monoamine level (ng/mg tissue) and turnover are indicated as mean ± SEM.
Sample numbers are indicated in each row.
NE, norepinephrine; MHPG, 3-methoxy-4-hydroxyphenylglycol.

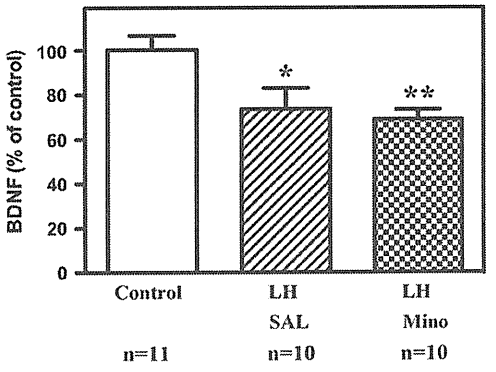

Fig. 3. Effects of minocycline on the BDNF expression in the hippocampus of LH rats. Minocycline (Mino) or saline (SAL) was administered via bilateral infusion into the cerebral ventricle of LH rats, and 4 days later, BDNF expression was examined. BDNF level (% control) are indicated as mean ± SEM. Sample numbers are indicated in each row. $F(2, 28) = 6.042$, $p = 0.0066$. *$p<0.05$, **$p<0.01$ when compared with controls (ANOVA followed by Tukey's test).

HT turnover in the nucleus accumbens under the steady state and in the prefrontal cortex under a stressful condition, although normal control rats did not show any alterations in DA or 5-HT turnover in the steady state or under a stressful condition (De La Garza and Mahoney, 2004). Therefore, LH rats might show further alterations in the levels of monoamines, metabolites and turnover under stressful conditions, and treatment with minocycline might block the monoaminergic changes induced by the stressful condition. Future studies will be needed to examine this question.

Finally, BDNF levels in the hippocampus of LH rat were lower than those of control rats, but the reduction in BDNF expression remained unchanged after treatment with minocycline. A reduction of BDNF in the hippocampus of LH rats was the expected result. A recent study on the effects of minocycline during in vitro hypoxia showed that minocycline suppressed the microglial activation and up regulation of pro-inflammatory mediators, but did not affect the hypoxic activation of BDNF (Lai and Todd, 2006). Microglia may supply neurons with BDNF (Kempermann and Neumann, 2003). Considering these results together, we may reasonably exclude the involvement of BDNF in the antidepressant-like effect of minocycline.

In a recent study, minocycline was effective as an antidepressant drug in an animal model of inflammatory-associated depressive disorders induced by lipopolysaccharide (LPS) (O'Connor et al., 2009). Pro-inflammatory cytokines, mainly interferonγ (IFN-γ) and TNF-α, induce Indoleamine 2,3-dioxygenase (IDO), which degrades tryptophan along the kynurenine pathway. Minocycline blocks IFN-γ-mediated protein kinase C phosphorylation and nuclear translocation of protein kinase C, which is necessary for IDO activation. The relationship between depression and inflammation remains to be elucidated. Future studies need to address the involvement of microglia in the antidepressant-like effect of minocycline.

In conclusion, infusion of minocycline into the cerebral ventricle of LH rats produced antidepressant-like effects, although infusion of minocycline into the cerebral ventricle of naïve rats did not increase locomotor activity in the open field tests. LH rats showed significant increased 5-HT turnover in the orbitofrontal cortex and decreased levels of BDNF in the hippocampus compared with control rats. However, these alterations in 5-HT turnover and BDNF expression remained unchanged after treatment with minocycline. Taken together, these results suggest that minocycline may be a therapeutic drug for the treatment of depression.

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Psychopharmacology
DOI 10.1007/s00213-011-2619-3

ORIGINAL INVESTIGATION

Protective effects of the antioxidant sulforaphane on behavioral changes and neurotoxicity in mice after the administration of methamphetamine

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Received: 19 June 2011 / Accepted: 12 December 2011

Abstract
Rationale Methamphetamine (METH) is a powerfully addictive stimulant associated with serious health conditions. Accumulating evidence suggests a role of oxidative stress in METH-induced behavioral abnormalities. Sulforaphane (SFN), found in cruciferous vegetables, is a potent antioxidant. It is of interest to determine whether SFN can attenuate behavioral and neuropathological changes associated with METH exposure.
Objectives This study was undertaken to examine the effects of SFN on behavioral changes and dopaminergic neurotoxicity in mice exposed to METH.
Methods The effects of SFN on acute hyperlocomotion and the development of behavioral sensitization induced by the administration of METH were examined. Levels of dopamine (DA) and its major metabolite 3,4-dihydroxyphenyl acetic acid (DOPAC) in the striatum were measured. In addition, DA transporter (DAT) immunoreactivity was also performed.
Results Pretreatment with SFN at 1, 3, and 10 mg/kg elicited a dose-dependent attenuation of acute hyperlocomotion in mice, after a single administration of METH (3 mg/kg). The development of behavioral sensitization after repeated administrations of METH (3 mg/kg/day, once daily for 5 days) was significantly reduced by pretreatment with SFN (10 mg/kg). In addition, the lowering of DA levels and DOPAC as well as DAT immunoreactivity in the striatum, usually seen after repeated administration of METH, was significantly attenuated by both pretreatment and the subsequent administration of SFN. Furthermore, SFN significantly reduced microglial activation in the striatum after repeated exposure to METH.
Conclusion It is therefore likely that SFN can be a useful drug for the treatment of signs associated with METH abuse in humans.

Keywords Sulforaphane · Dopamine · Methamphetamine · Microglia · Neurotoxicity · Sensitization

Abbreviations

METH	Methamphetamine
SFN	Sulforaphane
DA	Dopamine
DOPAC	3,4-Dihydroxyphenyl acetic acid
DAT	Dopamine transporter
PET	Positron emission tomography
Nrf2	NF-E2-related factor-2
ARE	Antioxidant responsive element
HPLC	High performance liquid chromatography

Electronic supplementary material The online version of this article (doi:10.1007/s00213-011-2619-3) contains supplementary material, which is available to authorized users.

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Published online: 27 December 2011

Introduction

Abuse of methamphetamine (METH) is an extremely serious and growing global problem, affecting the USA and Asian countries such as Japan, South Korea, Thailand, Philippines, and China (National Institute on Drug Abuse 2002; Yamamoto 2004; Barr et al. 2006; Hashimoto 2007; United Nations Office on Drug Use and Crime (UNODC) 2008; Gonzales et al. 2010; Chen et al. 2010; Colfax et al. 2010). METH is a powerfully addictive stimulant associated with serious health conditions, including memory loss, aggression, psychotic signs, and brain damage (Ujike and Sato 2004; Hashimoto 2007; Chen et al. 2010). However, there is currently no pharmacological treatment for the wide range of signs associated with METH exposure (Hashimoto 2007; Chen et al. 2010).

Repeated administration of METH is known to induce dopaminergic neurotoxicity in rodents and non-human primates, by producing long-term depletion of dopamine (DA) and its metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), as well as reducing the density of DA transporter (DAT) in the striatum (Davidson et al. 2001; Cadet et al. 2003; Fukami et al. 2004; Koike et al. 2005; Zhang et al. 2006; Hashimoto et al. 2004; 2007; Hagiwara et al. 2009). Furthermore, it has been reported that levels of dopamine nerve terminal markers, DA, tyrosine hydroxylase, and DAT are decreased in the striatum of post mortem brains (nucleus accumbens, caudate, putamen) of chronic METH users (Wilson et al. 1996). Moreover, brain imaging studies using PET show that the density of DAT in the caudate/putamen and nucleus accumbens of METH users is significantly lower than that of healthy controls (Sekine et al. 2001; Volkow et al. 2001). Although METH-induced neurotoxicity at dopaminergic terminals is well documented, the precise mechanisms of METH-induced neurotoxicity remain unknown (Cadet et al. 2003; Hashimoto 2007; Chen et al. 2010).

Multiple lines of evidence implicate oxidative stress in the METH-induced behavioral and neuropathological changes that damage brain dopaminergic neurons (Açikgöz et al. 2001; Fukami et al. 2004; Miyazaki et al. 2006; Hashimoto et al. 2004, 2007; Cadet et al. 2007; Yamamoto and Raudensky 2008; Chen et al. 2010). The potent antioxidant sulforaphane (SFN: 1-isothiocyanato-4-methylsulfinylbutane) is an organosulfur compound derived from a glucosinolate precursor found in cruciferous vegetables such as broccoli, Brussels sprouts, and cabbage (Zhang et al. 2005; Juge et al. 2007). A number of studies show that SFN is a very potent chemopreventative agent in numerous animal carcinogenesis and cell culture models (Juge et al. 2007; Cheung and Kong 2010; Kwak and Kensler 2010). The protection afforded by SFN is thought to be mediated via activation of the NF-E2-related factor-2 (Nrf2) pathway and subsequent up-regulation of phase II detoxification enzymes and antioxidant proteins, through an enhancer sequence referred to as the electrophilic responsive element or antioxidant responsive element (ARE) (Itoh et al. 2004; Kang et al. 2005; Cheung and Kong 2010; Kwak and Kensler 2010). Furthermore, SFN is known to exert neuroprotective effects against neurotoxicity caused by 6-hydroxydopamine, tetrahydrobiopterin, and ischemia/reperfusion, again through activation of the Nrf2–ARE pathway (Han et al. 2007; Danilov et al. 2009; Siebert et al. 2009; Ping et al. 2010). It has been reported that SFN increases Nrf2 protein levels in the striatum and affords protection against methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced death of nigral dopaminergic neurons (Jazwa et al. 2011). Taken together, it is likely that as a potent Nrf2 activator, SFN could protect against the death of dopaminergic neurons in the brains of Parkinson's disease patients suffering from oxidative stress-related neuropsychiatric diseases.

Given the potent antioxidant effects of SFN, it is of interest to determine whether SFN can attenuate behavioral and neuropathological changes associated with METH exposure. In this study, we investigated the effects of SFN on acute hyperlocomotion and the development of behavioral sensitization induced by the administration of METH. We also examined the effects of SFN on METH-induced neurotoxicity in the dopaminergic neurons of the mouse striatum.

Materials and methods

Animals

Male Balb/c AnNCrlCrlj (8 weeks old, 23–30 g body weight at the beginning of the experiment; Charles River Japan Inc., Tokyo, Japan) mice were housed under a 12-h light/12-h dark cycle (lights on from 07:00 to 19:00 hours) at room temperature (22±2°C; humidity, 55±5%) with free access to food and water. Balb/c mice were used, since this strain has a known sensitivity to METH-induced neurotoxicity (Kita et al. 1998; Koike et al. 2005; Zhang et al. 2006; Hagiwara et al. 2009). Experimental protocols were approved by the Institutional Animal Care and Use Committee of Chiba University.

Drugs

METH hydrochloride (d-methamphetamine; Dainippon Pharmaceutical Ltd., Osaka, Japan) was dissolved in physiological saline, and (*R,S*)-sulforaphane (SFN) (LKT Laboratories, Inc., St Paul, MN, USA) was dissolved in distilled water including 10% corn oil. All other chemicals were purchased from commercial sources. The dose of METH was expressed as a hydrochloride salt.

Behavioral evaluations

Effects of SFN on hyperlocomotion after a single administration of METH

In the acute behavioral experiments, the initial period of acclimation was 60 min. Either vehicle (10 ml/kg) or SFN at 1, 3, or 10 mg/kg was administered intraperitoneally (i.p.) to mice. Thirty minutes after the first injection, mice were injected subcutaneously (s.c.) with METH (3.0 mg/kg) or vehicle (10 ml/kg). Locomotor activity was measured over 3.5 h using an animal movement analysis system (SCANET SV-10; Melquest, Toyama, Japan), as reported previously (Zhang et al. 2006; Hagiwara et al. 2009).

Effects of SFN 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, i.p.)+vehicle (10 ml/kg, s.c.) group; a vehicle (10 ml/kg, i.p.)+METH (3 mg/kg, s.c.) group; a SFN (10 mg/kg, i.p.)+METH (3 mg/kg, s.c.) group; and a SFN (10 mg/kg, i.p.)+vehicle (10 ml/kg, s.c.) group. The interval between the first pretreatment injection and second test injection was 30 min. In this study, we used a 10-mg/kg dose of SFN in mice, as this was the most effective dose in the METH-induced hyperlocomotion experiments. After the second test injection, mice were returned to their home cages. This cycle of injections was repeated for each animal, on five consecutive days. One week after the final treatment, each mouse was given a low dose of METH (1 mg/kg, s.c.), and locomotion was measured over 3 h (including 1 h habituation) using an animal movement analysis system (SCANET SV-10), as described above (Zhang et al. 2006; Hagiwara et al. 2009).

METH-induced dopaminergic neurotoxicity in the striatum

We examined the effects of pretreatment and subsequent treatment with SFN on METH-induced neurotoxicity in mice. Thirty minutes after pretreatment injections of SFN (10 mg/kg, i.p.) or vehicle (10 ml/kg, i.p.), mice received three injections of METH (3 mg/kg, s.c.) or vehicle (10 ml/kg, s.c.) at 3-h intervals. Rectal temperatures were measured using a TD-320 thermometer coupled to a rectal probe (Shibaura Electronics Co., Ltd., Saitama, Japan), and temperatures were recorded 30 min before pretreatment injections and at 1, 4, and 7 h after the first injection of METH. Then, vehicle (10 ml/kg, i.p.) or SFN (10 mg/kg, i.p.) was administered to the mice 12 h after the first administration of vehicle or SFN (day 1). The mice received two daily (12-h intervals) injections of SFN (10 mg/kg, i.p.) or vehicle (10 ml/kg, i.p.) for two consecutive days (days 2 and 3). In this experiment, we used a treatment schedule to examine both prophylactic and therapeutic effects of SFN. Mice were sacrificed 3 days after the administration of METH for the measurement of DA and DOPAC levels. The brains were quickly removed and the striatum was dissected away on an ice-cold glass plate. Samples were stored at −80°C until use.

Measurement of DA and DOPAC by HPLC

Levels of DA and DOPAC in the mouse striatum were measured using high performance liquid chromatography (HPLC), coupled with electrochemical detection as reported previously (Koike et al. 2005; Zhang et al. 2006; Hagiwara et al. 2009). Briefly, tissue samples were homogenized in 0.2 M perchloric acid ($HClO_4$), containing 100 μM disodium EDTA and 100 ng/ml isoproterenol (internal standard), and were then centrifuged at 20,000×*g* for 15 min at 4°C. Supernatants were filtered through a 0.45-μm pore membrane (Millex-LH, 4 mm; Millipore, Tokyo, Japan). The HPLC system consisted of a liquid chromatograph pump (EP-300, Eicom, Kyoto, Japan), a degasser (DG-300, Eicom), a reversed phase column (Eicompak SC-5ODS 3.0×150 mm; Eicom), an ECD-300 electrochemical detector (Eicom), and a data processor (EPC-300, Eicom). The mobile phase consisted of 0.1 M acetate–citric acid buffer (pH 3.5) containing 16% methanol, 5 mg/l disodium EDTA, and 190 mg/l sodium octyl sulfate.

Immunohistochemistry for DAT and MAC1 in the brain

Immunohistochemistry on the mouse brain sections was performed as reported previously (Koike et al. 2005; Zhang et al. 2006; Hagiwara et al. 2009). Three days after the administration of METH (3 mg/kg×3 at 3-h intervals), mice were anesthetized with sodium pentobarbital (50 mg/kg) 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). Brains were removed from the skulls and 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 (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 50 mM 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, Cat. no: MAB 369, Chemicon International Inc., Temecula, CA, USA) or rat anti-MAC1 (CD11b; activated microglia) antibody (1:1,000, Cat. no: MCA74G, Serotec Ltd., Oxford, UK). The sections were washed twice in TBS and then processed using the avidin–biotin–peroxidase method (Vectastain Elite ABC,

Vector Laboratories, Inc., Burlingame, CA, USA). Sections were incubated for 5 min in a solution of 0.15 mg/ml diaminobenzidine containing 0.01% H_2O_2. Then, sections were mounted on gelatinized slides, dehydrated, cleared, and coverslipped under Permount® (Fisher Scientific, Fair Lawn, NJ, USA). The sections were imaged, and the staining intensity of DAT immunoreactivity in the anterior regions of the striatum was analyzed using a light microscope equipped with a CCD camera (Olympus IX70) and the SCION IMAGE software package. MAC1 immunoreactivity was quantified in the anterior regions (0.018 mm^2) of the striatum, in a blinded manner.

Statistical analysis

Data are presented as the mean±standard error of the mean (SEM). The results of the behavioral study and rectal temperature measurements 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, as well as the densities of DAT immunoreactivity and MAC1 (activated microglia)-immunoreactive staining cells and the behavioral study, were analyzed by one-way ANOVA, followed by the post hoc Bonferroni/Dunn test for multiple comparisons. For all analyses, p values of less than 0.05 were considered statistically significant.

Results

Effects of SFN on hyperlocomotion in mice after a single administration of METH

A single administration of METH (3 mg/kg, s.c.) markedly increased locomotion in mice. Two-way ANOVA analysis revealed significant differences among the six groups studied [F (5, 100)=11.18, $p<0.0001$]. Pretreatment with SFN (at 1, 3, or 10 mg/kg, i.p., 30 min before the administration of METH) attenuated METH-induced hyperlocomotion in mice, in a dose-dependent manner (Fig. 1). High dose of SFN (10 mg/kg) significantly attenuated METH-induced hyperlocomotion in mice (Fig. 1). In contrast, SFN (10 mg/kg) alone did not alter locomotion in mice when compared to vehicle controls (Fig. 1).

Effects of SFN on the development of behavioral sensitization after repeated administration of METH

Repeated administration of METH (3 mg/kg/day, once daily for five consecutive days) increased METH (1 mg/kg)-induced hyperlocomotion in mice previously treated with METH, compared with the results obtained from the control (vehicle+vehicle) group. These results indicated the development of behavioral sensitization by repeated treatment with METH (Fig. 2). Two-way ANOVA analysis revealed significant differences among the four groups [F (3, 51)= 5.22, $p<0.001$]. The post hoc analysis showed that repeated

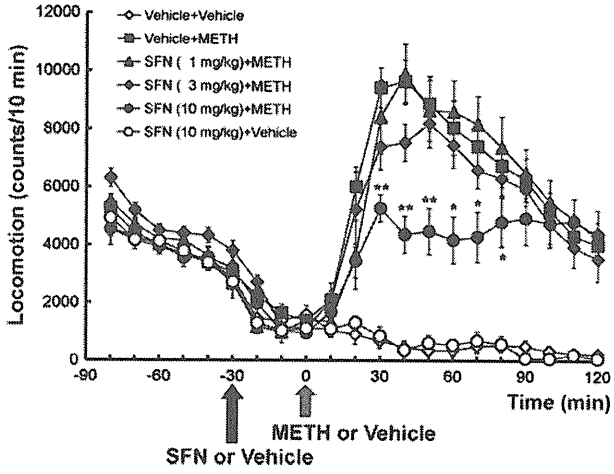

Fig. 1 Effects of SFN on hyperlocomotion in mice after a single administration of METH. Thirty minutes after i.p. injection of vehicle (10 ml/kg) or SFN (1, 3, or 10 mg/kg), METH (3 mg/kg) or vehicle (10 ml/kg) was administered s.c. to the mice. Behavior (locomotion) in the mice was evaluated. Each value is the mean±SEM (n=10–11 per group). *$p<0.05$, **$p<0.01$ as compared with the vehicle+METH group (Bonferroni/Dunn method)

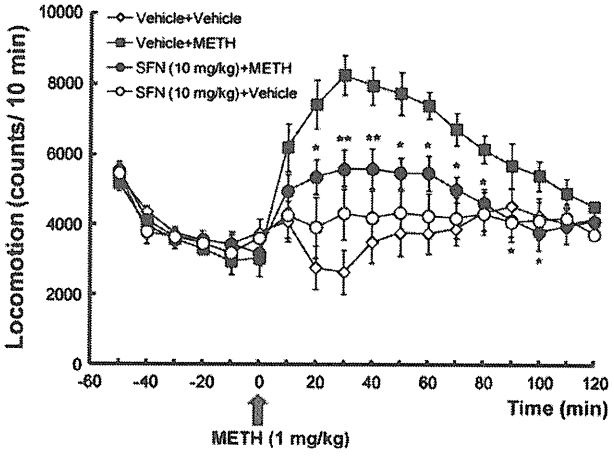

Fig. 2 Effects of SFN on the development of behavioral sensitization in mice after the repeated administration of METH. Vehicle (10 ml/kg)+vehicle (10 ml/kg) group, vehicle (10 ml/kg)+METH (3 mg/kg) group, SFN (10 mg/kg)+METH (3 mg/kg) group, and SFN (10 mg/kg)+vehicle (10 ml/kg) group were treated daily as noted 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. Behavior (locomotion) in the mice was evaluated. Each value is the mean±SEM (n=10 per group). *$p<0.05$, **$p<0.01$ as compared to the vehicle+METH group (Bonferroni/Dunn method)