between patients and normal controls, the Chi-squared test was used for the categorical variables, and Student's *t*-test and the Mann–Whitney *U* test were employed for the continuous variables. Pearson's and Spearman's correlation coefficients were examined to identify any correlations of serum BDNF levels with the clinical variables of patients and with the scores of neuropsychological tests of all subjects. The Shapiro–Wilk test was used to assess normal distribution. Significance for the results was set at $p<0.05$.

3. Results

3.1. Serum BDNF levels between schizophrenic patients and healthy controls

There was no significant difference in serum BDNF levels between normal controls (mean, 14.6 ng/ml [SD, 4.4]) and patients with schizophrenia (mean, 15.3 ng/ml [SD, 3.8]) ($U=1870.5$, $p>0.05$). Furthermore, we found no differences in BDNF levels between the residual and paranoid types of schizophrenia (Table 1), and no differences in serum BDNF levels among the four drug groups, i.e., the risperidone, olanzapine, quetiapine and the aripiprazole groups (Table 2).

3.2. Correlation between serum BDNF levels and cognitive functions

As shown in Table 3, significant differences were observed between patients with schizophrenia and normal controls in all neuropsychological tests.

Then, we examined the correlations between serum BDNF levels and scores of the cognitive function tests. Interestingly, we found significant negative correlations between serum BDNF levels and scores of DSDT in normal controls, but not in patients with schizophrenia (Table 4). There were no other correlations between serum BDNF levels and the scores of neuropsychological tests in patients or controls. Additionally, we found significant positive correlations between the Information subtest scores of WAIS-R and the scores of the DSDT in normal controls; without distractor ($r=0.332$, $p=0.016$) and with distractor ($r=0.259$, $p=0.064$) respectively, but not in patients with schizophrenia.

3.3. Correlation between serum BDNF levels and clinical variables

There was a significant positive correlation between serum BDNF levels and SANS total scores in patients with schizophrenia (Table 4). Of the five subscale symptom groups in SANS, significant positive correlations with the serum BDNF level were detected in two subscales (S1 affective flattering–blunting, S4 anhedonia-asociality), but not in the S2 alogia, S3 avolition-apathy or S5 attention impairment subscales (Table 4).

Table 3
Cognitive data of subjects.

	Controls	Patients	p
	n=52	n=63	
Estimated IQ	110.2 (12.0)	102.4 (13.9)	<.01[a]
Information	11.1 (2.6)	10.1 (2.7)	<.05
Digit span	11.7 (2.9)	10.6 (2.9)	<.05
Picture completion	11.0 (1.9)	10.5 (2.2)	NS
Letter fluency test, words	35.2 (9.0)	28.0 (8.9)	<.001
Category fluency test, words	49.1 (6.8)	39.9 (6.9)	<.001[a]
WCST, accomplished categories	4.9 (1.5)	3.3 (2.2)	<.001
WCST, perseverative errors	0.9 (1.8)	4.5 (6.7)	<.001
Trail making test A, sec	27.2 (7.7)	33.8 (10.1)	<.001
Trail making test B, sec	52.9 (16.0)	80.5 (27.1)	<.001
Stroop test part D, sec	12.7 (2.5)	14.2 (2.6)	<.01
Stroop test Part C,sec	18.6 (5.3)	22.7 (5.9)	<.001
DSDT without distractor, %	83.0 (14.9)	74.8 (17.5)	<.01
DSDT with distractor, %	92.4 (9.8)	82.8 (17.7)	<.001

Values represent mean (SD). NS, not significant.
Abbreviations: WCST, Wisconsin Card Sorting Test; DSDT, Digit Span Distraction Test.
[a] Student's *t*-test. The other p values are by Mann–Whitney *U*-test.

In patients with schizophrenia, among the three subtests in the short version of the WAIS-R, a significant correlation with serum BDNF levels was detected in the Information subtest scores, but not in the Digit Span or Picture Completion subtests (Table 4). Furthermore, we found no correlation between the Information subtest scores and SANS scores ($r=-0.009$, $p=0.944$). Therefore, the Information subtest scores and SANS scores were independently correlated with the serum BDNF levels.

The serum BDNF levels in patients were not correlated with the age of illness onset ($r=0.186$, $p=0.143$), the duration of illness ($r=0.069$, $p=0.590$) or the duration of untreated psychosis ($r=-0.059$, $p=0.646$). There were no significant correlations between serum BDNF levels and BPRS scores ($r=-0.001$, $p=0.991$) or DIEPSS scores ($r=0.054$, $p=0.673$). There was no significant correlation between serum BDNF levels and antipsychotic dosages ($r=0.066$, $p=0.606$).

4. Discussion

The primary finding of the present study is a positive correlation of serum BDNF levels with the SANS scores in the patients with schizophrenia. Furthermore, serum BDNF levels correlated with only negative dimensions (affective flattening, anhedonia) of the SANS subscales, but not with cognitive dimensions (alogia, attention) of the SANS subscales in the schizophrenic patients. This finding was in a good agreement with a previous study which demonstrated a positive correlation between serum BDNF levels and the negative subscale of the Positive and Negative Syndrome Scale (PANSS) in chronic patients with schizophrenia (Reis et al., 2008). Also, we

Table 2
Characteristics of the patients by the type of atypical antipsychotics.

Type of atypical antipsychotics	Total					p
	n=60	Risperidone	Olanzapine	Quetiapine	Aripiprazole	
		n=25	n=18	n=8	n=9	
Age, year	36.2 (8.2)	38.4 (7.0)	34.9 (10.1)	34.8 (7.4)	33.7 (7.5)	NS [a]
Onset, year	26.9 (7.1)	25.9 (5.6)	26.3 (9.3)	30.6 (5.9)	27.4 (7.1)	NS
Duration of illness, year	9.3 (7.4)	12.5 (7.9)	8.6 (7.4)	4.1 (2.1)	6.2 (5.1)	<0.01 [b]
Antipsychotic drug dose,[c] mg/day	313.7 (244.0)	228.0 (154.2)	230.6 (121.4)	791.7 (267.7)	293.3 (80.0)	<0.001 [d]
BDNF, ng/ml	15.4 (3.9)	16.6 (3.9)	15.3 (4.2)	14.6 (3.2)	12.7 (3.0)	NS

Values represent mean (SD). NS, not significant. Perospirone (n=2) and bronanserine (n=1) groups are excluded.
Abbreviation: BDNF, Brain Derived Neurotrophic Factor.
[a] P values are by ANOVA. The other p values are by Kurskal–Wallis test.
[b] Risperidone vs Quetiapine is significant by post hoc Bonferroni correction ($p<0.05$).
[c] Chlorpromazine equivalent dose.
[d] Risperidone vs Quetiapine and Olanzapine vs Quetiapine are each significant by post hoc Bonferroni correction ($p<0.05$).

Table 4
Correlation coefficients with serum BDNF levels.

	Controls	Patients
	n=52	n=63
Subtests of WAIS-R		
Information	−0.205	0.291*
Digit span	−0.037	0.185
Picture completion	−0.005	0.074
Neuropsychological tests		
Letter fluency test, words	−0.045	0.020
Category fluency test, words	−0.128	0.043[a]
WCST, accomplished categories	−0.177	0.002
WCST, perseverative errors	0.088	0.197
Trail making test A, sec	0.096	0.128
Trail making test B, sec	−0.052	0.048
Stroop test part D, sec	0.180	0.087
Stroop test part C, sec	−0.006	0.152
DSDT without distractor, %	−0.309*	0.048
DSDT with distractor, %	−0.353*	0.060
SANS total score	–	0.291[a,*]
S1 affective flattening	–	0.300[a,*]
S2 alogia	–	0.133[a]
S3 avolition apathy	–	−0.005
S4 anhedonia asociality	–	0.303*
S5 attention	–	0.108

No other clinical values significantly correlated with serum BDNF levels.
[a] Pearson's correlation coefficients. The other values are Spearman's correlation coefficients.
* $p<0.05$.

found a positive correlation between serum BDNF levels and the Information subtest scores of WAIS-R in patients with schizophrenia, but not in normal controls. This finding may further extend a recent study suggesting that measurement of serum BDNF levels may be of use to monitor for a successful training for cognitive enhancement in patients with schizophrenia (Vinogradov et al., 2009).

However, we found no differences in serum BDNF levels between normal controls and patients with schizophrenia. A recent meta-analysis demonstrated a reduction in blood BDNF levels in schizophrenic patients compared to healthy controls (Green et al., 2010). While some studies reported that BDNF levels in the serum of schizophrenic patients were not different from those of healthy volunteers (Huang and Lee, 2006; Jockers-Scherubl et al., 2004; Shimizu et al., 2003), other studies found a significant decrease (Grillo et al., 2007; Pirildar et al., 2004; Tan et al., 2005; Toyooka et al., 2002) or even an increase (Gama et al., 2007). Since the present study showed significant correlations of serum BDNF levels with the SANS scores and Information scores of WAIS-R in schizophrenia, the causes of heterogeneity across study results might include the SANS scores and Information scores of WAIS-R. Previous studies demonstrated a relationship between intelligence and serum BDNF in schizophrenia (Adcock et al., 2009; Vinogradov et al., 2009) and a significant association between BDNF levels and positive symptoms (Buckley et al., 2007, Rizos et al., 2008, 2010a, 2010b).

Additional causes of the heterogeneity across study results may include the different stages of illness, duration of untreated psychosis, subtype (hebephrenic vs paranoid) or influences of antipsychotic medication. In support, a recent study showed association of serum BDNF and duration of untreated psychosis in first episode schizophrenic patients (Rizos et al., 2010a, 2010b). A previous study reported associations between serum BDNF levels and clinical phenotypes in schizophrenic patients (Huang and Lee, 2006), although no differences in BDNF levels were found between the residual and paranoid types of schizophrenia in the present study (Table 1). Recent studies of schizophrenia reported that chronic treatment with olanzapine, lurasidone and risperidone increase BDNF levels (Chen and Huang, 2011; Czubak et al., 2009; Fumagalli et al., 2011) and that serum BDNF increase during antipsychotic treatment (Lee et al., 2011), although no differences in serum BDNF levels were found among the four drug groups (risperidone, olanzapine, quetiapine and aripiprazole groups) in the current study (Table 2).

Since previous studies suggested that serum BDNF levels correlate with gender, age, dietary profile or BMI (Green et al., 2010; Guimaraes et al., 2008; Zhang et al., 2007), these factors may influence the serum BDNF levels in subjects. The lack of these factors' information is considered as limitation.

Finally, the scores of the DSDT were negatively correlated with serum BDNF levels in normal controls, but not in patients with schizophrenia. The DSDT reflects verbal working memory (Green et al., 1997; Oltmanns and Neale, 1975). Thus, serum BDNF levels may associate with verbal working memory in healthy subjects. When we see a rule in normal controls but not in the patients, it is natural to think that the rule plays a role in the pathophysiology of the disease. Thus, the lack of association of serum BDNF levels with cognitive functions of schizophrenic patients in the present results might be related with the dysfunction of working memory, which is different from previous studies (Vinogrado et al., 2009; Carlino et al., 2011). Future study will be needed to elucidate this point.

We did not assess the proteolytic isoforms of BDNF (pro-BDNF, truncated BDNF, mature BDNF) in the serum. BDNF detected by an ELISA assay includes pro-BDNF and truncated-BDNF as well as mat-BDNF (Carlino et al., 2011). Thus, the alteration in serum BDNF in the present result might well include pro-BDNF, truncated-BDNF and mat-BDNF. Furthermore, the study by Carlino et al. (2011) demonstrated that mat-BDNF and pro-BDNF were increased whereas truncated-BDNF was decreased in the schizophrenic patients (Carlino et al., 2011). Therefore, the heterogeneity of serum BDNF examined by an ELISA assay might be due to the differential alteration of mat-BDNF, truncated-BDNF and pro-BDNF. The same study showed that cognitive impairments in patients with schizophrenia correlated with low serum truncated-BDNF isoform levels (Carlino et al., 2011). In the present study, the proteolytic isoforms of BDNF might influence the results of cognitive impairments. The question of why serum BDNF correlates positively with the autistic features of schizophrenia, and correlates negatively with cognitional tests might be answered by the differential alteration of the proteolytic isoforms of BDNF. Future study will be needed to elucidate these points.

5. Conclusion

In conclusion, there was a significant negative correlation between serum BDNF levels and verbal working memory in normal controls, but not in patients with schizophrenia. Meanwhile, the scores of negative symptoms and the scores of a verbal comprehension test each positively correlated with serum BDNF levels in chronic patients with schizophrenia. These findings suggest that the heterogeneity of serum BDNF levels may associate with that of negative symptoms and cognitive impairments in chronic patients with schizophrenia.

Declaration of interest

All authors declare that they have no conflicts of interest.

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Association between the Regulator of G-protein Signaling 9 Gene and Patients with Methamphetamine Use Disorder and Schizophrenia

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Abstract: The regulator of G-protein signaling (RGS) modulates the functioning of heterotrimeric G protein. RGS9-2 is highly expressed in the striatum and plays a role in modulating dopaminergic receptor-mediated signaling cascades. Previous studies suggested that the RGS9 gene might contribute to the susceptibility to psychotic diseases. Therefore, we investigated the association between the RGS9 gene and two related dopamine psychoses, schizophrenia and methamphetamine use disorders. The subjects comprised 487 patients of schizophrenia and 464 age- and sex-matched healthy controls and 220 patients of methamphetamine use disorder and 289 controls. We genotyped two nonsynonymous polymorphisms, rs12452285 (Leu225Ser) and rs34797451 (His498Arg), of the RGS9 gene. Rs34797451 showed monomorphism in the present Japanese population, but rs12452285 showed polymorphism. There were no significant differences in genotypic or allelic distributions of rs12452285 between patients with schizophrenia and the corresponding control or between patients with methamphetamine use disorder and the corresponding control. We also analyzed the clinical features of methamphetamine use disorder. We found a significant association in allelic distribution with the phenotypes of age at first consumption (p=0.047). The present study suggested that the RGS9 gene is unlikely to play a major role in schizophrenia and methamphetamine dependence liability and/or the development of methamphetamine induced psychosis, at least in a Japanese population.

Keywords: Substance abuse, methamphetamine, regulator of G-protein signaling 9, case-control association.

INTRODUCTION

Human and animal studies suggest that the D2-like dopamine receptors play a central role in the development of substance dependence and substance-induced psychotic disorders due to consumption of a diverse class of drugs, e.g., alcohol, nicotine, opioids, cannabinoids, cocaine, and amphetamines, and also of endogenous psychosis of schizophrenia [1-3]. Two animal models of schizophrenia, an amphetamine-sensitized rat and the phencyclidine-treated rat, showed marked dopamine supersensitivity and an increase in the proportion of striatal D2 receptors in the high-affinity state [4-7]. These findings indicate that the dopamine system is involved in the neural mechanisms of psychiatric disorders, substance use disorders, and schizophrenia.

The activity of the dopamine receptors is regulated by intricate mechanisms including the heterotrimeric G protein system. The regulator of G-protein signaling (RGS) modulates the functioning of heterotrimeric G protein in part by stimulating the GTPase activity of the G protein subunits [8, 9]. RGS9 is a member of the RGS family, and the RGS9 gene gives rise to two products, RGS9-1 and RGS9-2, *via* alternative splicing [10, 11]. RGS9-1 is expressed in the retina, and RGS9-2 is highly expressed in the striatum and plays a role in modulating dopaminergic-mediated signaling cascades [12, 13]. Previous studies showed that the expression of RGS9-2 in the postmortem brain of schizophrenia patients was lower than that of controls [14, 15]. RGS9 knockout mice showed supersensitivity to dopamine and marked elevation in the proportion of D2 high-affinity receptors [13]. These findings suggest that the RGS9 gene variation contributes to the sensitivity of D2 receptors in the brain and to development of psychotic disorders.

It is well known that consumption of methamphetamine, an indirect dopamine agonist, produces psychosis at a high rate. The symptoms of methamphetamine-induced psychosis are similar to those of schizophrenia, and the diseases show a cognate course of illness. Thus, it seemed that methamphetamine use disorder and schizophrenia share in part the mechanisms of their neural pathogenesis. Based on the above rationale, we investigated the association between the RGS9 gene and methamphetamine use disorder or schizophrenia in a Japanese population.

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METHODS

Subjects

The subjects comprised 220 unrelated patients with methamphetamine dependence (175 males and 45 females, average age 37.0 ± 11.8 years) who met the ICD-10-DCR criteria (F15.2), 486 schizophrenic patients (247 males and 240 females, average age 50.5 ± 12.8 years) who met F20, and two sets of corresponding age-, sex-, and geographical origin-matched healthy controls, 289 control subjects (225 males and 64 females, average age 37.1 ± 12.8 years) and 464 control subjects (225 males and 239 females, average age 51.3 ± 14.3 years) . Two hundred and eighteen of the patients with methamphetamine dependence have or had comorbid methamphetamine psychosis (F15.5). Among the schizophrenic patients, 221 were the paranoid subtype and 239 were the hebephrenic subtype. Diagnosis of methamphetamine use disorder and schizophrenia and determination of subtype was performed by two trained psychiatrists on the basis of all available information. Most of the control subjects were medical staff members who had no past history or family history of substance dependence or major psychotic disorders. All subjects were Japanese, born and living in restricted areas of Japan. This study was initiated after receiving the approval of the ethical committees of the participating institutions. Written informed consent was obtained from all participants.

Clinical Phenotypes

Clinical observation has revealed substantial inter-individual differences in certain phenotypes of methamphetamine-taking behavior and psychosis that seem to be regulated, at least in part, genetically. The rationale and methods of the subgrouping of phenotypes were previously described [16]. In brief, the patients with methamphetamine dependence and psychosis were divided into five subgroups according to the following clinical phenotypes: multisubstance-abuse status, age at first consumption of methamphetamine, latency to the onset of psychotic symptoms after the first consumption of methamphetamine, prognosis of psychosis after therapy, and the complication of spontaneous relapse to a psychotic state.

Genotyping

Peripheral blood was obtained from the subjects, and the genomic DNA was extracted from peripheral leukocytes using a standard procedure. We selected two nonsynonymous polymorphisms, rs12452285 (Leu225Ser) in exon 12 and rs34797451 (His498Arg) in exon 18, of the RGS9 gene. Genotyping was performed using TaqMan technology on an ABI7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA). All genotyping was performed in a blinded fashion, with the control and case samples mixed randomly.

Statistical Analysis

Statistical analysis of association was performed using SNPAlyze software (Dynacom Co., Mobara, Chiba, Japan). Deviation from Hardy-Weinberg equilibrium and the case-control study were tested using the χ^2 test. The differences between subgroups were evaluated on Fisher's exact test. Statistical significance was set at 0.05.

RESULTS

Rs34797451 showed monomorphism in the present Japanese population, but rs12452285 showed polymorphism. Accordingly, subsequent analyses were done on rs12452285 only. The genotype distribution and allele frequencies for the polymorphism of patients with methamphetamine use disorder and control subjects are shown in Table **1** and those for the patients of schizophrenia in Table **2**. The genotype distributions of patients and control subjects did not deviate from

Table 1. Genotype and Allele Distributution of rs12452285 of the RGS9 Gene with METH Use Disorder and Controls

		Genotype(%)			*P*	Allele(%)		*P*
rs12452285	**N**	**C/C**	**C/T**	**T/T**		**C**	**T**	
Controls	289	266(92.0)	23(8.0)	0(0.0)		555(96.0)	23(4.0)	
Patients	220	200(90.9)	18(8.2)	2(0.9)	0.27	418(95.0)	22(5.0)	0.43

Table 2. Genotype and Allele Distribution of rs12452285 of the RGS9 Gene in Patients with Schizophrenia and Controls

		Genotype(%)			*P*	Allele(%)		*P*
rs12452285	**N**	**C/C**	**C/T**	**T/T**		**C**	**T**	
Controls	464	422(91.0)	39(8.4)	3(0.6)		883(95.2)	45(4.8)	
Schizophrenics	487	428(87.9)	57(11.7)	2(0.4)	0.02	913(93.7)	61(6.3)	0.18
Paranoid type	221	190(86.0)	30(13.6)	1(0.4)	0.11	410(92.8)	32(7.2)	0.07
Hebephrenic type	239	215(90.0)	23(9.6)	1(0.4)	0.81	453(94.8)	25(5.2)	0.76

Hardy-Weinberg equilibrium at the polymorphism. We found no significant differences in genotypic or allelic distribution of rs12452285 between methamphetamine use disorder and the corresponding controls or between schizophrenia and the corresponding controls.

To investigate further the roles of the RGS9 gene in the pathophysiology of psychosis and drug-taking behaviors, we examined the association of the RGS9 gene with several clinical phenotypes of methamphetamine dependence and psychosis, i.e., the age at first consumption of methamphetamine, latency to onset of psychosis after abuse, prognosis of psychosis after therapy, spontaneous relapse even without reconsumption of methamphetamine, and multiple substance abuse status, which show individual variation and may in part be regulated genetically. There was a significant difference in allelic distribution between the two subgroups divided by age of first use of methamphetamine (p=0.047, Table **3**) but not in genotypic distribution. For the other clinical phenotypes, there was no significant association with genotypic or allelic distributions of rs12452285 of the RS9 gene.

DISCUSSION

The present study showed that the RGS9 gene is not associated with susceptibility to methamphetamine use disorder or schizophrenia. There was a significant difference in allelic distribution in the phenotype of age at first consumption of methamphetamine (P=0.047), but the significance was marginal, and there was no significant difference in its genotypic distribution, indicating a necessity for confirmation by replication analyses.

It was well known that three different conditions, psychostimulant-induced behavioral sensitization in rodents, psychostimulant-induced psychoses in humans, and chronic schizophrenia show similar longitudinal alternations, progressively enhanced susceptibility to abnormal behaviors, psychotic state, and relapse [17]. Many studies suggest that the susceptibility to dopamine release that developed in the striatum and accumbens is the most direct and common mechanism for the behavioral sensitization phenomenon in rodents after administration of psychostimulants including methamphetamine, amphetamine, and cocaine [18-22]. Two independent groups found approximately twice as much amphetamine-induced dopamine efflux in the striatum of patients with schizophrenia in comparison to healthy controls [23, 24]. These data support a hypothesis that a process of endogenous sensitization of dopaminergic systems is involved in the pathogenesis of schizophrenia.

There are two splice isoforms of the RGS9 gene. RGS9-1 is the short splice isoform and expressed in the retina, and RGS9-2 is the long splice isoform, which is highly expressed in striatum [10, 11]. RGS9-2 was present in a large fraction of D2 receptor-containing neurons and co-expression of RGS9-2 accelerated D2-mediated channel activation [13]. It was revealed that a single injection of amphetamine reduced the RGS9 mRNA levels [25]. RGS9 knockout mice showed heightened locomotor and rewarding responses to cocaine and a marked increase in the proportions of $D2^{High}$ receptors

Table 3. Subgroups of METH Dependence and Psychosis by Clinical Characteristic

Clinical Phenotype		Genotype(%)			*P*	Allele(%)		*P*
rs12452285	**N**	**C/C**	**C/T**	**T/T**		**C**	**T**	
Multisubstance abuse								
Yes	155	141(91.0)	14(9.0)	0(0.0)	0.06	296(95.5)	14(4.5)	0.34
No	59	53(89.8)	4(6.8)	2(3.4)		110(93.2)	8(6.8)	
Age at first consumption								
≤20	117	103(88.0)	13(11.1)	1(0.9)	0.08	219(93.6)	15(6.4)	0.047
>20	101	96(95.0)	4(4.0)	1(1.0)		196(97.0)	6(3.0)	
Latency to onset of psychosis								
≤3 years	101	93(92.1)	8(7.9)	0(0.0)	0.33	194(96.0)	8(4.0)	0.36
>3 years	92	83(90.2)	7(7.6)	2(2.2)		173(94.0)	11(6.0)	
Prognosis of psychosis								
Transient	112	104(92.9)	7(6.2)	1(0.9)	0.61	215(96.0)	9(4.0)	0.34
Prolonged	90	80(88.9)	9(10.0)	1(1.1)		169(93.9)	11(6.1)	
Spontaneous relapse of psychosis								
Yes	93	85(91.4)	6(6.5)	2(2.1)	0.21	176(94.6)	10(5.4)	0.75
No	117	106(90.6)	11(9.4)	0(0.0)		223(95.3)	11(4.7)	

[4, 13]. In particular, RGS9 knockout mice showed conditioned place preference after lower doses of cocaine than the wild type [13]. Consistent with the result, overexpression of RGS9-2 induced by injection of a herpes simplex virus reduced the dopamine sensitivity [13]. The other group reported that mice lacking RGS9 show a 10-fold increase in sensitivity to the rewarding effects of morphine [26]. These lines of evidence suggest that RGS9 plays an important role in substance use disorder by modulating the dopamine pathway *via* D2 receptors. Recently, Seeman *et al.* reported that expression of RGS9-2 was reduced by 22% in the hippocampus of humans with schizophrenia [15], and the result is consistent with the previous study that reported a 40% reduction of RGS9 in the prefrontal cortices in schizophrenia [14]. They also found that the expression of RGS9-2 in the amphetamine-sensitized rat striatum was reduced by 11% [15].

The RGS9 gene is located on chromosome region 17q21-25 [11], and the region was implicated in major mental illness susceptibility through linkage studies [27-30]. We analyzed two SNPs, rs12452285 (Leu225Ser) and rs34797451 (His498Arg), of the RGS9 gene because these are the only two nonsynonymous polymorphisms in the RGS9 gene registered in the NCBI SNP database. Recently, Liou *et al.* examined a possible association between the RGS9 gene and tardive dyskinesia using seven single nucleotide polymorphisms, rs8077696, rs8070231, rs2292593, rs2292592, rs9916525, rs1122079 and rs4790953. In haplotype analyses, they found a significant association with the haplotype consisting of rs8077696, rs8070231, and rs2292593 of the RGS9 gene [31]. Tardive dyskinesia is an abnormal involuntary movement disorder usually caused by chronic antipsychotic treatment, which blocks D2 dopamine receptors and may induce subsequent supersensitivity of D2 receptors. Therefore, it is possible that the haplotype consisting of these markers is associated with functional changes in RGS9, and a possible candidate for RGS9 analyses for methamphetamine use disorder or schizophrenia.

In conclusion, this study showed that the RGS9 gene is unlikely play a major role in schizophrenia and methamphetamine use disorder liability and/or the development of methamphetamine induced psychosis, at least in a Japanese population.

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ORIGINAL INVESTIGATION

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

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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

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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

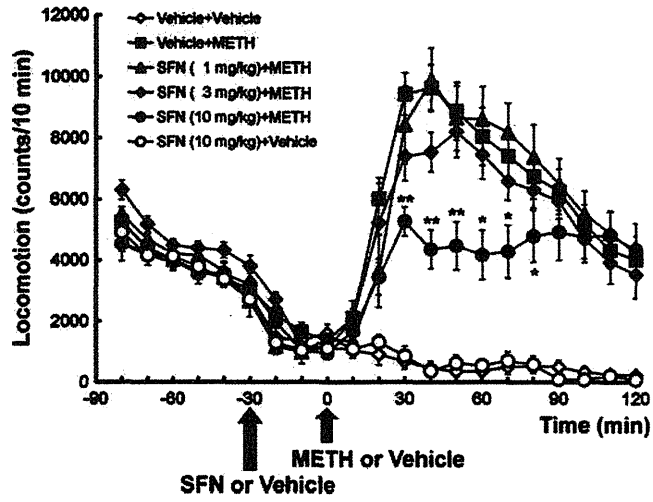

Fig. 1 Effects of SFN on hyperlocomotion in mice after a single administration of METH. Thirty minutes after i.p. injection of vehicle (10 ml/kg) or SFN (1, 3, or 10 mg/kg), METH (3 mg/kg) or vehicle (10 ml/kg) was administered s.c. to the mice. Behavior (locomotion) in the mice was evaluated. Each value 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)

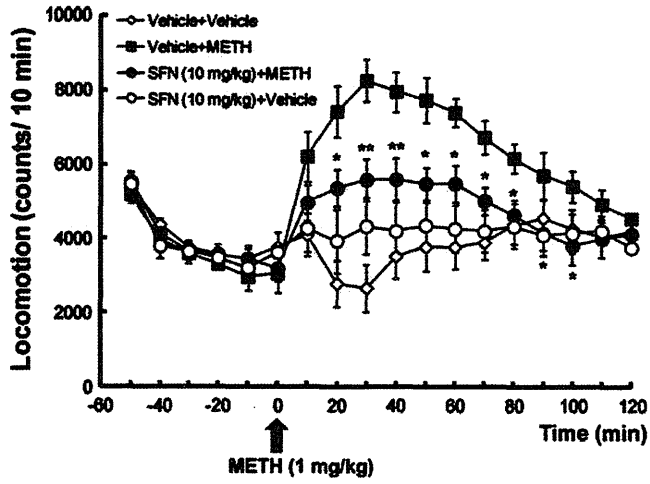

Fig. 2 Effects of SFN on the development of behavioral sensitization in mice after the repeated administration of METH. Vehicle (10 ml/kg)+vehicle (10 ml/kg) group, vehicle (10 ml/kg)+METH (3 mg/kg) group, SFN (10 mg/kg)+METH (3 mg/kg) group, and SFN (10 mg/kg)+vehicle (10 ml/kg) group were treated daily 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)

administration of METH significantly ($p<0.001$) increased METH (1 mg/kg)-induced hyperlocomotion in mice previously treated with METH, compared with the control group. The post hoc analysis also showed that pretreatment with SFN (10 mg/kg) significantly [F (3, 27)=6.13, $p<0.05$] attenuated the development of sensitization after the administration of METH. In contrast, locomotion in the SFN (10 mg/kg)+vehicle group did not differ from that of the control (vehicle+vehicle) group (Fig. 2).

Effects of SFN on hyperthermia induced by the administration of METH

Two-way ANOVA analysis revealed significant differences among the four groups [F (3, 9)=38.51, $p<0.0001$]. Repeated injections of METH (3 mg/kg×3 at 3-h intervals) produced significant hyperthermia in these mice (Fig. 3). However, pretreatment with SFN (10 mg/kg) did not affect the induction of METH-induced hyperthermia in the mice (Fig. 3). Furthermore, SFN (10 mg/kg) alone did not alter rectal temperatures in these mice.

Effects of SFN on the reduction in DA and DOPAC levels in the striatum by the repeated administration of METH

One-way ANOVA analysis revealed that striatal DA [F (3, 38)=12.95, $p<0.0001$] and DOPAC [F (3, 38)=5.60, $p=0.0028$] levels were significantly different among the four groups studied. Pretreatment and a subsequent dose of SFN (10 mg/kg) significantly attenuated the reduction of DA and DOPAC in the striatum typically observed after repeated administration of METH (Fig. 4). Furthermore, treatment

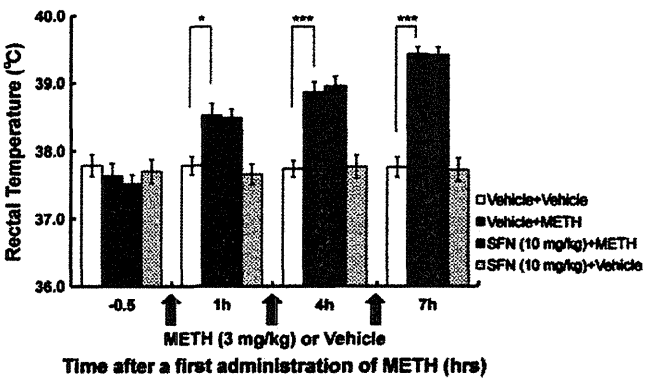

Fig. 3 Effect of SFN on METH-induced hyperthermia in mice. Mice received three injections of vehicle (10 ml/kg, 3-h intervals, s.c.) or METH (3 mg/kg, 3-h intervals, s.c). Vehicle (10 ml/kg, i.p.) or SFN (10 mg/kg i.p.) was injected into the mice 30 min prior the first injection of METH or vehicle. Rectal temperature was recorded 30 min before the first injection of METH or vehicle and 1, 4, or 7 h after the first METH (or vehicle) injection. Each value is the mean± SEM (n=8–9 per group). *$p<0.05$, ***$p<0.001$ as compared to the vehicle+METH group (Bonferroni/Dunn method)

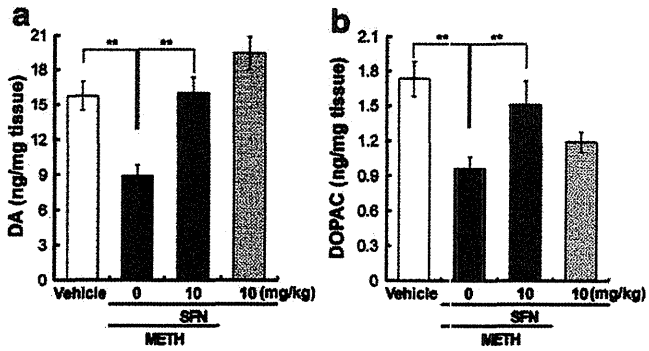

Fig. 4 Effects of SFN on DA (**a**) and DOPAC (**b**) levels in the mouse striatum after the repeated administration of METH. Thirty minutes after i.p. injection of SFN (10 mg/kg) or vehicle (10 ml/kg), mice received three injections of METH (3 mg/kg, s.c.) or vehicle (10 ml/kg, s.c.) at 3-h intervals (day 1). 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). 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). Mice were sacrificed 3 days after the administration of METH (day 4). Values are the mean±SEM (n=10–11 per group). **$p<0.01$ as compared to the vehicle+METH group (Bonferroni/Dunn method)

with SFN (10 mg/kg) alone did not alter levels of DA and DOPAC in the mouse striatum (Fig. 4).

DAT immunohistochemistry

Repeated administration of METH (3 mg/kg×3 at 3-h intervals) markedly decreased the density of DAT in the mouse striatum (Fig. 5). One-way ANOVA analysis showed significant differences in DAT immunoreactivity in the striatum [F (3, 30)=55.93, $p<0.0001$] among the four groups. The post hoc analysis indicated that pretreatment and subsequent administration of SFN (10 mg/kg) significantly ($p<0.0001$) attenuated the reduction of DAT immunoreactivity in the mouse striatum usually seen after repeated administration of METH (Fig. 5). The administration of SFN (10 mg/kg) alone did not alter the density of DAT immunoreactivity in the mouse striatum (Fig. 5).

MAC1 immunohistochemistry

Three days after the repeated dose of METH (3 mg/kg×3 at 3-h intervals), MAC1 immunoreactivity in the mouse striatum was markedly increased (Fig. 6). One-way ANOVA analysis revealed significant differences among the four groups [F (3, 31)=277.41, $p<0.0001$], and the post hoc analysis showed that pretreatment and a subsequent administration of SFN (10 mg/kg) significantly ($p<0.001$) attenuated the increase in MAC1 immunoreactivity in the striatum associated with the administration of METH. Treatment with SFN (10 mg/kg) plus vehicle had no effect on MAC1 immunoreactivity in the mouse striatum (Fig. 6).

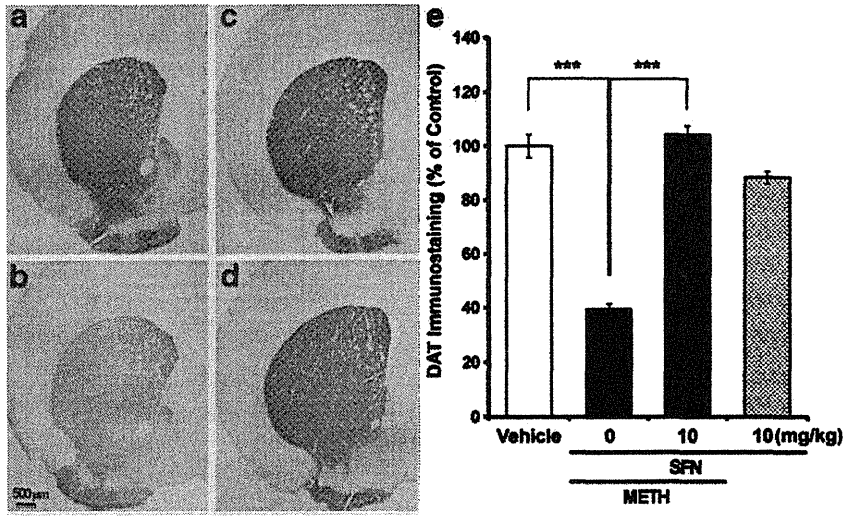

Fig. 5 Effects of SFN on the reduction of DAT density in the mouse striatum after repeated administration of METH. **a** Vehicle+vehicle, **b** vehicle+METH, **c** SFN+METH, and **d** SFN+vehicle. Thirty minutes after i.p. injection of SFN (10 mg/kg) or vehicle (10 ml/kg), the mice received three injections of METH (3 mg/kg, s.c.) or vehicle (10 ml/kg, s.c.) at 3-h intervals (day 1). 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). The mice were perfused 3 days after the administration of METH (day 4). **a–d** Representative photomicrographs showing the DAT immunoreactivity in the striatum of the mice. *Scale bar* was 500 μm. The mean value for DAT immunoreactivity staining was determined for each group and was expressed as a percentage of that of matched control group (**e**). Each value is the mean±SEM (n=7–9 per group). ***p<0.001 as compared to the vehicle+METH group (Bonferroni/Dunn method)

Discussion

A major finding of this study is that SFN ameliorated behavioral changes such as acute hyperlocomotion and the development of behavioral sensitization typically induced by the administration of METH. It also showed that SFN protects against METH-induced dopaminergic neurotoxicity in the mouse striatum. SFN occurs naturally in cruciferous vegetables like broccoli, cabbage, watercress, and Brussels sprouts, in its precursor form, glucosinolate. On chewing, the glucose moiety of this glucosinolate precursor is hydrolyzed by myrosinase into the corresponding isothiocyanate (Fenwick et al. 1983). Interestingly, dietary SFN-rich sprouts reduce colonization and attenuate gastritis in *Helicobacter pylori*-infected humans (Yanaka et al. 2009; Yanaka 2011). In addition, a phase II study of SFN (200 μmol (35 mg) daily) in patients with recurrent prostate cancer is now in progress (NCT01228084). Taken together, our results suggest that SFN could be a promising, therapeutic drug for the treatment of multiple signs associated with METH abuse in humans, particularly as it is safe for human consumption.

The acute pharmacological effects of amphetamines such as METH are linked to their capacity to increase extracellular DA levels via the release of DA from presynaptic terminals and by the inhibition of DA re-uptake (Seiden et al. 1993). In this study, we found that pretreatment with SFN attenuated acute hyperlocomotion in mice, induced by a single dose of METH. This study does not identify the precise cellular mechanisms for the actions of SFN on METH-induced behavioral effects; however, the findings at least in part suggest that SFN acts by decreasing extracellular DA levels in the mouse striatum. SFN is widely reported to induce Nrf2-dependent gene expression, although its molecular targets have not been fully characterized (Juge et al. 2007; Cheung and Kong 2010; Kwak and Kensler 2010). It remains unclear as to whether activation of the Nrf-2-electrophile-responsive element/ARE pathway accounts for the ability of SFN to diminish the acute behavioral effects induced by METH in mice. Further studies on the ability of this agent to ameliorate METH-associated acute neurochemical and behavioral effects will be necessary.

Repeated exposure to METH results in a progressively enhanced and enduring behavioral response to the drug, a phenomenon known as behavioral sensitization. A number of behavioral, neurochemical, biochemical, and molecular studies have shown that the initiation of this complex process involves the interaction of several neurotransmitters, neuropeptides, neurotrophic factors, and their associated receptor signaling pathways (Robinson and Becker 1986; Pierce and Kalivas 1997; White and Kalivas 1998; Licata and Pierce 2003; Vanderschuren and Kalivas 2000; Scholl et al. 2009). Determining the full interplay of these factors remains elusive. Several studies propose the involvement of the mesolimbic dopamine system, including the ventral tegmental area, nucleus accumbens, and associated brain regions such as the striatum in the development of behavioral sensitization. Previously, we reported that antioxidants

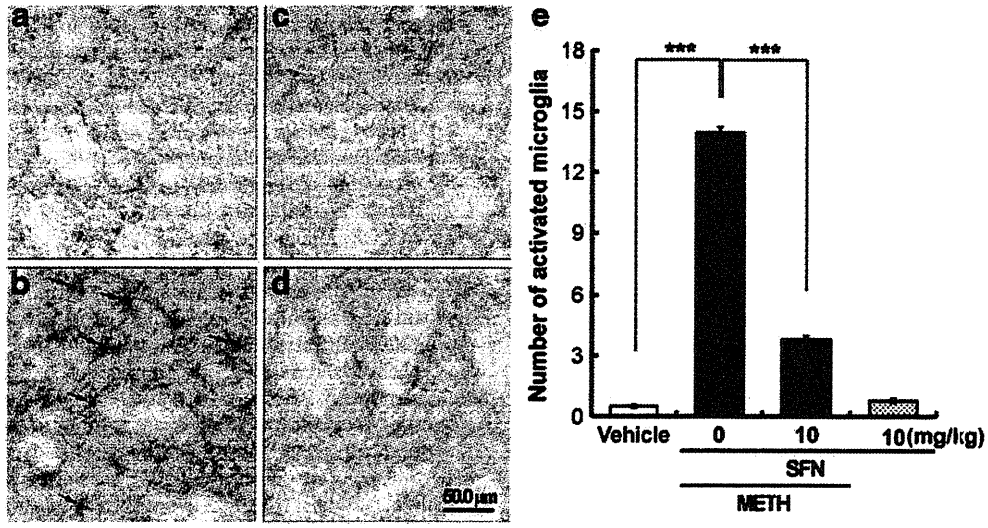

Fig. 6 Effects of SFN on microglial activation in the mouse striatum after the repeated administration of METH. **a** Vehicle+vehicle, **b** vehicle+METH, **c** SFN+METH, and **d** SFN+vehicle. Thirty minutes after i.p. injection of SFN (10 mg/kg) or vehicle (10 ml/kg), the mice received three injections of METH (3 mg/kg, s.c.) or vehicle (10 ml/kg, s.c.) at 3-h intervals (day 1). 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). Mice were perfused 3 days after the administration of METH (day 4), and MAC1 immunohistochemistry was performed. **a–d** Representative photomicrographs depicting MAC1 immunoreactivity (activated microglia) in the striatum of mice. Resting state microglia was observed in the striatum of the control mice (**a**). Activated microglia was observed in the striatum of METH-treated mice (**b**). *Scale bar* was 50 μm. The mean value representing the staining of activated microglia was determined for each group and was expressed as a percentage of that of matched control mice (**c**). The number of activated microglia was counted based on one microscopic view (×200) (**e**). Each value is the mean±SEM (n=8–9 per group). ***p<0.001 as compared to vehicle+METH group (Bonferroni/Dunn method)

such as *N*-acetyl-L-cysteine and minocycline attenuate the development of METH-induced behavioral sensitization in rodents (Fukami et al. 2004; Zhang et al. 2006). It is therefore likely that the potent antioxidant properties of SFN play a role in its actions, although further detailed studies are needed to confirm this.

In this study, we found that pretreatment and subsequent administration of SFN (10 mg/kg) significantly attenuated the METH-induced reduction of DA and DOPAC levels as well as DAT immunoreactivity in the mouse striatum. These protective mechanisms may block the neurotoxic effects on DA neurons. Further studies will still be necessary to fully define these mechanisms. In the treatment paradigm of METH-induced neurotoxicity (3 mg/kg×3 at 3-h intervals), we performed behavioral evaluations in mice after three injections of METH. We found that behavioral abnormalities in mice after the first administration of METH are similar to the results (Fig. 1) of acute METH administration (Supplemental Fig. 1). In contrast, we found that behavioral abnormalities in mice after the third administration of METH were similar between the groups, vehicle+METH and SFN+METH, indicating that the effect of SFN was not detectable in behavioral abnormalities after the third administration of METH (Supplemental Fig. 1). Thus, it is unlikely that the biochemical effects of SFN on METH-induced neurotoxicity are similar to the behavioral effects of SFN on METH-induced behavioral abnormalities.

A number of studies show that the METH-induced neurotoxic effect on DA nerve endings within the striatum is associated with microglial activation (Escubedo et al. 1998; Pubill et al. 2003; Guilarte et al. 2003; LaVoie et al. 2004; Thomas et al. 2004; Thomas and Kuhn 2005; Zhang et al. 2006). The temporal and dosage relationships between METH-induced neurotoxicity and microglial activation suggest that this activation might contribute to METH-induced neurotoxicity in the striatum (Thomas et al. 2004). Interestingly, SFN reduces lipopolysaccharide-induced microglial activation in the mouse brain, suggesting that SFN is a potent inhibitor of microglial activation (Innamorato et al. 2008). We conclude that, in part, SFN reduces METH-induced neurotoxicity in the mouse striatum by inhibiting microglial activation. Additional studies on the role of microglial activation in METH-induced dopaminergic neurotoxicity are warranted.

A number of studies indicate that neurotoxic doses of METH cause hyperthermia and that hypothermia can suppress METH-induced neurotoxicity, suggesting a role for body temperature in METH-induced dopaminergic neurotoxicity (Albers and Sonsalla 1995; Ali et al. 1996). However, we found no evidence that SFN altered METH-induced hyperthermia in mice. Therefore, it is unlikely that body temperature plays a role in the protective effect of SFN on METH-induced neurotoxicity in mice.

Recently, it has been reported that SFN increases Nrf2 protein levels in the mouse striatum and protects against MPTP-induced death of nigral dopaminergic neurons in a cell culture model of Parkinson's disease (Jazwa et al. 2011). In the neonatal hypoxia–ischemia rat model, pretreatment with SFN increases the expression of Nrf2 immunoreactivity, while decreasing the number of TUNEL-positive neurons and microglial activation in the rat brain (Ping et al. 2010), suggesting that its neuroprotective effect is mediated through increased Nrf2 expression. It is likely that SFN protects against dopaminergic neurotoxicity in the mouse striatum by increasing Nrf2 expression, although this needs to be confirmed.

In conclusion, this study demonstrated that in mice, SFN inhibited METH-induced behavioral changes such as acute hyperlocomotion and the development of behavioral sensitization and dopaminergic neurotoxicity in mice. This makes SFN a promising therapeutic agent for the treatment of multiple signs associated with METH abuse, since it is a naturally occurring compound found in cruciferous vegetables.

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Conflicts of interest All authors had no potential conflict of interest.

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The antibiotic minocycline prevents methamphetamine-induced rewarding effects in mice

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ABSTRACT

Repeated use of methamphetamine (METH) causes dependence in humans, and to date, there are no effective medication treatments for METH addiction. We previously reported that the antibiotic minocycline attenuated behavioral abnormalities (hyperactivity and behavioral sensitization) and dopaminergic neurotoxicity in mice and monkeys, after the administration of METH. In this study, we examined the effect of minocycline on METH-induced rewarding effects in mice using the conditioned place preference (CPP) paradigm. Minocycline (40 mg/kg, IP) significantly attenuated METH (1.0 mg/kg, SC)-induced place preference in mice. *In vivo* microdialysis experiments using free-moving mice, showed that minocycline (40 mg/kg, IP) significantly attenuated the increased extracellular dopamine (DA) levels within the nucleus accumbens, typically seen after the administration of METH (1.0 mg/kg, SC). These findings suggest that minocycline may block METH-induced rewarding effects by down regulating extracellular DA levels in the nucleus accumbens of mice. This would make minocycline a potential therapeutic drug for the treatment of METH induced disorders.

1. Introduction

Abuse of methamphetamine (METH) is a worldwide, public health problem (Hashimoto, 2007; Gonzales et al., 2010; Karila et al., 2010; Chen et al., 2010). The abuse of METH has significant psychiatric and medical consequences, including psychosis, dependence, overdose, and death. However, there are currently no pharmacological treatments for METH-induced symptoms (Hashimoto, 2007; Sofuoglu and Sewell, 2009; Gonzales et al., 2010; Karila et al., 2010; Sofluoglu, 2010; Chen et al., 2010).

Minocycline is a second-generation tetracycline that readily crosses the blood–brain barrier, and has powerful anti-inflammatory and neuroprotective properties (Domercq and Matute, 2004; Hashimoto, 2007; Chen et al., 2010). Previously, we reported that minocycline attenuated hyperlocomotion and the development of behavioral sensitization in mice, after the administration of METH (Zhang et al., 2006a). Furthermore, the reduction of dopamine (DA) and DA transporter (DAT) in the striatum of mouse and monkey brains associated with repeated METH administration, was significantly attenuated by subsequent treatment with minocycline (Zhang et al., 2006a; Hashimoto et al., 2007). Moreover, minocycline ameliorated 3,4-methylenedixoymethamphetamine (MDMA)-induced neurotoxicity of serotonergic neurons within mouse brains (Zhang et al., 2006b). It has also been reported that minocycline alleviated symptoms (e.g., hyperlocomotion, prepulse inhibition deficits, cognitive deficits) in mouse and rat models of schizophrenia, after the administration of *N*-methyl-D-aspartate (NMDA) receptor antagonists, such as, phencyclidine (PCP) and dizocilpine (Fujita et al., 2008; Zhang et al., 2007; Levkovitz et al., 2007). These findings suggest that minocycline may represent a promising therapeutic drug, for the treatment of long-term symptoms associated with METH abuse (Hashimoto, 2007, 2008a; Chen et al., 2010).

In this study, we examined the effect of minocycline on METH-induced rewarding effects in mice, using the conditioned place preference (CPP) paradigm. We also examined the effects of minocycline on METH induced increases of extracellular levels of DA in the nucleus accumbens, since DA levels in this brain region play an important role in METH-induced rewarding effects (Ikemoto, 2007).

2. Materials and methods

2.1. Animals

C57BL/6 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) in groups of 5 or 6 per cage, under a controlled 12/12-h light–dark cycle (lights on from

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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 food pellets. The experimental procedure was approved by the Animal Care and Use Committee of Chiba University.

2.2. Conditioned place preference (CPP)

The place conditioning paradigm (CPP; Brain Science Idea Inc., Osaka, Japan) was used for studying METH-induced rewarding effects. The groups were Control (vehicle + vehicle) group (n = 22), vehicle + METH group (n = 24), minocycline + METH group (n = 24), and minocycline + vehicle group (n = 21). The test mouse was allowed to move freely between transparent and black boxes for a 15 min session once a day, for 3 days (days 1–3) as preconditioning (Fig. 1). On day 3, the time spent in each box was measured. There was no significant difference between time spent in the black compartment with a smooth floor and the white compartment with a textured floor, indicating that there was no place preference before conditioning. On days 4, 6, and 8, minocycline (40 mg/kg, IP, Wako Chemical Pure Ltd., Tokyo, Japan) or vehicle (10 ml/kg, IP) was injected 30 min before the administration of vehicle (physiological saline: 10 ml/kg, SC) or METH (1.0 mg/kg, SC, Dainippon Pharmaceutical Ltd., Osaka, Japan), and then mice were confined to either the transparent or black box for 30 min (Fig. 1). The dose (40 mg/kg) of minocycline used was previously reported to be effective in METH-induced behavioral abnormalities and PCP-induced cognitive deficits (Zhang et al., 2006a, 2007; Fujita et al., 2008). On days 5, 7, and 9, mice were given saline and placed in the opposite METH-conditioning box for 30 min. On day 10, the post-conditioning test was performed without drug treatment, and the time spent in each box was measured for 15 min (Fig. 1). A counterbalanced protocol was used in order to nullify any initial preference by the mouse. The CPP score was designated as the time spent in the drug-conditioning sites, minus the time spent in the saline-conditioning sites.

2.3. Measurement of extracellular dopamine (DA) levels

Mice were anesthetized with sodium pentobarbital prior to the stereotaxic implantation of a probe into the nucleus accumbens (+1.1 mm anteroposterior, +1.0 mm mediolateral from the bregma, and −4.0 mm dorsoventral from the dura), according to the Franklin and Paxinos Atlas (1997). 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 and free-moving 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. Vehicle (10 ml/kg, IP) or minocycline (40 mg/kg, IP) was administered into mice. The dialysate was collected in 30-min fractions. After *in vivo* microdialysis experiments, the position of probe in the nucleus accumbens was confirmed in all mice. The DA levels in each fraction were measured by high performance liquid chromatography (HPLC), with electrochemical detection using a reversed phase column (EICOMPAK PP-ODS, 4.6 × 30 mm, Eicom, Kyoto, Japan) and a mobile phase 1% MeOH/100 mM phosphate buffer (pH 6.0) including 50 mg/L disodium EDTA disodium and 500 mg/L dodium decane-1-sulfonate.

2.4. Statistical analysis

Data are presented as the mean ± standard error of the mean (S.E.M.). CPP data were analyzed by one-way analysis of variance (ANOVA), followed by the Bonferroni/Dunn test. The results of extracellular DA levels were also analyzed by repeated one-way ANOVA, followed by the Student's *t*-test. Significance was set at $P<0.05$.

3. Results

3.1. Effects of minocycline on METH-induced rewarding effects in mice

We investigated the effects of minocycline on the rewarding effects of METH, using the CPP paradigm, which measures the rewarding properties of abused drugs. One-way ANOVA revealed significant differences among the four groups ($df = 3.87$, $F = 5.402$, $p = 0.002$)(Fig. 2). *Post hoc* analysis showed that METH (1.0 mg/kg, SC) significantly increased CPP scores in mice, compared with the vehicle-treated group. When mice were treated with minocycline (40 mg/kg, IP) 30 min before receiving METH (1.0 mg/kg, SC), METH-induced place preference was significantly attenuated. Minocycline (40 mg/kg, IP) alone failed to induce place preference in mice (Fig. 2).

3.2. Effects of minocycline on extracellular DA levels in the nucleus accumbens after the administration of METH

To explore how minocycline inhibits METH-induced rewarding effects, we examined the effect of minocycline on extracellular DA levels in the nucleus accumbens, after administering METH, using an *in vivo* microdialysis technique. A single dose of METH (1.0 mg/kg, SC) stimulated a marked increase in extracellular DA levels in the nucleus accumbens of mice (Fig. 3). Repeated ANOVA analysis showed a significant difference relative to controls (Time × Group, $F = 4.957$,

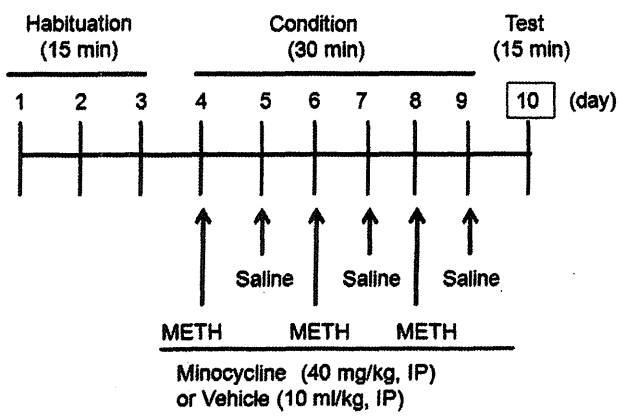

Fig. 1. Experiment schedule for the CPP paradigm. Mice were treated with vehicle (10 ml/kg, IP) or minocycline (40 mg/kg, IP) 30 min before receiving METH (1 mg/kg, SC) or saline (10 ml/kg, SC). Detailed procedure was shown in the Materials and methods section.

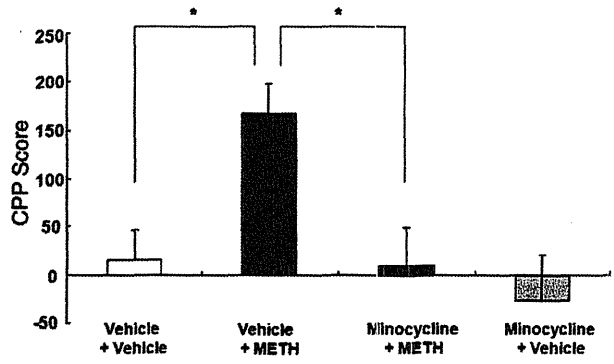

Fig. 2. The effects of minocycline on METH-induced rewarding effects in mice. Thirty minutes after administration of vehicle (10 ml/kg, IP) or minocycline (40 mg/kg, IP), METH (1.0 mg/kg, SC) or saline (10 ml/kg, SC) was administered to mice. A detailed treatment schedule is shown in the Materials and methods section. Vehicle + vehicle group (n = 22), vehicle + METH group (n = 24), minocycline + METH group (n = 24), minocycline + vehicle group (n = 21). Each value is the mean ± S.E.M. *P<0.05 when compared with the vehicle + METH treated group.

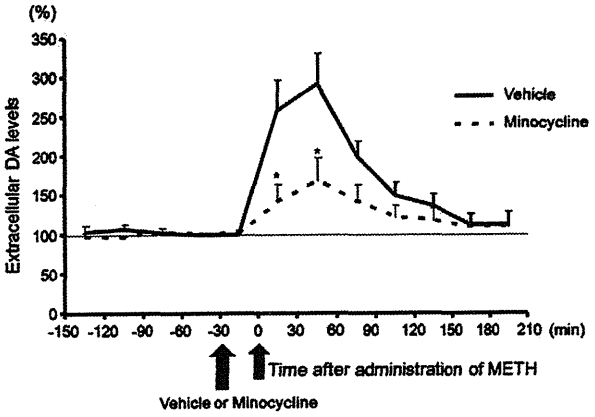

Fig. 3. The effects of minocycline on METH induced increases of extracellular DA levels in the nucleus accumbens. Thirty minutes after the administration of vehicle (10 ml/kg, IP) or minocycline (40 mg/kg, IP), METH (1.0 mg/kg, SC) was injected into mice. The dialysate was collected in 30-min fractions, and DA levels were measured by HPLC. Basal extracellular DA levels in the nucleus accumbens were 4.17 ± 0.101 nmol/L (n = 16, mean ± SEM). The values are the mean ± SEM of 8 mice. *P<0.05 when compared with the control (vehicle) group.

P<0.001). Treatment with minocycline (40 mg/kg, IP) significantly attenuated this increase within the nucleus accumbens (Fig. 3).

4. Discussion

In this study, we found that minocycline attenuated both METH-induced rewarding effects and the marked increases of extracellular DA in the nucleus accumbens, induced by METH administration. It is well known that increased DA signaling in the nucleus accumbens after METH exposure plays an important role in the development of METH-induced rewarding effects (Ikemoto, 2007; Lüscher and Malenka, 2011). Therefore, it is likely, that by inhibiting the increases in extracellular DA within the nucleus accumbens, minocycline may mediate a reduction in METH-induced rewarding effects in mice. The precise mechanisms by which minocycline inhibits METH-induced DA release are currently unknown. The vesicular monoamine transporter (VMAT2) is an essential cellular component, contributing to METH-induced increases in extracellular DA levels and rewarding effects (Patel et al., 2003). However, minocycline did not bind to either VMAT2 or DAT (Hashimoto, data not shown), suggesting that it acts through another route.

A recent double-blind, placebo-controlled, crossover study demonstrated that minocycline (200 mg/day) significantly attenuated some of the subjective-rewarding effects and increases in plasma cortisol, after the administration of d-amphetamine in healthy controls (Sofuoglu et al., 2011). In addition, a case report showed that minocycline was effective in treating psychotic symptoms in a female patient with METH use disorder (Tanibuchi et al., 2010). Furthermore, there are case and open reports showing that minocycline is efficacious in treating schizophrenia (Ahuja and Carroll, 2007; Miyaoka et al., 2008; Chaves et al., 2010; Hashimoto, 2010). Another randomized, double-blind, placebo-controlled study, demonstrated that adjunctive minocycline therapy was effective at treating negative and cognitive symptoms in early-phase schizophrenia (Levkovitz et al., 2010). In totality, minocycline would appear to be a potential therapeutic drug for neuropsychiatric disorders, including METH use disorder and schizophrenia (Hashimoto, 2008a,b; Miyaoka, 2008; Chen et al., 2010).

In contrast, it has been reported that, in a drug discrimination experiment, pretreatment with minocycline produced leftward shifts in the PCP dose–response curve although minocycline did not substitute for PCP (Munzar et al., 2002). Furthermore, minocycline also produced leftward shifts in the dose–response curve for dizocilpine in the same subjects (Munzar et al., 2002). These findings suggest that minocycline may enhance the behavioral effects of the NMDA receptor antagonists, inconsistent with previous reports (Fujita et al., 2008; Zhang et al., 2007; Levkovitz et al., 2007). Therefore, further studies on the effects of minocycline on the behavioral changes by the NMDA receptor antagonists will be necessary.

Very recently, we reported that minocycline, but not tetracycline, potentiated nerve growth factor (NGF)-induced neurite outgrowth in PC12 cells, and that the eukaryotic translation initiation factor eIF4AI plays a role in this mechanism (Hashimoto and Ishima, 2010), suggesting that minocycline may have neurotrophic and neuroprotective effects. Given the role of neuronal plasticity in drug dependence (Lüscher and Malenka, 2011), it seems that minocycline's role in neuronal plasticity may provide a partial explanation for the reduction of METH-induced rewarding effects in mice, although further studies are needed.

5. Conclusion

This study suggests that minocycline can block METH-induced rewarding effects in mice. Therefore, minocycline would appear to be a potential therapeutic drug for METH use disorder, although further studies of minocycline in patients with METH use disorder will be necessary

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