Fig. (3) shows the cytotoxicity of methylone and methamphetamine in CHO cells stably expressing rDAT, rNET and rSERT, together with mGAT1 as a control. Methylone did not induce the release of LDH from any cell line except that expressing SERT (Fig. 3). Furthermore, in combination with methamphetamine it caused a significant increase in the release of LDH in the cells stably expressing the monoamine transporters but not in the control CHO cells or cells expressing GAT (Fig. 3).

## DISCUSSION

The present study demonstrated that methylone acts as a non-selective inhibitor of the monoamine neurotransmitter transporters DAT, NET and SERT, with a rank order in terms of potencies of NET > DAT >> SERT. Kinetic analyses revealed the characteristics of the inhibition to resemble those by methamphetamine. In addition, methylone induced a reversal of transport similar to methamphetamine. Methamphetamine, like amphetamine, is transported by DAT and NET, and probably SERT [13]. The present study demonstrated that methylone had similar properties to methamphetamine not only in inhibiting the uptake by transporters but also in reversing the direction of transport, suggesting that it too is likely to be a transportable inhibitor. However, methylone itself was cytotoxic only at high concentrations, though in combination with methamphetamine it had a significant effect on cells expressing the monoamine transporters but not the control CHO cells or cells expressing GAT. These results suggest that the transport of methylone through monoamine transporters underlies the cytotoxicity.

The effectiveness of methylone and methamphetamine in inhibiting the uptake of substrates by monoamine transporters demonstrated here was well consistent with previous findings [5, 6]. Cozzi *et al.* [5] found that methylone inhibited DAT more than NET, while Nagai *et al.* [6] and ourselves observed a more potent effect on NET than DAT. On the other hand, Nagai *et al.* [6] reported that methylone inhibited DAT and SERT equally, while Cozzi *et al.* [5] and ourselves found the effect to be weaker at SERT than DAT. The discrepancy may be due to the different preparations or concentrations of labeled substrates used.

The concentrations of methylone in the brain at dosages at which the drug is abused, 100 – 200 mg [14], are unknown. Experiments with rats demonstrated the intraperitoneal administration of methylone at 5 mg/kg to be followed by a rapid increase in the plasma concentration ranging from 700 to 1500 ng/mL within 15 or 30 min [11]. These values seem compatible with those for MDMA [7]. Therefore, the present findings suggest that methylone may inhibit monoamine transporters in the CNS at concentrations relevant to its abuse.

The present study demonstrated cytotoxicity at high concentrations, as assessed from the amount of LDH released in

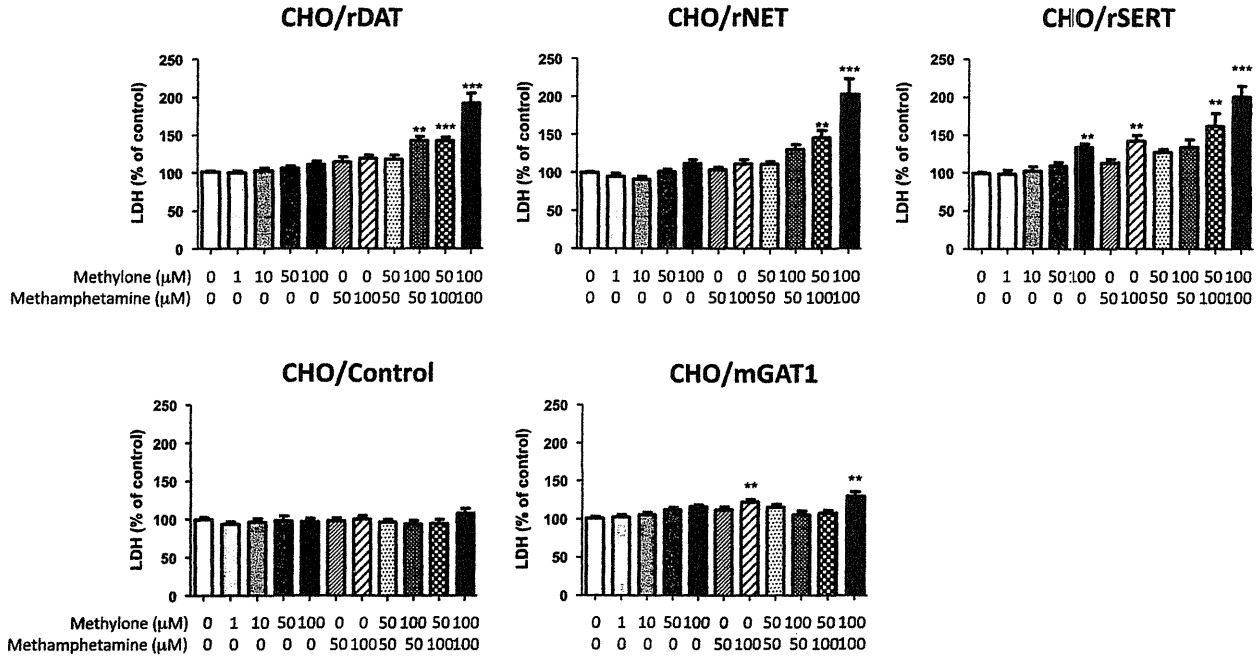


**Fig. (3). Effect of methylone and methamphetamine on viability of CHO cells stably expressing monoamine and GABA transporters.** Cells were seeded on 96-well culture plates and cultured for 24 h prior to treatment with the drug under examination. They were then incubated with various concentrations of methylone and/or methamphetamine for 24h, after which the amount of LDH released into the medium was determined. Values are expressed as a percentage of the control (absence of drugs), and represent the mean ± SEM, n=6-16. **P<0.01, ***P<0.001 vs control.

CHO cells. This effect was observed in cells expressing the monoamine transporters, especially those expressing SERT, but not cells expressing mGAT1, suggesting a relationship with the transport of methylone, although the affinity of the transporter for methylone does not explain the potency of methylone's toxicity. In addition, the present study demonstrated that methylone and methamphetamine combined had a supra-additive effect on the release of LDH in CHO cells expressing monoamine transporters. The cytosolic accumulation of monoamines or methamphetamine may cause oxidative stress, resulting in cell death [15]. Therefore, one may assume that methylone modulates the toxic effects of other monoaminergic agents, such as methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA), through interaction at monoamine transporters. Recently, Shimizu *et al.* [14] reported a case study of a 27-year-old male who took methylone and 5-MeO-MIPT after ingesting a drug powder called pure methylone obtained via the internet, suggesting that substance-related disorders may be complicated by the combined use of psychoactive drugs. According to recent analyses, "ecstacy" and other designer drugs consist of mixtures of MDMA and other substances [16, 17]. Therefore, it is important to note their cytotoxicity when taken simultaneously. Further study is needed to clarify this issue.

In summary, we investigated the effects of methylone on monoamine transporters. The ability of methylone to inhibit transporter function, and damage cells heterologously expressing monoamine transporters, suggests that the transport of methylone underlies its cytotoxicity.

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# Association Between 5HT1b Receptor Gene and Methamphetamine Dependence

H. Ujike[1,2,*], M. Kishimoto[1], Y. Okahisa[1], M. Kodama[1], M. Takaki[1], T. Inada[2,3], N. Uchimura[2,4], M. Yamada[2,5], N. Iwata[2,6], M. Iyo[2,7], I. Sora[2,8] and N. Ozaki[2,9]

[1]*Department of Neuropsychiatry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan;* [2]*JGIDA (Japanese Genetics Initiative for Drug Abuse), Japan;* [3]*Seiwa Hospital, Tokyo, Japan;* [4]*Department of Neuropsychiatry, Kurume University Graduate School of Medicine, Kurume, Japan;* [5]*Department of Psychogeriatrics, National Institute of Mental Health, National Center of Neurology and Psychiatry, Kodaira, Japan;* [6]*Department of Psychiatry, Fujita Health University School of Medicine, Houmei, Japan;* [7]*Department of Psychiatry, Chiba University Graduate School of Medicine, Chiba, Japan;* [8]*Department of Neuroscience, Division of Psychobiology, Tohoku University Graduate School of Medicine, Sendai, Japan;* [9]*Department of Psychiatry, Nagoya University Graduate School of Medicine, Nagoya, Japan*

**Abstract:** Several lines of evidence implicate serotonergic dysfunction in diverse psychiatric disorders including anxiety, depression, and drug abuse. Mice with a knock-out of the 5HT1b receptor gene (*HTR1B*) displayed increased locomotor response to cocaine and elevated motivation to self-administer cocaine and alcohol. Previous genetic studies showed significant associations of *HTR1B* with alcohol dependence and substance abuse, but were followed by inconsistent results. We examined a case-control genetic association study of *HTR1B* with methamphetamine-dependence patients in a Japanese population. The subjects were 231 patients with methamphetamine dependence, 214 of whom had a comorbidity of methamphetamine psychosis, and 248 age- and sex-matched healthy controls. The three single nucleotide polymorphisms (SNPs), rs130058 (A-165T), rs1228814 (A-700C) and rs1228814 (A+1180G) of *HTR1B* were genotyped. There was no significant difference in allelic and genotypic distributions of the SNPs between methamphetamine dependence and the control. Genetic associations of *HTR1B* were tested with several clinical phenotypes of methamphetamine dependence and/or psychosis, such as age at first abuse, duration of latency from the first abuse to onset of psychosis, prognosis of psychosis after therapy, and complication of spontaneous relapse of psychotic state. There was, however, no asscocation between any SNP and the clinical phenotypes. Haplotype analyses showed the three SNPs examined were within linkage disequilibrium, which implied that the three SNPs covered the whole *HTR1B*, and distribution of estimated haplotype frequency was not different between the groups. The present findings may indicate that *HTR1B* does not play a major role in individual susceptibility to methamphetamine dependence or development of methamphetamine-induced psychosis.

**Keywords:** Methamphetamine dependence, association study, *HTR1B*, haplotype.

## INTRODUCTION

Family and twin studies have provided evidence that genetic factors can influence individual differences in vulnerability to substance abuse and dependence [1, 2]. We previously reported that patients with methamphetamine use disorders showed substantial individual differences in psychotomimetic and psychotogenic effects of methamphetamine consumption, e.g., intensity of subjective euphoric effects, latency to onset of methamphetamine-induced psychosis, and prognosis of psychosis after discontinuance of methamphetamine use [3], whose clinical variations should be affected by individual genetic background.

Pharmacological manipulation of serotonergic signaling can modulate the activity of brain reward pathways, and thus the effects of substance dependence to diverse classes of drugs. Fluoxetine, a selective serotonin uptake inhibitor, reduced self-administration of cocaine [4]. Ethanol intake decreased after the administration of 5-HT precursors, 5-HT uptake inhibitors, intracerebral 5-HT, and postsynaptic 5-HT agonists in animals and humans [5]. These serotonergic effects against drug abuse could be mediated by 5HT1b receptors at least. Administration of a 5HT1b agonist, CP-94,253, reduced ethanol self-administration and alcohol-induced aggressive behaviors *via* activation of postsynaptic 5HT1b receptors [6, 7]; in contrast, it facilitated cocaine reward by reducing 5HT release *via* 5HT1b autoreceptor stimulation at presynaptic sites [8]. Mice lacking 5HT1b receptors displayed increased locomotor response to and self-administration of cocaine [9], and elevated alcohol consumption [10].

Previous genetic studies indicated that the 5HT1b receptor gene (*HTR1B*, MIM 182131) was associated with drug dependence and related behaviors. Thus, *HTR1B* polymorphisms were reported to be associated significantly with al-

*Address correspondence to this author at the Department of Neuropsychiatry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan;
Tel: +81-86-235-7151; Fax: +81-86-235-7246;
E-mail: hujike@cc.okayama-u.ac.jp

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coholism with antisocial behaviors [11, 12], whole alcoholism [13, 14], substance dependence [15] and heroin addiction [16], although there were also several inconsistent reports [17, 18]. Therefore, in order to investigate the roles of *HTR1B* in substance dependence, we examined a possible genetic association of *HTR1B* with methamphetamine dependence in a Japanese population.

## METHODS

### Subjects

The subjects consisted of 231 patients with methamphetamine dependence (184 male, 47 female; mean age±SD, 36.6±11.8) and 248 age-, sex-, and geographical origin-matched healthy controls (198 male, 50 female; mean age±SD, 36.6±10.6), who have no individual or family history of drug dependence or major psychotic disorders such as schizophrenia and bipolar disorders. Almost all patients (N=214) are or were co-morbid with methamphetamine-induced psychosis. All subjects were unrelated Japanese. Consensus diagnoses of methamphetamine dependence were made by two trained psychiatrists according to the ICD-10 criteria on the basis of interviews and medical records. The study protocol and purpose were explained to all subjects participating in the study, and written informed consent was obtained from all subjects. This study was approved by the Ethics Committee of each participating institute of Japanese Genetics Initiative for Drug Abuse (JGIDA) [19].

The patients with methamphetamine dependence and/or psychosis were divided into subgroups according to several clinical phenotypes that may indicate indirectly the severity of and liability to dependence and psychosis: 1) age at first abuse of methamphetamine: younger than 20 years old, which is underage in Japan, or older; 2) latency to onset of psychotic state after initial methamphetamine consumption: divided into two groups by median latency of 3 years; 3) Duration of psychotic state after discontinuance of abuse and therapy with antipsychotics: transient type and prolonged type, which were defined as psychosis that subsides within one month or lasts longer than one month, respectively; 4) complication of spontaneous psychosis after remission of methamphetamine-induced psychosis, and 5) multi-substance abuse status.

### Genotyping

*HTR1B* consists of single exon and is a relatively small gene of about 11.7 Kb. HapMap data indicates that there is only one single nucleotide polymorphism (SNP) in the exon, rs 6297, which was proven polymorphic in a Japanese population. However, we did not examine this SNP because it is synonymous, Val3Val, indicating no or less physiological involvement. Instead, we genotyped three SNPs flanking the the gene, rs6297 (A-700C) and rs130058 (A-161T) in the 5' flanking region and rs1228814 (A+1180G) in the 3' flanking region, which have potential to be functional. Genotyping was performed by the PCR-RFLP method. The genomic DNA was extracted from peripheral leukocytes using a standard method. Each polymorphic site was amplified by PCR (the PCR primer sequence of each SNP is available on request) in a 15-ml volume containing 3% dimethyl sulfoxide and 0.75 units of Taq DNA polymerase (Promega Co., Japan) using a unique primer set. The PCR reaction was performed under the following conditions: 95°C for 5 min, then 35 cycles of 30 s of denaturing at 95°C, 1 min of annealing at the appropriate temperature, and 30 s of extension, and final elongation at 72°C for 10 min. The PCR products were digested with the corresponding restriction enzyme for each polymorphism, Nde*I* for rs6297, Nla*III* for rs130058 and Cfro13*I* for rs1228814, and then electrophoresed on 3.0% agarose gels and stained with GelStar (Takara Co., Japan). All genotyping was performed in a blinded fashion, with the control and cases samples mixed randomly. The genotyping of the SNPs were confirmed in part by direct sequencing.

### Statistical Analysis

Statistical analysis of association was performed using SNPAlyze software (Dynacom Co., Japan). Deviation from Hardy-Weinberg equilibrium and the case-control study were tested using the $\chi^2$ test for goodness of fit and $\chi^2$ test for dependence, respectively. Linkage disequilibrium (LD) was tested using the $\chi^2$ test, and D' and $r^2$ values were made the index in the authorization of LD. Case-control haplotype analysis was performed by the permutation method, and permutation *p*-values were calculated based on 100,000 replications.

## RESULTS

The genotype distribution and allele frequencies of the each polymorphism are shown in Table **1**. The genotype distributions of patients and control subjects did not deviate from Hardy-Weinberg equilibrium at any SNP examined. We found no significant difference between the patients and controls in the frequencies of the genotype or allele at any SNP of *HTR1B* (rs6297: allele: p=0.37, genotype: p=0.38, rs130058: allele: p=0.30, genotype: p=0.33 rs1228814: allele: p=0.14, genotype: p=0.47).

We estimated the pairwise LD between the three SNPs of *HTR1B* using the D' and $r^2$ values as an index. A D' of more than 0.7 was found between all the SNPs (0.8455 between rs6297 and rs130058, 1.000 between rs6297 and rs1228814, 0.8216 between rs130058 and rs1228814) indicating that the three SNPs are in linkage disequilibrium (LD) and located within one LD block. Then, we performed case-control haplotype analysis (Table **2**). There were 5 kinds of haplotypes consisting of the three SNPs. There was no significant difference in distribution of haplotype between methamphetamine dependence and controls (overall permutation $p$=0.81). Neither haplotype consisting of the two SNPs in the promoter region (rs6297 and rs130058) showed a significant difference in distribution between the groups.

Additional analyses of subgroups of patients with methamphetamine dependence/psychosis stratified by five items of clinical phenotypes (Table **3**) revealed that there was no significant association of any SNP of *HTR1B* with any clinical phenotype of methamphetamine dependence and/or psychosis.

## DISCUSSION

The 5HT1b receptors are expressed in the brain of rodents, and homologous 5HT1Dβ receptors are expressed in the human brain. The 5HT1b receptors are located at nerve

**Table 1. Case-Control Association Analyses of *HTR1B***

| Loci Groups | N | Genotype (%) | | | *p* | Allele (%) | | *p* |
|---|---|---|---|---|---|---|---|---|
| SNP1 (rs6297) | | A/A | A/G | G/G | | A | G | |
| Control | 248 | 73.4 | 23.8 | 2.8 | | 85.3 | 14.7 | |
| MAP-dependence | 228 | 68.9 | 27.6 | 3.5 | 0.37 | 82.7 | 17.3 | 0.38 |
| SNP2 (rs130058) | | T/T | T/A | A/A | | T | A | |
| Control | 227 | 87.2 | 12.8 | 0 | | 93.6 | 6.4 | |
| MAP-dependence | 229 | 89.5 | 10.5 | 0 | 0.3 | 94.8 | 5.2 | 0.33 |
| SNP3 (rs1228814) | | C/C | C/A | A/A | | C | A | |
| Control | 246 | 73.6 | 25.2 | 1.2 | | 86.2 | 13.8 | |
| MAP-dependence | 225 | 70.7 | 27.5 | 1.8 | 0.14 | 84.4 | 15.6 | 0.47 |

**Table 2. Haplotype Analysis of *HTR1B* in Methamphetamine Dependence**

| Haplotype | Controls | MAP-Dependence | *p* |
|---|---|---|---|
| A-T-C | 0.6973 | 0.6721 | 0.41 |
| G-T-C | 0.1480 | 0.1673 | 0.43 |
| A-T-A | 0.0902 | 0.1095 | 0.33 |
| A-A-A | 0.0520 | 0.0461 | 0.68 |
| A-A-C | 0.0094 | 0.0034 | 0.26 |

Global permutation *p* value =0.81 ($c^2$=3.20).

terminals of various pathways and act as autoreceptors that are involved in the regulation of release of diverse neurotransmitters, including serotonin itself [20]. The 5HT1b receptors are also located at postsynaptic sites. A lot of studies suggest that 5HT1b receptors are implicated in several physiological functions, behaviors, and neuropsychiatric disorders including migraine, aggression, anxiety, depression, and substance dependence [20].

Genetic associations of HTR1B have been examined with various psychiatric conditions such as antisocial behaviors, suicide, depression, and schizophrenia. As to substance dependence, Lappapleinene *et al.* [11] found that rs6296, a synonymous SNP in exon 1 (G861C, Val3Val), was associated with antisocial alcoholism in two independent populations of alcoholic patients for the first time, but this was followed by consistent [12] and inconsistent results [15, 17, 18, 21]. Finally, Fehr *et al.* [13] reported that the risk allele of 861C reported by Lappapleinene *et al.* [11] was protective in their patients with alcoholism. These inconsistencies among alcoholism studies may indicate that status of co-morbidity with other substance abuse could influence the results because *HTR1B* was shown to be associated with substance abuse [15] and heroin addiction [16].

In the present study, we examined three SNPs in the 5' and 3' flanking regions of *HTR1B*, rs6297 (A-700C), rs130058 (A-161T), and rs1228814 (A+1180G) in patients with methamphetamine dependence and found no association at any loci. Neither was any association found with several clinical phenotypes, such as initial abuse of methamphetamine at a younger age, rapid onset of psychotic state induced by methamphetamine, longer duration of psychosis after discontinuance of methamphetamine abuse, complication of spontaneous relapse of psychosis after remission, and multi-substance abuse status. Haplotype analysis of the three SNPs also showed no significant difference in haplotype distribution between the patients and controls. As the LD block consisting of the three SNPs covers the whole of *HTR1B*, it is unlikely that any untested polymorphism including G861C in *HTR1B* could be associated with methamphetamine dependence or its clinical phenotypes. Our findings are consisted with a study of cocaine, another psychostimulant, which showed that T-261G, A-161T, and G861C of *HTR1B* was not associated with cocaine abuse [17].

Duan *et al.* [22] examined effects of common SNPs in the promoter region of *HTR1B* on its transcription activity by *in vitro* reporter assay and revealed that T-261G and A-161T (rs130058) potently affected gene expression. The haplotypes consisting of -261G and -161A enhanced transcriptional activity 2.3-fold compared with major haplotype consisting of -261T and -161T. The A-161T polymorphism altered characteristics of binding to AP-1 transcription factor.

**Table 3. Association of *HTR1B* with Clinical Phenotypes of Methamphetamine Dependence and Psychosis**

| SNP1 (rs6297) | N | Genotype (%) | | | *p* | Allele (%) | | *p* |
|---|---|---|---|---|---|---|---|---|
| | | A/A | A/G | G/G | | A | G | |
| Age at first use | | | | | | | | |
| 20y <= | 111 | 0.68 | 0.26 | 0.05 | | 0.82 | 0.18 | |
| 19y >= | 113 | 0.68 | 0.30 | 0.02 | 0.37 | 0.83 | 0.17 | 0.38 |
| Latency of psychosis | | | | | | | | |
| 3y > | 103 | 0.64 | 0.31 | 0.05 | | 0.80 | 0.20 | |
| 3y <= | 83 | 0.70 | 0.28 | 0.02 | 0.57 | 0.84 | 0.16 | 0.31 |
| Prognosis of psychosis | | | | | | | | |
| Transient | 114 | 0.68 | 0.30 | 0.03 | | 0.82 | 0.18 | |
| Prolonged | 84 | 0.69 | 0.26 | 0.05 | 0.65 | 0.82 | 0.36 | 0.94 |
| Spontaneous relapse of psychotic symptoms | | | | | | | | |
| + | 84 | 0.62 | 0.36 | 0.02 | | 0.80 | 0.20 | |
| − | 129 | 0.71 | 0.24 | 0.05 | 0.15 | 0.83 | 0.17 | 0.35 |
| Poly-substance abuse | | | | | | | | |
| + | 158 | 0.69 | 0.28 | 0.03 | | 0.83 | 0.17 | |
| − | 63 | 0.68 | 0.27 | 0.05 | 0.85 | 0.82 | 0.18 | 0.77 |
| **SNP2 (rs130058)** | **N** | **Genotype (%)** | | | *p* | **Allele (%)** | | *p* |
| | | T/T | T/A | A/A | | T | A | |
| Age at first use | | | | | | | | |
| 20y <= | 111 | 0.86 | 0.14 | 0.00 | | 0.93 | 0.07 | |
| 19y >= | 114 | 0.92 | 0.08 | 0.00 | 0.31 | 0.96 | 0.04 | 0.33 |
| Latency of psychosis | | | | | | | | |
| 3y > | 103 | 0.91 | 0.09 | 0.00 | | 0.96 | 0.04 | |
| 3y <= | 84 | 0.90 | 0.10 | 0.00 | 0.85 | 0.95 | 0.05 | 0.86 |
| Prognosis of psychosis | | | | | | | | |
| Transient | 115 | 0.88 | 0.12 | 0.00 | | 0.94 | 0.06 | |
| Prolonged | 84 | 0.93 | 0.07 | 0.00 | 0.24 | 0.96 | 0.04 | 0.26 |
| Spontaneous relapse of psychotic symptoms | | | | | | | | |
| + | 85 | 0.92 | 0.08 | 0.00 | | 0.96 | 0.04 | |
| − | 129 | 0.87 | 0.13 | 0.00 | 0.26 | 0.93 | 0.07 | 0.28 |
| Poly-substance abuse | | | | | | | | |
| + | 159 | 0.90 | 0.10 | 0.00 | | 0.95 | 0.05 | |
| − | 63 | 0.87 | 0.13 | 0.00 | 0.57 | 0.94 | 0.06 | 0.58 |

**Table 3. contd....**

| SNP3 (rs1228814) | N | Genotype (%) | | | *p* | Allele (%) | | *p* |
|---|---|---|---|---|---|---|---|---|
| | | C/C | C/A | A/A | | C | A | |
| Age at first use | | | | | | | | |
| 20y <= | 110 | 0.72 | 0.25 | 0.04 | | 0.84 | 0.16 | |
| 19y >= | 111 | 0.68 | 0.32 | 0.00 | 0.14 | 0.84 | 0.16 | 0.47 |
| Latency of psychosis | | | | | | | | |
| 3y > | 100 | 0.76 | 0.22 | 0.02 | | 0.87 | 0.13 | |
| 3y <= | 83 | 0.66 | 0.33 | 0.01 | 0.27 | 0.83 | 0.17 | 0.23 |
| Prognosis of psychosis | | | | | | | | |
| Transient | 113 | 0.71 | 0.28 | 0.01 | | 0.85 | 0.15 | |
| Prolonged | 82 | 0.73 | 0.24 | 0.02 | 0.59 | 0.85 | 0.15 | 0.91 |
| Spontaneous relapse of psychotic symptoms | | | | | | | | |
| + | 81 | 0.73 | 0.27 | 0.00 | | 0.86 | 0.14 | |
| − | 129 | 0.69 | 0.28 | 0.03 | 0.27 | 0.83 | 0.17 | 0.34 |
| Poly-substance abuse | | | | | | | | |
| + | 156 | 0.70 | 0.28 | 0.02 | | 0.84 | 0.16 | |
| − | 62 | 0.69 | 0.29 | 0.02 | 0.98 | 0.84 | 0.16 | 0.98 |

Therefore, our negative findings may be significant and indicate that higher or lower density of the 5HT1b receptor due possession of -161A or -161T of *HTR1B* does not affect individual susceptibility to methamphetamine dependence and psychosis.

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**Current Topics**

# Translational Research in Neurodevelopmental Disorders: Development of Etiology-Based Animal Models

## Animal Models of Attention-Deficit/Hyperactivity Disorder

Yosefu ARIME, Yumiko KUBO, and Ichiro SORA*

*Department of Biological Psychiatry, Tohoku University Graduate School of Medicine; 1–1 Seiryo-machi, Sendai 980–8574, Japan.*

Received June 13, 2011

**Attention-deficit hyperactivity disorder (AD/HD) is a clinically heterogenous disorder including hyperactivity, impulsivity, and inattention. Both psychostimulant and non-psychostimulant drugs such as methylphenidate and atomoxetine, respectively, to modulate catecholeamine neurotransmission are used as current pharmacotherapies for AD/HD. Multiple lines of evidence suggest that genetic factors play major roles in the etiology of AD/HD. *meta*-Analyses and pooled data analyses have suggested associations between AD/HD and polymorphisms in genes encoding monoamine neurotransmission molecules. There has been considerable research on this disorder using genetic, pharmacological, and neuroimaging approaches, and several animal models of AD/HD such as spontaneously hypertensive rat (SHR), dopamine transporter (DAT) knockout mice, coloboma mutant mouse, and Grin1 mutant mouse have been reported. These animal models are valuable tools for investigating molecular, cellular, and behavioral mechanisms as well as the neural development and circuit mechanisms of AD/HD. Here, we review the recent literature on animal models of AD/HD and discuss their advantages and limitations.**

**Key words** dopamine; norepinephrine; transporter; spontaneously hypertensive rat; coloboma mutant mouse

### 1. INTRODUCTION

Attention-deficit hyperactivity disorder (AD/HD) is a clinically heterogenous and highly heritable disorder. AD/HD is defined by the core symptoms of hyperactivity, impulsivity, and impaired sustained attention.[1] According to the Diagnostic and Statistical Manual of Mental Disorders 4th Edition, Text Revision (DSM-IV-TR), there are three subtypes of AD/HD such as predominantly inattentive, predominantly hyperactive/impulsive, and combined type. Both psychostimulant drugs such as methylphenidate and D-amphetamine and non-psychostimulant medications such as atomoxetine are used as current pharmacotherapy for AD/HD.[2,3] To date, although various clinical studies such as molecular genetic studies and neuroimaging analyses have been reported, the etiology of AD/HD has yet to be revealed. Animal models can be useful to facilitate our understanding of this disorder because they are helpful in studying the behavioral consequences. In this review, we focus on current animal models of AD/HD.

### 2. HUMAN STUDIES OF AD/HD

Multiple lines of evidence suggest that genetic factors play major roles in the etiology of AD/HD; the heritability for this disorder was found 0.85—0.90.[4] AD/HD is among the most common neurodevelopmental disorders affecting 5—8% of children, and often persists into adolescence and adulthood.[5,6] Despite this high prevalence rate, it was found that the concordance rate of AD/HD is 81% in monozygotic twins, compared with 29% in dizygotic twins.[7] Moreover, the few genome-wide scans and candidate gene studies conducted so far are not conclusive.[8]

*meta*-Analyses and pooled data analyses have suggested associations between AD/HD and polymorphisms in the dopamine receptor (DRD4 and DRD5), dopamine transporter (DAT), synaptosomal-associated protein (SNAP-25; 25 kDa), and serotonin transporter.[9] Other gene variants also have been suggested to be associated with AD/HD such as genes that encode monoamine oxidase A, dopamine $\beta$-hydroxylase, norepinephrine transporter (NET), and $\alpha$2-adrenoceptor.[10—12] It is also thought that the gene-environment interactions exist in the etiology of AD/HD. The most consistent and robustly associated results are prenatal exposure to nicotine from smoking *in utero*.[13—15]

### 3. ANIMAL MODELS OF AD/HD

The diagnostic criteria of AD/HD like many other psychiatric disorders depend on behavior. Animal models of AD/HD are postulated to show phenomenological similarities, *i.e.* they should mimic the three core symptoms of hyperactivity, impulsivity, and impaired sustained attention (face validity: the procedure must mimic clinical symptomatology), they should be improved by treatment of therapeutic agents such as methylphenidate and atomoxetine (predictive validity: the procedure must be capable of predicting therapeutic effects in patients), and they should conform to a theoretical rationale for AD/HD such as genetic and neuropathological abnormalities (construct validity: the procedure must reproduce etiological factors of the disease).[16,17] Table 1 lists modification and behavioral characteristics of animal models of AD/HD introduced in this review.

**Spontaneously Hypertensive Rat (SHR)** Sponta-

* To whom correspondence should be addressed. e-mail: sora@med.tohoku.ac.jp

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Table 1. Modification and Behavioral Characteristics of Animal Models of AD/HD

| Subject | Modification | Hyperactivity | | | | | Impulsivity | Inattentiveness |
|---|---|---|---|---|---|---|---|---|
| | | In novel environment | In familiar environment | Decreased by therapeutics treatment | | | | |
| | | | | MPD | AMP | ATX | | |
| AD/HD (human) | | No | Yes | Yes | Yes | Yes | Yes | Yes |
| SHR | Bred for hypertension | No[51,52] | Yes[52] | Yes[27] | Yes[26] | Yes[53] | Yes[18] | Yes[18] |
| DAT KO | Knockout of the DAT gene | Yes[54] | Yes[54] | Yes[36] | Yes[36] | Yes[a] | Yes[a] | ND |
| Coloboma mutant | Mutation on the SNAP-25 gene, *etc.* | Yes[40,41] | Yes[40,55] | No[41] | Yes[41] | Yes[56] | Yes[55] | ND |
| Grin1 mutant | Mutation on the Grin1 gene | Yes[44] | Yes[44] | Yes[44] | ND | ND | ND | ND |

AD/HD, attention-deficit/hyperactivity disorder; SHR, spontaneously hypertensive rat; DAT KO, dopamine transporter knockout mouse; MPD, methylphenidate; AMP, amphetamine; ATX, atomoxetine; ND, not determined; *a*) our unpublished data.

neously hypertensive rat (SHR) is the most widely studied animal model for AD/HD and was developed by inbreeding rats of the Wistar-Kyoto (WKY) strain. SHR shows several major AD/HD-like symptoms such as impulsivity, hyperactivity, and poor sustained attention compared with WKY. This impulsivity develops over time and is seen as inability to inhibit a response during the extinction phase of an operant task as well as inability to delay a response so as to obtain a larger reward.[18,19] The hyperactivity shown in this model is not present in a novel environment and non-threatening conditions and also develops over time when reinforcers are infrequent.[18,20] In addition to behavioral alterations, SHR exhibits impaired dopamine release in the prefrontal cortex, nucleus accumbens systems and striatum.[21—23] Not only dopamine systems but also norepinephrine seem altered such as disturbed norepinephrine transmission. SHR shows enhanced norepinephrine release by glutamate compared with WKY but normal release by electrical stimulus.[24,25] It was reported that the hyperactivity was ameliorated by treatment of methylphenidate and D-amphetamine[26,27] and this phenomenon is associated with abnormalities in DAT.[28] The $\alpha$-adrenoceptor agonists clonidine and guanfacine have proven to be efficacious in the treatment of AD/HD.[29,30] In this model rat, the selective $\alpha$2A-adrenoceptor agonist guanfacine improves hyperactivity, impulsivity, and impaired sustained attention.[31]

Although SHR satisfies a lot of AD/HD validation criteria it also shows high blood pressure. Hypertension in non-medicated patients with AD/HD has not been reported. To separate the hyperactivity from hypertention, WKHA rat was developed by crossbreeding SHR and WKY.[32] The WKHA rat is hyperactive and hypersensitive to stress but not hypertensive. However, methylphenidate does not decrease hyperactivity in this rat, but rather increased locomotion.[33] Thus WKHA rat has face validity but does not have predictive validity as an animal model of AD/HD. Hypertension in SHR develops in adulthood and prepubertal SHR (4—5 weeks old) does not show hypertension but does show hyperactivity. Therefore it would be appear that the SHR fulfills many aspects of validity of animal models of AD/HD especially in children.

**Dopamine Transporter Knockout Mice** DAT knockout (KO) mice lack the DAT gene, which is responsible for clearance of released extracellular dopamine[34]; in these mice extracellular dopamine levels both in the nucleus accumbens and striatum are about 10 times higher than wild-type levels.[35] DAT is a major target for methylphenidate and amphetamine; DAT-1 gene is associated with AD/HD.[9] DAT KO mice show hyperlocomotion in novel environment[34] and impaired learning and memory.[36,37] The predictive validity of DAT KO mice includes the findings that methylphenidate and amphetamine are effective in reducing hyperactivity.[36] Whether other therapeutic agents such as selective norepinephrine reuptake inhibitor atomoxetine and $\alpha$2A-adrenoceptor agonist guanfacine reduce hyperactivity in this model remains unclear.

There are limitations in the DAT KO mice for assessing AD/HD-like symptoms and pathology. As described above, DAT KO mice show extremely elevated dopamine levels in the striatum and nucleus accumbens whereas there is no evidence for hyperdopaminergic tone in AD/HD patients. Moreover, pharmacological approaches by psychostimulants such as methylphenidate and amphetamine have some disadvantages in this model because this mouse does not have DAT protein, which is a primary target of these drugs. Recent study using manganese-enhanced magnetic resonance imaging (MEMRI) suggested that DAT KO mice have alterations in mesocortical circuitry that originate from the prefrontal cortex to subcortical regions.[38] Therefore the mechanism of amelioration of hyperactivity in DAT KO mice is not simply the result of elevated dopamine functions in the striatum and nucleus accumbens.

**Coloboma Mutant Mouse** The coloboma mutant mouse was developed as a product of neutron irradiation.[39] The coloboma mouse exhibits profound spontaneous locomotor hyperactivity that was remarkably reduced by amphetamine.[40,41] This mouse has mutations in the gene encoding SNAP-25 and phospholipase C beta-1.[42] However, methylphenidate does not decrease hyperactivity in this mouse, but rather increases locomotion.[41] Since the hyperactivity in coloboma mutant mouse is rescued by overexpression of SNAP-25, low levels of SNAP-25 expression appear to be related with this hyperactivity. The SNAP-25 protein is essential for docking and fusion of synaptic vesicles necessary for synaptic transmission and several findings suggest possible association between AD/HD and SNAP-25.[9] However, coloboma mutant mice have delayed attachment of the lens and microphthalmia.[43] Therefore data of behavioral tests using this mouse must be carefully interpreted.

**Grin1 Mutant Mouse** Many existing genetic animal models were created by deletion or overexpression of genes implicated in psychiatric disorders including AD/HD. How-

ever, in many patients psychiatric disorders do not result from a complete absence or duplication/triplication of a gene. Further research progress in the field of animal models, which have nonsense mutation and missense mutation and have better construct validity, is expected. Recently, mice having a missense mutation R844C in the Grin1 gene, which encodes *N*-methyl-D-aspartate (NMDA) receptor subunit 1, were generated in the RIKEN large-scale *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis project. These mice show increased spontaneous locomotor activity that was ameliolated by methylphenidate treatment.[44] However, it is unclear whether the Grin1 mutation associates with AD/HD and Grin1 mutant mice have predictive validity such as responses to amphetamine and atomoxetine.

## 4. CONCLUSION

Recently, multiple lines of evidence support that structural abnormalities exist in AD/HD patients. A large number of studies have reported reduced brain volume in patients with AD/HD, particularly the prefrontal cortex, anterior cingulate cortex, basal ganglia, and cerebellum.[45,46] Moreover, cortical development in children with AD/HD, particularly in the lateral prefrontal cortex, lagged behind that of typically developing children by several years[47] and adults with AD/HD had thinner cortices in inferior parietal lobe and lateral prefrontal and anterior cingulate cortices.[48] Dopamine plays roles in attention, working memory, and reward processes in the central nervous system.[49] Recent neuroimaging study using positron emission tomography (PET) reported that D2/D3 receptor availability in left caudate was lower in adult AD/HD patients than in healthy controls and blunted dopamine increases by methylphenidate in caudate were associated with symptoms of inattention.[50]

Animal models are valuable tools for investigating molecular, cellular, and behavioral mechanisms as well as the neural development and circuit mechanisms of child-onset psychiatric disorders such as AD/HD. We think that one future direction is to focus on neural development and circuits by the re-evaluating above-described animal models of AD/HD using GENSAT BAC transgenic mouse line expressing green fluorescent protein (GFP) and neural tracers such as biocytin, fluorogold, and carbocyanine dyes to provide new insights to identify the etiology of this disorder and potential targets for development of new treatments.

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# 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

Shiho Arakawa [a], Yukihiko Shirayama [b,*], Yuko Fujita [b], Tamaki Ishima [b], Mao Horio [b], Katsumasa Muneoka [a], Masaomi Iyo [a], Kenji Hashimoto [b]

[a] Department of Psychiatry, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chiba 260-8670, Japan
[b] Division of Clinical Neuroscience, Chiba University Center for Forensic Mental Health, 1-8-1 Inohana, Chiba 260-8670, Japan

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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.

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## 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

* Corresponding author at: Department of Psychiatry, Teikyo University Chiba Medical Center, 3426-3 Anesaki, Ichihara 299-0111, Japan. Tel.: +81 436 62 1211; fax: +81 436 62 1511.
*E-mail address:* shirayama@rapid.ocn.ne.jp (Y. Shirayama).

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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).

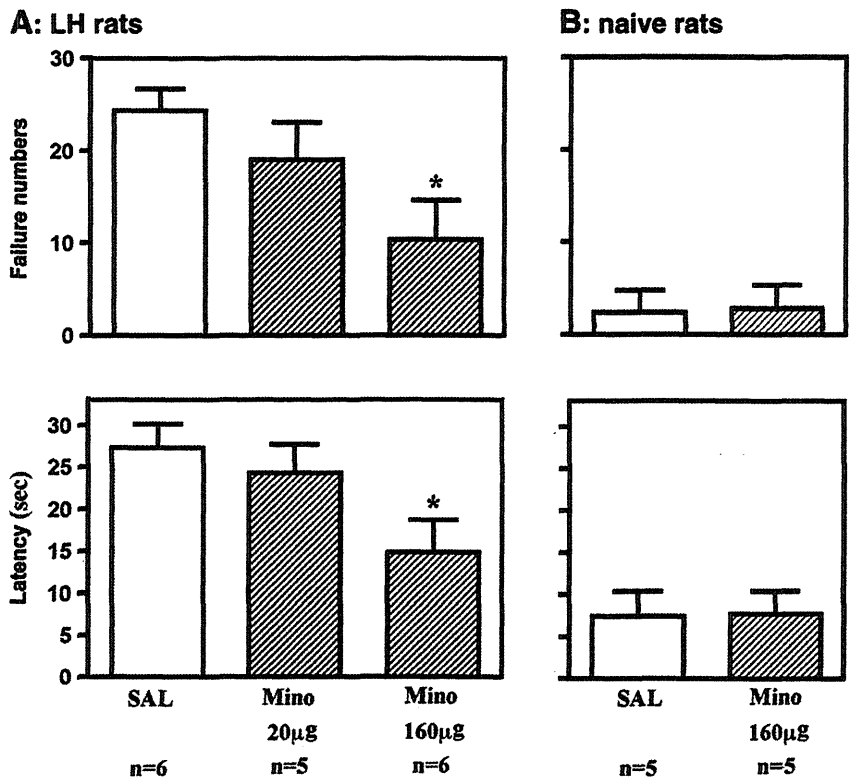


**Fig. 1.** Minocycline decreased escape failure in the LH paradigm. Minocycline (Mino) or saline (SAL) was administered via bilateral infusion into the cerebral ventricle, and animals were subjected to a conditioned avoidance test 4 days later. Escape failure and latency to escape were determined. The 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.

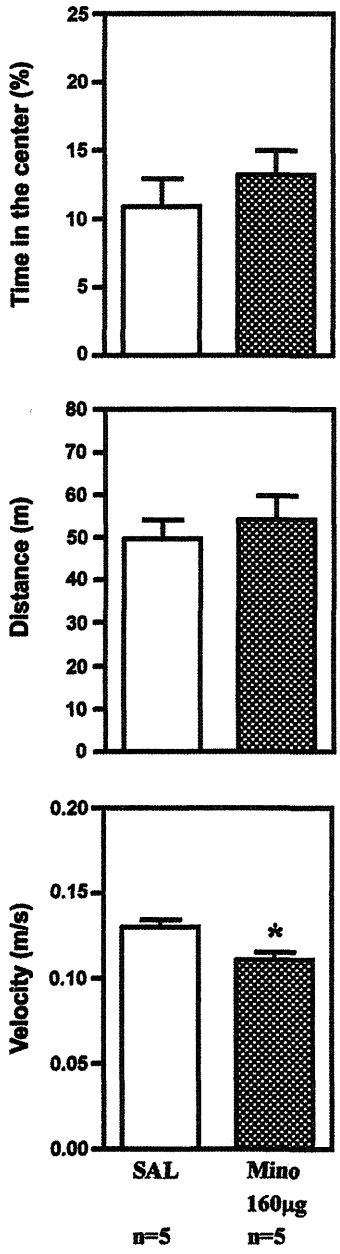


**Fig. 2.** Effects of minocycline infusion into the cerebral ventricle of naïve rats on locomotor activity. Minocycline (Mino) or saline (SAL) was administered via bilateral infusion into the cerebral ventricle, and 4 days later, the time spent in the center, distance traveled, and velocity in an open 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.

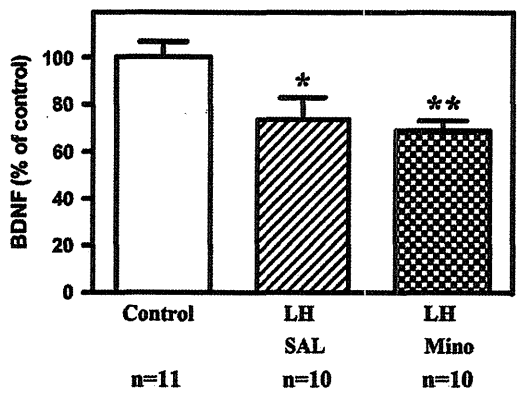


**Fig. 3.** Effects of minocycline on the BDNF expression in the hippocampus of LH rats. Minocycline (Mino) or saline (SAL) was administered via bilateral infusion into the cerebral ventricle of LH rats, and 4 days later, BDNF expression was examined. BDNF level (% control) are indicated as mean±SEM. 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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# Associations of serum brain-derived neurotrophic factor with cognitive impairments and negative symptoms in schizophrenia

Tomihisa Niitsu [a], Yukihiko Shirayama [a,b,*], Daisuke Matsuzawa [c], Tadashi Hasegawa [a], Nobuhisa Kanahara [a], Tasuku Hashimoto [a], Tetsuya Shiraishi [a], Akihiro Shiina [a], Goro Fukarni [a], Mihisa Fujisaki [a], Hiroyuki Watanabe [a], Michiko Nakazato [a], Makoto Asano [d], Sho Kimura [e], Kenji Hashimoto [f], Masaomi Iyo [a]

[a] *Department of Psychiatry, Chiba University Graduate School of Medicine, Chiba, Japan*
[b] *Department of Psychiatry, Teikyo University Chiba Medical Center, Chiba, Japan*
[c] *Department of Cognitive Behavioral Physiology, Chiba University Graduate School of Medicine, Chiba, Japan*
[d] *Chiba Psychiatric Medical Center, Chiba, Japan*
[e] *Kimura Hospital, Chiba, Japan*
[f] *Division of Clinical Neuroscience, Chiba University Center for Forensic Mental Health, Chiba, Japan*

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ABSTRACT

Brain-derived neurotrophic factor (BDNF) may be involved in the pathophysiology of schizophrenia. The aim of this study was to examine the associations of serum BDNF levels with the cognition and clinical characteristics in patients with schizophrenia. Sixty-three patients with schizophrenia and 52 age- and sex-matched healthy controls were examined with neuropsychological tests. Serum BDNF levels were determined by enzyme-linked immunosorbent assay (ELISA). There were no significant differences in serum BDNF levels between normal controls and patients with schizophrenia. Serum BDNF levels of normal controls showed negative correlations with verbal working memory, but this was not the case with schizophrenic patients. Meanwhile, serum BDNF levels of schizophrenic patients showed positive correlations with the scores of the Scale for the Assessment of Negative Symptoms (SANS) and the Information subtest scores of Wechsler Adult Intelligence Scale Revised (WAIS-R). Serum BDNF levels are related with the impairment of verbal working memory and negative symptoms in patients with schizophrenia.

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## 1. Introduction

Schizophrenia is characterized by three distinct symptom clusters: positive symptoms, negative symptoms, and cognitive impairments. Negative symptoms, an important and enduring component of the psychopathology of schizophrenia (Stahl and Buckley, 2007), include blunted affect, alogia, asociality, anhedonia and avolition (Andreasen, 1982; Kirkpatrick et al., 2006). Negative symptoms predict quality of life, social functioning and overall outcome measures in patients with schizophrenia (Bow-Thomas et al., 1999; Dickerson et al., 1999; Milev et al., 2005). Negative symptoms and cognitive impairments are involved in the prefrontal cortex (Ingvar and Franzen, 1974; Weinberger, 1988) and share many features, but are separable domains of illness (Harvey et al., 2006). While positive symptoms are greatly improved with atypical antipsychotic medication, negative symptoms and cognitive impairments are not sufficiently improved (Erhart et al., 2006; Keefe et al., 2007).

The cognitive impairments are the core features of schizophrenia, with both working memory and attention being characteristically impaired in patients with schizophrenia (Elvevag and Goldberg, 2000; Reichenberg, 2010). Cognitive deficits are related to community outcome, social problem solving and skill acquisition (Green, 1996), and therefore might predict the functional outcome in schizophrenic patients (Green et al., 2004).

Accumulating evidence suggests that brain-derived neurotrophic factor (BDNF) plays a role in the pathophysiology of psychiatric diseases, including depression and schizophrenia (Angelucci et al., 2005). It is well documented that BDNF is involved in neuronal survival, differentiation and outgrowth during brain development (Numakawa et al., 2010). Recently, a meta-analysis study showed that blood levels of BDNF were reduced in medicated and drug-naïve patients with schizophrenia (Green et al., 2010). However, the significant heterogeneity across the study results remained unexplained.

*Abbreviations:* BDNF, Brain-derived neurotrophic factor; BMI, Body-mass index; BPRS, Brief psychiatry rating scale; DIEPSS, Drug induced extrapyramidal symptoms scale; DSDT, Digit span distraction test; ELISA, Enzyme-linked immunosorbent assay; IQ, Intelligence quotient; PANSS, Positive and negative syndrome scale; SANS, Scale for the assessment of negative symptoms; WAIS-R, Wechsler adult intelligence scale revised; WCST, Wisconsin card sorting test.

* Corresponding author at: Department of Psychiatry, Teikyo University Chiba Medical Center, 3426-3 Anesaki, Ichihara 299-0111, Japan. Tel.: +81 436 62 1211; fax: +81 436 62 1511.
*E-mail address:* shirayama@rapid.ocn.ne.jp (Y. Shirayama).

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In this study, we examined the associations of serum BDNF levels with negative symptoms and cognitive impairments in patients with schizophrenia. To assess the cognitive functioning of the prefrontal cortex, 5 neuropsychological tests, verbal fluency, Wisconsin card sorting test (WCST), Stroop test, digit span distraction test (DSDT), and trail making test were administered. The rationale for choosing these tests stems from the hypothesis that each test works on a region-dominant part (medial or dorsolateral portions) of the brain and could examine the region-related functions.

## 2. Methods

### 2.1. Subjects

Sixty-three Japanese patients with schizophrenia (age: mean, 35.9 [SD, 8.2]; education: mean, 13.8 [SD, 2.3]; 26 men and 37 women) were recruited from the outpatients of the Chiba University Hospital and its affiliated hospitals, Chiba, Japan. Fifty-two age- and sex-matched healthy Japanese subjects also participated in this study as normal controls. Characteristics of the subjects are shown in Table 1. All subjects provided written informed consent for participation in the study after the procedure had been fully explained. The ethics committee of Chiba University Graduate School of Medicine approved the present study.

All patients were diagnosed according to the DSM-IV criteria for schizophrenia, and had no other psychiatric disorders, assessed by two senior level psychiatrists. Of the patients, 44 were diagnosed as the residual type and 19 were the paranoid type. They had been clinically stable for at least 3 months. All patients had been receiving monotherapy with a stable dose of a second-generation antipsychotic drug for at least 8 weeks prior to entry into the study. The antipsychotic drugs were risperidone (n = 25), olanzapine (n = 18), quetiapine (n = 8), perospirone (n = 2), aripiprazole (n = 9), and bronanserine (n = 1). The chlorpromazine-equivalent dose was 306 ± 240 (means ± SD) mg/day (Woods, 2003). Normal controls were recruited from the local community around the Chiba University Hospital. None of the normal controls presented with a personal history of psychiatric or neurological disorder, assessed by two senior level psychiatrists.

### 2.2. Clinical assessments

Clinical symptoms were assessed by using the Brief Psychiatry Rating Scale (BPRS) (Overall and Gorham, 1962) and the Scale for the Assessment of Negative Symptoms (SANS) (Andreasen, 1982). Drug-induced extrapyramidal symptoms were evaluated by using the Drug Induced Extrapyramidal Symptoms Scale (DIEPSS), because cognitive functions are influenced by extrapyramidal motor side effects (Inada et al., 2002). Intelligence quotient (IQ) scores were estimated by using the short version of the Japanese Wechsler Adult Intelligence Scale Revised (WAIS-R) (Misawa et al., 1993; Nakamura et al., 2000), which consisted of the Information, Digit Span, and the Picture Completion subtests. Age at onset, duration of illness and duration of untreated psychosis were evaluated.

### 2.3. Enzyme immunoassay

Blood samples of the participants were collected between 10:00 and 13:00 h. Serum was then separated by centrifugation at 3000 rpm for 7 min and stored at −80 °C until assay. Serum BDNF levels were measured by using a BDNF Emax Immunoassay System kit (Promega, Madison, WI).

### 2.4. Neuropsychological assessments

In the Verbal Fluency Test (letter, category), the number of words produced in 1 min for each trial was recorded for evaluation (Sumiyoshi et al., 2005). In the WCST, the number of achieved categories and perseverative errors were assessed (Shad et al., 2006). We used the short version of the WCST (Keio version; 48 cards) to shorten the procedural time (Hori et al., 2006; Igarashi et al., 2002). In the Trail Making Test Part A and Part B, the time taken to complete each part of the test was assessed in seconds (Reitan and Wolfson, 1993). In the Stroop Test, a list of 24 colored dots (Part D) and 24 colored words incongruent with the color (Part C) were used (Carter et al., 1995; Chan et al., 2004). The reaction time taken to complete each part of the test was assessed in seconds. In the DSDT, subjects were asked to remember a tape-recorded string of digits read by a female voice while ignoring the digits read by a male voice (distractor) (Green et al., 1997; Oltmanns and Neale, 1975). The percentages of digits correctly recalled under the condition with and without a distractor were assessed separately.

### 2.5. Statistical analysis

All statistical analyses were performed by using SPSS software (SPSS version 18.0J; SPSS, Tokyo, Japan). For the comparisons

**Table 1**
Demographic characteristics and serum BDNF levels of subjects.

| | Controls | Patients | | | | Controls vs patients |
|---|---|---|---|---|---|---|
| | n = 52 | n = 63 | Subtype | | Residual vs paranoid | |
| | | | Residual | Paranoid | | |
| | | | n = 44 | n = 19 | p | p |
| Gender (male/female) | 25/27 | 26/37 | 19/25 | 7/12 | NS [a] | NS [a] |
| Age, year | 34.9 (7.3) | 35.9 (8.2) | 36.7 (8.3) | 34.1 (8.1) | NS [b] | NS [c] |
| Education, year | 14.7 (2.7) | 13.8 (2.3) | 13.8 (2.4) | 13.7 (2.1) | NS [b] | NS [b] |
| Smoking (Non-smoker/smoker) | 43/9 | 45/18 | 33/11 | 12/7 | NS [a] | NS [a] |
| Age at onset of illness, year | – | 26.8 (7.0) | 27.5 (7.3) | 25.2 (6.1) | NS [c] | – |
| Duration of illness, year | – | 9.1 (7.3) | 9.2 (6.8) | 9.0 (8.6) | NS [b] | – |
| Duration of untreated psychosis, month | – | 8.1 (13.4) | 7.3 (9.6) | 9.8 (19.9) | NS [b] | – |
| BPRS | – | 25.5 (7.5) | 23.7 (7.1) | 29.6 (6.9) | < 0.05 [b] | – |
| SANS | – | 70.4 (11.8) | 68.1 (12.0) | 75.7 (9.5) | < 0.05 [b] | – |
| DIEPSS | – | 2.7 (2.7) | 2.5 (2.5) | 3.3 (3.3) | NS [b] | – |
| BDNF, ng/ml | 14.6 (4.4) | 15.3 (3.8) | 14.9 (3.6) | 16.2 (4.2) | NS [c] | NS [b] |

Values represent mean (SD). NS, not significant.
Abbreviation: BPRS, Brief Psychiatric Rating Scale; SANS, Scale for the Assessment of Negative Symptoms; DIEPSS, Drug Induced Extra-Pyramidal Symptoms Scale; BDNF, Brain-Derived Neurotrophic Factor.
[a] $\chi^2$ test.
[b] Mann–Whitney *U*-test.
[c] Student's *t*-test.