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Behavioural Pharmacology

Impaired spatial working memory and decreased frontal cortex BDNF protein level in dopamine transporter knockout mice

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ABSTRACT

Brain-derived neurotrophic factor (BDNF), one of the key brain neurotrophins, has been implicated in neuronal plasticity and memory. Recent studies document the importance of BDNF for normal long-term memory functions. However, there are few studies of the roles of BDNF in short-term memory. Dopamine is likely to play important roles in BDNF gene expression in specific brain regions, including frontal cortical regions that are implicated in short-term working memory processes that include spontaneous alternation. We have thus tested spatial working memory in dopamine transporter knockout (DAT KO) and wild-type mice. Spontaneous alternation in the Y-maze, an index of short-term spatial working memory in mice, was significantly decreased in DAT KO mice compared to wild-type mice. BDNF protein was significantly decreased in frontal cortex, though not in striatum or hippocampus, of the DAT KO mice. The data support the hypothesis that impaired spatial working memory in DAT KO mice may be related to decreased frontal cortical BDNF in these animals, and document apparent roles for BDNF in a short-term memory process.

1. Introduction

Brain-derived neurotrophic factor (BDNF) is a small dimeric protein that is widely expressed in the adult mammalian brain (Murer et al., 2001). There is abundant evidence that BDNF is involved in synaptic plasticity and memory processes (Poo, 2001; Tyler et al., 2002), particularly as BDNF relates to hippocampal dependent memory. BDNF has been suggested to be essential for normal persistence of long-term memory storage (Bekinschtein et al., 2008) and endogenous BDNF is required for long-term memory formation in the rat parietal cortex (Alonso et al., 2005). Lower levels of frontal cortical BDNF have been associated with impaired working memory performance in Ts65Dn mice, which are considered to be an animal model of Down's syndrome (Bimonte-Nelson et al., 2003). Reducing BDNF expression through intracerebroventricular infusion of BDNF antisense impairs performance in radial arm maze tests (Mizuno et al., 2000). BDNF has been implicated in long-term potentiation, an electrophysiological concomitant memory acquisition (Korte et al., 1998; Lessmann, 1998).

There is less data concerning the effects of BDNF on spatial working memory, although BDNF has been closely related to dopamine pathways and implicated in dopaminergic function (Berton et al., 2006; Fumagalli et al., 2003; Li et al., 2006; Li et al., 2007a,b). Lesions and other manipulations of mesocortical dopamine pathways can change performance in spontaneous alternation paradigms (Pioli et al., 2008) and alter BDNF gene expression in frontal cortical regions (Fumagalli et al., 2003). Dopamine has also been shown to directly regulate BDNF expression in striatal cells *in vitro* (Küppers and Beyer, 2001). These data therefore collectively implicate a dopamine mediated regulation of BDNF in prefrontal cortex dependent memory function.

We have produced and extensively characterized a line of DAT KO mice that display hyperlocomotion (Sora et al., 1998, 2001, 2009) as well as increased extracellular dopamine levels (Shen et al., 2004). These and other lines of DAT KO mice display altered performance in 8-arm radial maze testing, reduced prepulse inhibition and increased prefrontal cortical BDNF levels (Gainetdinov et al., 1999; Yamashita et al., 2006; Fumagalli et al., 2003). Each of these results suggests that DAT KO mice might also display alterations in spatial working memory due to increased dopaminergic tone, perhaps mediated by alterations in BDNF function. We thus now report results of spatial working memory Y-maze testing and evaluation of BDNF expression in DAT KO mice. We discuss ways in which this data, taken together, is consistent with the idea that direct and indirect effects of this knockout, including the altered BDNF expression, could contribute to

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the altered performance in this index of short-term memory function (Krejcova et al., 2004).

2. Materials and methods

2.1. Animals

DAT KO mice were produced as described (Sora et al, 2001), bred at the Animal Laboratory Institute of Tohoku University Graduate School of Medicine and maintained on a mixed genetic background combining C57BL/6 and 129Sv/J mouse strains. Offspring from heterozygote crosses were weaned at 28 days postnatal and housed in groups of two to five (segregated by sex), in an animal room maintained under a 12 h/12 h light/dark cycle with lights on from 8:00. Food and water were available *ad libitum*. Mice were genotyped using multiplex polymerase chain reaction methods on DNA extracted from tail biopsies, as previously described (Shen et al, 2004). Behavioral testing was conducted in 8–11 week old mice. All animal experiments were performed in accordance with the Guidelines for the Care of Laboratory Animals of Tohoku University Graduate School of Medicine.

2.2. Y-maze test

The Y-maze consisted of 3 arms (14×4.5×40 cm). Each mouse was placed at the end of one arm and allowed to move freely through the maze during an 8-min session. The total number of arm entries (locomotor activity) and alternation behavior were recorded using a video camera. The percentage of alternation was calculated as (total of alternation/total arm entries − 2). This measure is considered to reflect short-term memory in mice (Mamiya and Ukai, 2001). Additionally, the number of total arm entries was calculated as an index of locomotor activity (Ma et al., 2007).

2.3. ELISA for measuring BDNF protein concentration

Animals were sacrificed by decapitation. The brains were quickly removed and dissected on ice. Samples taken from the frontal cortex, caudate putamen and hippocampus were frozen at −80 °C before homogenization. Brain samples were diluted (hippocampus, 1:30; frontal cortex and striatum, 1:20) and homogenized in a lysis buffer (137 mM NaCl, 20 mM TRIS, 1% NP40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.5 mM sodium vanadate. The homogenates were centrifuged at 10,000 g for 20 min, and the supernatants were collected and processed for quantification of BDNF by ELISA using a BDNF Emax Immuno Assay kit (Promega, Madison, Wis., USA) according to the manufacturer's instructions (Schaaf et al., 1998) and carried out as described previously (Li et al., 2006, 2007a; Amano et al., 2007). Nunc Maxisorp 96-well immunoplates were coated with 100 μl/well of anti-BDNF monoclonal antibody (mAb) and incubated overnight at 4 °C. The plates were incubated in a block and sample buffer at room temperature for 1 h. Then, the samples were added to the coated wells (100 μl) and shaken for 2 h at room temperature. Following this the plates were incubated with an anti-human BDNF polyclonal antibody (pAb) for 2 h at room temperature with shaking and then incubated with an anti-IgY antibody conjugated to horseradish peroxidase for 1 h at room temperature. The plates were then incubated with tetramethylbenzidine solution for 15 min and 1 M hydrochloric acid was added to the wells. The colorimetric reaction product was measured at 450 nm. BDNF standards ranging from 7.8 to 500 pg/ml were used for quantification. Standard curves were plotted for each plate (correlation coefficient; $r=0.99$). Detection limit was 15.6 pg/ml, and cross-reactivity with other related neurotrophic factors was less than 3%.

2.4. Statistical analysis

The significance of the data was analyzed using unpaired *T*-tests. $P<0.05$ was considered significant.

3. Results

3.1. Behavioral results in the Y-maze test

Fig. 1 shows spontaneous alternation in Y-maze testing of homozygous DAT KO and wild-type littermate mice. Spontaneous alternation was decreased in DAT KO mice compared to wild-type mice ($P<0.05$). Despite these differences, there were no significant differences in the number of total arm entries between DAT KO mice and wild-type mice.

3.2. Changes of BDNF protein

Fig. 2 shows changes of BDNF protein in DAT KO mice compared to wild-type littermates. The concentration of BDNF protein was significantly decreased, by approximately 50%, in the frontal cortex of DAT KO mice compared to wild-type mice ($P<0.05$). However, there were no significant changes in BDNF level in the caudate putamen or hippocampus of DAT KO mice.

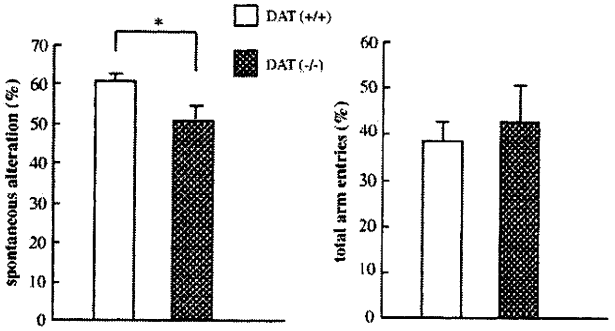

Fig. 1. Comparison of spontaneous alternation between DAT (+/+) and DAT (−/−) mice in Y-maze test. Each mouse was placed at the end of one arm and allowed to move freely through the maze during an 8-min session. Spontaneous alternation but not number of total arm entries was decreased in DAT (−/−) mice compared to DAT (+/+) mice ($P<0.05$). Columns represent the mean ± S.E.M., $n=10$–11, *$P<0.05$, unpaired *T*-test.

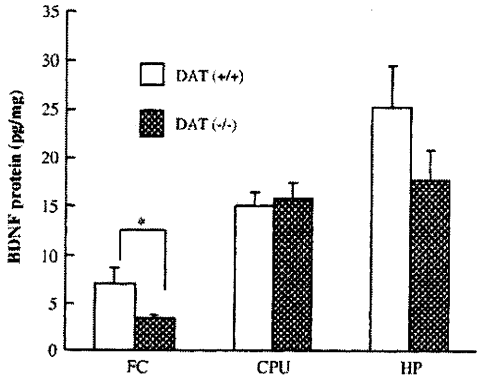

Fig. 2. Changes of BDNF protein in DAT (+/+) and DAT (−/−) mice. BDNF level was significantly decreased in frontal cortex of DAT (−/−) mice compared to DAT (+/+) mice ($P<0.05$). However, there were no changes in caudate putamen and hippocampus in DAT (−/−) mice. FC, frontal cortex; CPU, caudate putamen; HP, hippocampus. Columns represent the mean ± S.E.M., $n=8$, *$P<0.05$, unpaired *T*-test.

4. Discussion

Observation of decreased Y-maze spontaneous alternation in DAT KO mice is potentially consistent with the idea that these animals have impaired working memory function. Since the number of total arm entries was not significantly different between the two genotypes, confounding influences of locomotor hyperactivity in DAT KO mice seems unlikely. Documentation of significantly decreased frontal cortical BDNF in the DAT KO mice provides a plausible potential mechanism for impairments in spontaneous alternation.

However, although it would seem most parsimonious to attribute differences in performance in DAT KO mice to differences in working memory function, it is possible that other behavioral changes primarily underlie these differences. Although prefrontal cortex lesions impair spontaneous alternation (Mogensen and Divac, 1993), manipulations of several other brain regions also affect spontaneous alternation (Lalonde, 2002), and performance in spontaneous alternation tests is open to attentional and motivational confounds (Hughes, 2004). Attentional impairments have already been noted in DAT KO mice (Yamashita et al., 2006) as well as differences in motivational function (Hironaka et al., 2004). In particular the latter study demonstrated normal operant responses for food under many conditions, but impaired extinction behavior. One way to interpret such changes is as a perseverative response. Similar perseveration of dominant or initial response tendencies has been suggested to underlie alterations in DAT KO behavior in the forced swim test (Perona et al., 2008), and delayed acquisition of the Morris Water Maze (Hall, Sora, and Uhl, unpublished observations). Perseverative behavior has been associated with dopamine function and is enhanced by amphetamine in a manner that is dependent on the baseline probability of a particular response (Evenden and Robbins, 1983). Furthermore, locomotor sensitization induced by repeated DA agonist administration is also associated with reduced spontaneous alternation (Einat and Szechtman, 1995). This circumstance might be considered to apply also to DAT KO mice that have enhanced extracellular dopamine function in the striatum and nucleus accumbens (Shen et al., 2004), although it must be considered to what extent these differences are not just mediated by differences in striatal dopamine function, but altered balance of dopamine function in the nucleus accumbens and prefrontal cortex.

There is substantial support for the idea that intact prefrontal dopamine function is important for certain mnemonic functions. Blocking dopaminergic transmission in rat the mediofrontal cortex degrades spatial choice performance in Y-maze testing (Kozlov et al., 2001), and the prefrontal cortex has been postulated to play key roles in short-term memory (Goldman-Rakic, 1996; Kesner and Rogers, 2004). Dopamine agonists can improve short-term spatial memory in human volunteers (Mehta et al., 2001), in ways that are postulated to involve frontal cortex (Egan et al, 2002). These postulated prefrontal mnemonic roles thus add to traditional roles for dopamine transmission in the prefrontal cortex that include influences on higher motor functions, motivation, and cognition (Egan and Weinberger, 1997; Lewis et al., 1998; Yang et al., 1999).

At least some of these dopaminergic effects may involve D_1 receptors. In the prefrontal cortex of rodents and monkeys, both the amount of receptor mRNA and the number of receptor-binding sites are significantly greater for the dopamine D_1 receptor than for the other dopamine receptor subtypes (Lidow et al., 1991; Gaspar et al., 1995; Goldman-Rakic et al., 1992). Disrupting dopamine transmission in the prefrontal cortex caused by infusions of dopamine D_1 receptor antagonists or by excitotoxic lesions impairs working memory in nonhuman primates (Sawaguchi and Goldman-Rakic, 1991, 1994). Although extracellular dopamine levels in the prefrontal cortex are not affected by DAT knockout (Shen et al., 2004), further studies are needed to determine whether there are postsynaptic differences in dopaminergic function, and in particular whether differences in D_1 receptor function might contribute to differences in spontaneous alternation in DAT knockout mice that are reported here.

There is also substantial support for dopamine effects on BDNF. Dopaminergic agonists can regulate BDNF mRNA and protein levels (Küppers and Beyer, 2001). BDNF mRNA expression is also reduced in the frontal cortex of another line of DAT KO mice (Fumagalli et al., 2003). Influences in the opposite direction may be more modest; Chourbaji et al. (2004) reported that tissue content of dopamine was unchanged in the frontal cortex of BDNF heterozygous mice. These data, combined with our current results, suggest that dopamine could contribute to reduced synaptic formation and impaired spatial working memory, in part, through reductions in neurotrophin expression. Associations between lower frontal cortical BDNF protein levels and impaired working memory in Ts65Dn Down's syndrome mice are also consistent with this idea (Bimonte-Nelson et al., 2003). However, this study also showed that the effect of BDNF on working memory is maybe related to cholinergic degeneration. However, whether impaired spatial working memory in DAT KO mice is related to cholinergic degeneration needs further study. Another study revealed that performance in the complex maze was better in wild-type than APP23 animals. This difference is maybe related to decreased hippocampal BDNF levels on training in APP23 animals (Hellweg et al., 2006). This brain region differs from ours (frontal cortex). However it still confirms that a change of BDNF level plays a role in maze behavior like in our study.

Our current data found that BDNF levels were reduced by approximately 50% in the frontal cortex of our DAT KO mice, extending results obtained in other strains of DAT KO mice (Fumagalli et al., 2003). While extracellular dopamine levels in the frontal cortex of DAT KO mice are similar to those of wild-type mice (Shen et al., 2004), their frontal cortical dopamine content is approximately 50% of wild-type levels (Sora et al., 2001) which may indicate changes in synaptic content that may relate directly to both differences in BDNF levels and spontaneous alteration.

While the findings that spatial working memory deficits and frontal cortical BDNF deficits in DAT KO mice are consistent with the possibility that these two observations are linked, there is no direct evidence for such a linkage. The hippocampus, for example, expresses abundant BDNF (Li et al., 2006) and is closely tied to spatial working memory (Luine et al., 1994). Conceivably, the trend toward decreased BDNF in this region might contribute to the behavioral observations made here. It is possible that the changes in dopamine alone, by affecting working memory or some other function as discussed above, may make large contributions to the behavioral phenotype in ways that make the BDNF findings coincidental. However, it seems unlikely that the robust changes in BDNF levels that are observed here would be without behavioral consequences.

In conclusion, spontaneous alternation in the Y-maze was impaired in DAT KO mice compared to wild-type mice. Concomitant changes in the expression of BDNF protein were observed in the frontal cortex but not in the caudate putamen or hippocampus of DAT KO mice. Taken together, these observations are at least consistent with the hypothesis that impaired working memory in the Y-maze in these mice may receive contributions from the decreased frontal cortex BDNF found in DAT KO mice.

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The adenosine A2A receptor is associated with methamphetamine dependence/psychosis in the Japanese population

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Abstract

Background: Several lines of evidence suggest that the dopaminergic nervous system contributes to methamphetamine (METH) dependence, and there is increasing evidence of antagonistic interactions between dopamine and adenosine receptors. We therefore hypothesized that variations in the A2A adenosine receptor (*ADORA2A*) gene modify genetic susceptibility to METH dependence/psychosis.

Methods: We first analyzed variations in the exons and exon-intron boundaries of the *ADORA2A* gene in METH dependent/psychotic patients. Then an association analysis between these single nucleotide polymorphisms and METH dependence/psychosis was performed using a total of 171 METH dependent/psychotic patients and 229 controls.

Results: We found 6 variations, of which one single nucleotide polymorphism (SNP) was novel. Significant associations were observed between the allelic and genotypic frequencies of the Exon2+751 (rs5751876) SNP and METH dependence/psychosis. These associations were observed especially in females. In the clinical feature analyses, significant associations were observed between the SNP and the patient subgroup using METH alone (i.e., without concomitant use of other substances of abuse).

Conclusions: These results suggest that the *ADORA2A* gene could be a vulnerability factor for METH dependence/psychosis, especially in females and/or in patients using only METH.

Background

Methamphetamine (METH) is a psychomotor stimulant with high liability for abuse, and METH has become a very serious social problem not only in Japan [1] but also worldwide, including in the United States [2]. Use of METH induces a strong psychological dependence, and repeated usage frequently results in psychotic states, the symptoms of which are similar to those of paranoid-type schizophrenia [3,4]. Chronic METH abusers have been shown to have persistent dopaminergic deficits [5,6].

Amphetamines are thought to produce their stimulant effects mainly via the dopamine system [7,8], although other systems may also be involved. Dopamine D1 and D2 receptors exist as heterodimers with adenosine A1 and A2A receptors, respectively, which modulate their responsiveness [9,10], suggesting that responses to amphetamines may also be dependent on adenosinergic function.

Several lines of evidence suggest that adenosine A2A receptors (ADORA2A) play a role in inhibiting the effects of METH. ADORA2A antagonists have been shown to significantly increase the action of amphetamine-induced locomotor activity in mice [11] and to mimic the discriminative-stimulus effects of METH in rats [12]. ADORA2A agonists reduced amphetamine-induced locomotor activity in mice [11] and attenuated amphetamine-induced stereotypy in rats [13]. *ADORA2A* gene knockout mice showed attenuation in locomotor responses [14] and no development of locomotor sensitization to amphetamine [15]. These reports suggest the

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pharmacological potential of ADORA2A adenosinergic agents to modulate adaptive responses to METH exposure.

There have been no association analyses between *ADORA2A* gene polymorphisms and METH dependence/psychosis. In the present study, we analyzed all coding exons and exon-intron boundaries of the *ADORA2A* gene to reveal the variations in the Japanese population and examined the associations between novel and reported polymorphisms in the *ADORA2A* gene and METH dependent/psychotic patients in Japan.

Methods

Subjects

One-hundred seventy-one unrelated patients with METH dependence/psychosis (138 males and 33 females; mean age 37.5 ± 12.0 years) meeting ICD-10-DCR criteria (F15.2 and F15.5) were used as case subjects; they were outpatients or inpatients of psychiatric hospitals. The 229 control subjects (119 males and 110 females; mean age 41.2 ± 12.3 years) were mostly medical staff members who had neither personal nor familial history of drug dependence or psychotic disorders, as verified by a clinical interview. All subjects were Japanese, born and living in the northern Kyushu, Setouchi, Chukyo, Tokai, and Kanto regions. This study was approved by the ethical committees of each institute of the Japanese Genetics Initiative for Drug Abuse (JGIDA), and all subjects provided written informed consent for the use of their DNA samples for this research [3]. After informed consent was obtained, blood samples were drawn and genomic DNA was extracted by the phenol/chloroform method.

Defining variants of the *ADORA2A* gene

Initially, DNA samples from 16 METH dependent/psychotic patients were used to identify nucleotide variants within the *ADORA2A* gene (NCBI accession numbers: AP000355 and NT_011520). Exons 1, 2 and exon-intron boundaries were amplified by polymerase chain reaction (PCR) using a thermal cycler (Astec, Fukuoka, Japan), and the products were sequenced in both directions using BigDye terminators (Applied Biosystems, Foster City, CA) by an ABI Genetic analyzer 3100 (Applied Biosystems). The primers used for amplifying exon 1 and its exon-intron boundaries were 1F and 4R, and those used for sequencing were 1F, 1R, 2F, 2R, 3F, 3R, 4F, and 4R (Table 1). The primers used for amplifying exon 2 and its exon-intron boundaries were 5F and 8R, and those used for sequencing were 5F, 5R, 6F, 6R, 7F, 7R, 8F, and 8R.

Genotyping of both Exon1+179 (rs13306114) and Exon1+219 was performed by PCR amplification using 3F and 4R primers followed by sequencing with 3F and 3R primers. Genotyping of IVS1+64 (rs13306116) was performed by PCR amplification using 4F and 4R primers followed by restriction enzyme *Bcn*I digestion (PCR-restriction fragment length polymorphism; PCR-RFLP). Genotyping of Exon2+751 (rs5751876) and IVS2 +28 (rs34923252) was performed by PCR amplification using 5F and 8R primers followed by sequencing with 6F and 6R primers or 8F and 8R primers, respectively.

Clinical category analysis

For the clinical category analysis, the patients were divided into two subgroups by four different clinical features. As some subjects had missing data for some clinical features, the sum of each subgroup was not total to 171.

(A) Latency of psychosis from first METH intake: less than 3 years (n = 64, average = 0.76y) or more than 3 years (n = 70, average = 9.4y). The course of METH psychosis varied among patients, with some patients showing psychosis sooner after the first METH intake, as previously reported [3,16]. Because the median latency was 3 years, this time point was used as the cut-off in defining the two groups. (B) Duration of psychosis after the last METH intake: transient (< 1 month) or prolonged (≥ 1 month). Some patients showed continuous psychotic symptoms even after METH discontinuation, as previously reported [3,16]. Psychotic symptoms disappeared in the patients with the transient-type psychosis within one month after the discontinuation of METH consumption and the beginning of treatment with neuroleptics. Patients with the prolonged-type psychosis had symptoms that continued for more than one month even after the discontinuation of METH consumption and the beginning of neuroleptic treatment. (C) Spontaneous relapse: present or not. It has been well documented that once METH psychosis has developed, patients in the remission phase are liable to spontaneous relapse without reconsumption [3,16]. (D) Multiple drug usage: mono-drug (use of METH without concomitant use of other drugs of abuse), or poly-drugs. Some patients consumed multiple drugs in addition to METH. They consumed at least either one of cocaine, marihuana, morphine, heroin, LSD, alcohol or paint thinner in addition to METH.

Statistical analysis

The Hardy-Weinberg (HW) equilibrium of genotypic frequencies in each SNP was tested by the chi-square test. The allelic and genotypic frequencies of patients and control groups were compared using the chi-square test. The level of statistical significance was set at α = 0.05. The odds ratio (OR) and its 95% confidence interval (CI) were calculated as a measure of the association between Exon2+751 (rs5751876) alleles and METH

Table 1 Primers used in this study

Exon	Forward		Reverse	
Exon 1	1F:	GAGGTCCATTTGGATCCAGACCAT	1R:	TTCTCTCGCCAGGCCAACTTCTCA
	2F:	ACATCCTTCCACATCCGAGCTCCA	2R:	CAGGCCAGGTGTCAGCCTGAGGAT
	3F:	AGTCTCAGCGGGAATTCTATTGGA	3R:	GACGAAGCAGGCAATGAAGAGGCA
	4F:	TACATCACGGTGGAGCTGGCCATT	4R:	ACAGCTCCCTGCTAAGCCCAATGC
Exon 2	5F:	ACTACTCAATGACCATCTGGGCAT	5R:	TGATGGCCAGTGACTTGGCAGCAT
	6F:	TGCTGCTCATGCTGGGTGTCTATT	6R:	TCTTCTCCCAACGTCACTGGTCAA
	7F:	TTAGCCATGAGCTCAAGGGAGTGT	7R:	CTCTGGCACTGCTCTGTTACAACT
	8F:	TCACTCTCTGGCTGCTGGGTCTGC	8R:	GTCACAGTTCTGAGAAGGTAACAT

dependence/psychosis. Haplotype frequencies were calculated by the Arlequin program available from http://anthropologie.unige.ch/arlequin[17]. A global test of differentiation among samples based on haplotype frequencies was performed using the Arlequin program.

Results

Analysis of *ADORA2A* gene variants

To identify polymorphisms in the *ADORA2A* gene, all coding exons and exon-intron boundaries were analyzed using genomic DNA from 16 Japanese METH dependent/psychotic subjects. Six single nucleotide polymorphisms (SNPs) were identified, of which one was novel (Exon1+219) (Table 2). Consistent with the previous study in Japanese samples [18], we could not find 405C/T, 432C/T, or 1018C/T polymorphisms. Two SNPs at Exon2+751 (rs5751876) and Exon2+1360 (rs35060421) were in linkage disequilibrium (LD) in the sense that the genotypic patterns of the 16 samples examined were the same. This LD has also been reported in a Caucasian population [19]. We chose Exon2+751 (rs5751876) to represent these SNPs. Exon1+179 (rs13306114), Exon1+219, IVS1+64 (rs13306116), Exon2+751 (rs5751876) and IVS2+28 (rs34923252) were chosen for further analyses.

Relationship between *ADORA2A* gene SNPs and METH dependence/psychosis

Association analyses between these SNPs in the *ADORA2A* gene and METH dependence/psychosis were performed using DNA samples from 171 METH dependent/psychotic subjects and 229 control subjects. The genotypic frequencies in these SNPs in controls were within the Hardy-Weinberg expectations. Significant differences were observed in both the genotypic (P = 0.018) and the allelic (P = 0.0057) frequencies of the Exon2+751 (rs5751876) SNP between METH dependent/psychotic patients and controls (Table 3). When Bonferroni correction was performed according to the number of SNPs examined, the corrected significance level was $p < 0.01$ (0.05/5 = 0.1). Significance was still observed in the allelic frequencies. Odds ratio of the allelic frequencies of the Exon2+751 (rs5751876) SNP between METH dependent/psychotic patients and controls was 1.50 (95% CI: 1.13 to 1.99).

The SNPs having minor allele frequencies of over 5%, Exon2+751 (rs5751876) and IVS2+28 (rs34923252), were used for a global test of differentiation among samples based on haplotype frequencies using the Alrequin program. Significant association with METH dependence/psychosis was observed (P = 0.027).

Gender-dependent association analyses were performed. In female samples, the Exon2+751 (rs5751876) SNP showed significant associations with METH dependence/psychosis in genotypic (P = 0.0078) and allelic (P = 0.014) distributions (Table 4). When Bonferroni correction was performed by gender (i.e., using two categories: male or female), the corrected significance level was $P < 0.025$. Female samples still showed significance.

Subcategory analyses were performed on the clinical parameters (latency of psychosis, prognosis psychosis, spontaneous relapse, and multiple drug usage) on the Exon2+751 (rs5751876) SNP (Table 5). Nominal

Table 2 *ADORA2A* gene variants found in the Japanese population

Location	Variants	rs#	Function	Reference
Exon1+179	C/T	rs13306114	untranslated	
Exon1+219	C/T		untranslated	
IVS1+64	G/A	rs13306116	intron	
Exon2+751	C/T	rs5751876	synonymous (Tyr- > Tyr)	1083C/T[30], 1976T/C[19]
Exon2+1360	T6/T7	rs35060421	untranslated	2592C/Tins[19]
IVS2+28	T/A	rs34923252	intron	

Table 3 Genotype and allele frequencies of the *ADORA2A* gene SNPs in patients and controls

SNP	Group	N	Genotype (%)			P	Allele (%)		P
Exon1+179 (rs13306114)			C/C	C/C	C/T		T/T	C	T
	Control	229	229 (100.0%)	0 (0.0%)	0 (0.0%)		458 (100.0%)	0 (0.0%)	
	METH	171	168 (98.2%)	3 (1.8%)	0 (0.0%)	0.132	339 (99.1%)	3 (0.9%)	0.154
Exon1+219			C/C	C/T	T/T		C	T	
	Control	229	228 (99.6%)	1 (0.4%)	0 (0.0%)		457 (99.8%)	1 (0.2%)	
	METH	171	169 (98.8%)	2 (1.2%)	0 (0.0%)	0.701	340 (99.4%)	2 (0.6%)	0.807
IVS1+64 (rs13306116)			G/G	G/A	A/A		G	A	
	Control	229	219 (95.6%)	10 (4.4%)	0 (0.0%)		448 (97.8%)	10 (2.2%)	
	METH*[1]	171	162 (94.7%)	8 (4.7%)	1 (0.6%)	0.504	332 (97.1%)	10 (2.9%)	0.663
Exon2+751 (rs5751876)			T/T	T/C	C/C		T	C	
	Control	229	70 (30.6%)	114 (49.8%)	45 (19.7%)		254 (55.5%)	204 (44.5%)	
	METH	171	35 (20.5%)	85 (49.7%)	51 (29.8%)	0.018	155 (45.3%)	187 (54.7%)	0.0057
IVS2+28 (rs34923252)			T/T	T/A	A/A		T	A	
	Control	229	181 (79.0%)	43 (18.8%)	5 (2.2%)		405 (88.4%)	53 (11.6%)	
	METH	171	144 (84.2%)	26 (15.2%)	1 (0.6%)	0.258	314 (91.8%)	28 (8.2%)	0.146

*1: METH sample: Hardy-Weinberg Equilibrium p = 0.0214

N: Number of samples

P: Significance values between METH samples and controls.

significance was observed in the transient course of prognosis of psychosis (P = 0.036), absence of spontaneous relapse (p = 0.046), and use of only METH (P = 0.0099), when they were compared to controls. When Bonferroni correction was performed according to the number of subcategories (2), the corrected significance level was p < 0.025. Significance was still observed in the subjects using only METH. But these differences were not observed between these patient subgroups.

Discussion

We analyzed *ADORA2A* gene variations in a Japanese population and found six SNPs in exons and exon-intron boundaries. Significant associations were observed between the Exon2+751 (rs5751876) SNP and METH dependence/psychosis. The Exon2+751 (rs5751876) SNP showed significant associations with METH dependence/psychosis in female samples and with subjects using only METH.

This is the first report of an association analysis between *ADORA2A* gene polymorphisms and METH dependence/psychosis. The result that METH dependence/psychosis was significantly associated with *ADORA2A* gene polymorphism was in line with the findings in animal studies. In animals, the pharmacological effects of psychostimulants like cocaine and amphetamine were counteracted by ADORA2A agonists and potentiated by ADORA2A antagonists [20-23].

Exon2+751 (rs5751876) SNP showed a significant association with METH dependence/psychosis. As Exon2+751 (rs5751876) SNP is a synonymous variant, this SNP cannot include amino acid substitutions. However, we found one untranslated region SNP, Exon2+1360 (rs35060421, 2592C/Tins [19]), in LD with Exon2+751 (rs5751876). This LD was also reported in a Caucasian population [19]. While there is no evidence of functional alterations of these SNPs at this time point, one may hypothesize that untranslated region SNPs would change gene expression

Table 4 Gender-dependent association analyses of the Exon2+751 (rs5751876) SNP in patients and controls

Gender	Group	N	Genotype (%)			P	Allele (%)		P
Male									
			T/T	T/C	C/C		T	C	
	Control	119	31 (26.1%)	59 (49.6%)	29 (24.4%)		121 (50.8%)	117 (49.2%)	
	METH	138	27 (19.6%)	73 (52.9%)	38 (27.5%)	0.456	127 (46.0%)	149 (54.0%)	0.315
Female									
			T/T	T/C	C/C		T	C	
	Control	110	39 (35.5%)	55 (50.0%)	16 (14.5%)		133 (60.5%)	87 (39.5%)	
	METH	33	8 (24.2%)	12 (36.4%)	13 (39.4%)	0.0078	28 (42.4%)	38 (57.6%)	0.014

N: Number of samples

P: Significance values between METH samples and controls.

Table 5 Association analyses between the Exon2+751 (rs5751876) SNP and clinically subcategorized METH subjects

Samples	Subgroups	N	Genotype (%)			P1	P2
			T	T/C	C		
Control		229	70 (31%)	114 (50%)	45 (20%)		
METH	Latency of Psychosis						
	< 3 years	64	15 (23%)	32 (50%)	17 (27%)	0.366	
	≥ 3 years	70	17 (24%)	30 (43%)	23 (33%)	0.068	0.663
	Prognosis of Psychosis						
	Transient (< 1 month)	91	21 (23%)	40 (44%)	30 (33%)	0.036	
	Prolonged (≥ 1 month)	56	11 (20%)	34 (61%)	11 (20%)	0.231	0.115
	Spontaneous Relapse						
	Not present	104	22 (21%)	50 (48%)	32 (31%)	0.046	
	Present	60	12 (20%)	31 (52%)	17 (28%)	0.167	0.905
	Multiple Drug Usage						
	None (METH use only)	52	6 (12%)	29 (56%)	17 (33%)	0.0099	
	Poly-drugs	112	26 (23%)	54 (48%)	32 (29%)	0.127	0.214

N: Number of samples

P1: Significance values between METH samples and controls.

P2: Significance values between the subgroups in each clinical category

in a transcriptional and/or translational level, that lead to the pathophysiology of METH dependence/psychosis. Further analyses are necessary to study the functions of these SNPs or other SNPs in LD.

Significant difference was observed between the subgroup of patients using only METH and the controls in the variation of Exon2+751 (rs5751876) SNP, while no difference was observed between the subgroup of patients using poly-drugs and the controls nor within patient subgroups. The reason why the significant association was observed only in the patient subgroup using exclusively METH compared to controls is not known; however, further analyses in this patient subgroup could reveal the reasons for their predilection for METH alone. Actually, an association has been reported between the *ADORA2A* variants in the 1976C/T (Exon2 +751) and 2592C/T(ins) (Exon2+1360) polymorphisms and the anxiogenic response to amphetamine in healthy subjects [24]. Hohoff and colleagues found that its effect on anxiety was stronger at the lower amphetamine dose and discussed that relatively small genetic effects are more relevant at lower doses of amphetamine and can be overcome by increasing the amphetamine dose [24]. Emotional alterations and/or the dose of METH use might be mediated by these SNPs and that will likely be one of the key to understanding METH dependence/psychosis.

We found a significant association between the female subjects with METH dependence/psychosis and Exon2 +751 (rs5751876) SNP. These gender different associations between METH dependence/psychosis and SNPs have also been reported in several genes [25-28]. It has well been known and studied that male and female differ markedly with regard to their use of, and responses to, METH and related amphetamines [29]. Dluzen and colleagues summarized the data from published articles on gender differences in various parameters of METH use and responses and concluded that women seemed more dependent on and committed to METH but showed diminished (amphetamine-stimulated) dopamine responses and a decreased degree of toxicity [29]. Although larger replication studies need to be conducted on the association analysis between the SNP and METH dependence/psychosis in females, our results offer a potential means for understanding the differences between female and male in the progression to METH dependence/psychosis.

Limitations

The limitation of our study is its moderate sample size, especially in gender-dependent association analyses and in clinical category analyses. Although the number of our study subjects is generally regarded as small for association studies, it is hard to obtain many patient samples due to the illegality of METH use in Japan. We therefore organized the Japanese Genetics Initiative for Drug Abuse (JGIDA) to collect samples. Based on these efforts, we could finally obtain this moderate number of patient samples. Nevertheless, our study needs to be reproduced in a larger sample size regarding the associations between these SNPs and METH dependence/psychosis.

Conclusions

Our results suggest that the *ADORA2A* gene could be a vulnerability factor for METH dependence/psychosis,

especially in females and/or in patients using only METH. Further investigations of the role of the *ADORA2A* gene in the development of METH dependence/psychosis are warranted.

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Authors' contributions
HK conceived of this study, genotyped samples, analyzed data and drafted the manuscript. HU organized the Japanese Genetics Initiative for Drug Abuse (JGIDA). HU, NI, TI, MY, YS, NU, MI, NO and MI collected genome samples and informed consents. IS supervised all managements, analysis and interpretation and revised the manuscripts to give final approval of the version to be published. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Association Analysis of the Tryptophan Hydroxylase 2 Gene Polymorphisms in Patients with Methamphetamine Dependence/Psychosis

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Abstract: There is a growing evidence that serotoninergic systems modulate dopaminergic neurotransmission. We analyzed the association between the variations in the brain tryptophan hydroxylase 2 (*TPH2*) gene, a rate limiting enzyme for serotonin biosynthesis, and methamphetamine (METH) dependence/psychosis in a Japanese population. We found ten single nucleotide polymorphisms (SNPs) and two polynucleotide polymorphisms in *TPH2* gene exons and exon-intron boundaries. A total of 162 patients and 243 controls were used for the association analysis between these polymorphisms and METH dependence/psychosis. No significant differences were observed in either genotypic or allelic frequencies between METH dependence/psychotic patients and controls. A global test of differentiation among samples based on haplotype frequencies showed no significant association. With respect to latency of psychosis, prognosis of psychosis, and spontaneous relapse, we found no significant association with these SNPs. These results suggest that the *TPH2* gene variants may not be a factor in vulnerability to METH dependence/psychosis.

Keywords: Single nucleotide polymorphism, SNP, variation, serotonin, human, Japanese, MAP, abuse.

INTRODUCTION

Methamphetamine (METH) is a psychomotor stimulant with high liability for abuse, and METH abuse has become a very serious social problem in Japan [1]. Chronic METH abusers have been shown to have persistent dopaminergic deficits [2, 3]. In animals, amphetamine elevates extracellular dopamine levels in the mesolimbic circuits [4, 5]. There is growing evidence that serotonergic systems modulate dopaminergic neurotransmission. For example, the mesocorticolimbic dopamine system is under inhibitory control by the serotonin system, which exerts its actions *via* serotonin receptor subtypes [6, 7].

Acute and chronic administration of METH markedly decreases the activity of tryptophan hydroxylase (TPH) [8, 9], the rate-limiting enzyme in the biosynthesis of serotonin [10]. TPH2 (or neuronal TPH) was identified as a second isoform of TPH in 2003 [11, 12]. In contrast to TPH1, which is expressed predominantly in the pineal gland and the periphery, TPH2 mRNA is expressed in the raphe nuclei [11]. Since the identification of TPH2, there have been numerous association analyses between *TPH2* gene variants and psychiatric diseases. For example, associations have been observed between *TPH2* variants and bipolar disorder [13-18], suicidal behavior in major depression [19-21], the response to selective serotonin reuptake inhibitors (fluoxetine and/or citalopram) [22, 23] and emotional regulation in healthy subjects [24-28]. These reports indicate that polymorphic variants in the *TPH2* gene may have a role in the pathophysiology of a wide range of psychiatric disorders and emotional regulation. A recent study of heroin addiction also showed an association with *TPH2* variants in Hispanics and African-Americans [29].

The purpose of this study was (1) to identify novel sequence variations in all coding exons as well as exon-intron boundaries of the *TPH2* gene in Japanese, and (2) to investigate whether these polymorphisms and/or haplotypes were associated with METH dependence/psychosis.

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MATERIALS AND METHODS

Subjects

One-hundred sixty-two unrelated patients with METH dependence/psychosis (130 males and 32 females; mean age 37.4±12.0 years) meeting ICD-10-DCR criteria (F15.2 and F15.5) were used as case subjects; they were outpatients or inpatients of psychiatric hospitals. The 243 control subjects (168 males and 75 females; mean age 35.4±11.5 years) were mostly medical staff members who had neither personal nor familial history of drug dependence or psychotic disorders, as verified by a clinical interview. All subjects were Japanese, born and living in the northern Kyushu, Setouchi, Chukyo, Tokai, and Kanto regions. This study was approved by the ethical committees of each institute of the Japanese Genetics Initiative for Drug Abuse (JGIDA), and all subjects provided written informed consent for the use of their DNA samples for this research [30]. After informed consent was obtained, blood samples were drawn and genomic DNA was extracted by the phenol/chloroform method.

Defining Variants of the *TPH2* Gene

Initially, 16 METH dependent/psychotic patient samples were used to identify nucleotide variants within the *TPH2* gene (GenBank accession no. AC090109). Exons 1 to 11 and exon-intron boundaries were amplified by polymerase chain reaction (PCR) using a thermal cycler (Astec, Fukuoka, Japan), and the products were sequenced in both directions using BigDye terminators (Applied Biosystems, Foster City, CA) by an ABI Genetic analyzer 3100 (Applied Biosystems).

Genotyping of each polymorphism except in exon 11 was performed by PCR amplification using the relevant primers listed in Table **1** followed by sequencing using the same primers in both directions. Genotyping of polymorphisms in exon 11 was performed by PCR amplification using 9F and 11R primers followed by sequencing using 10F, 11F, and 11R primers.

Patient Subgroups

For the clinical category analysis, the patients were divided into two subgroups by three different clinical features. (A) Latency of psychosis from first METH intake: less than 3 years or more than 3 years. The course of METH psychosis varied among patients, with some patients showing psychosis sooner after the first METH intake, as previously reported [30, 31]. Because the median latency was three years, this time point was used as the cutoff in defining the two groups. (B) Duration of psychosis after the last METH intake: transient (<1 month) or prolonged (≧1 month). Some patients showed continuous psychotic symptoms even after METH discontinuation, as previously reported [30, 31]. Patients with the transient type showed a reduction of psychotic symptoms within one month after the discontinuation of METH consumption and the beginning of treatment with neuroleptics. Patients with the prolonged type showed a psychotic symptoms continued for more than one month even after the discontinuation of METH consumption and the beginning of neuroleptic treatment. (C) Spontaneous relapse: present or not. It has been well documented that once METH psychosis has developed, patients in the remission phase are liable to spontaneous relapse without reconsumption [30, 31].

Statistical Analysis

The Hardy-Weinberg equilibrium of genotypic frequencies in each SNP was tested by the chi-square test. The level of statistical significance was set at $\alpha = 0.05$. The allelic and genotypic frequencies of patients and control groups were compared using the chi-square test. Locus by locus linkage disequilibrium (LD) was evaluated by D' and r^2, which were calculated by the haplotype frequencies using the appropriate formula in the Excel program. A global test of differentiation among samples based on haplotype frequencies was performed using the Arlequin program available from http://anthropologie.unige.ch/arlequin [32].

Table 1. Primers Used in this Study

Exon	Forward		Reverse	
Exon 1	1F	CCT TAT GTA TTG TTC TCC ACC ACC	1R	GTT GAG CAC GCA GTG ATT GGC ACA
Exon 2	2F	CCA CTA GAT GAT GTC TTA GAC CAT	2R	CTG ACC TCC TAA CCT GGC AAT AGT
Exon 3,4	3F	GTA CTT GGC ACC TTG CTT AAG ATG	3R	TGG AAG TCT GCT CTC AGT TAT GGG
Exon 5	4F	GCT CAA CTA AGC CAT TCT GCT TAC	4R	GTA GCA CTT GGC ATG TGG CTC ACA
Exon 6	5F	GAT CCT TTC AGA CGC TCA TGT GCT	5R	CAT ACT CAT GTA GCC CAG CAC AGC
Exon 7	6F	GTG CGG TAA GCA TCA CTT TCG ATT	6R	CAG ATG AGG AGT CTG ATC CTT CAG
Exon 8	7F	GAA GTC CCA GCA TTG ATG AAC TGT	7R	GGC TAA GCT GAG TAA TTC TGA CAG
Exon 9	8F	CAG GAA GCG TAA GAC TCT TAG TAG	8R	GTC AGT AGG ATC ACT GCT AGC TCA
Exon 10, 11	9F	CCT GCA CAC AGG AGA GTT CCA TAT	9R	CAT GCT GGC AAC AAC ATA GTT CCA
	10F	CAA TCC CTA CAC ACA GAG TAT TGA	10R	CAT TCC AAC TGC TGT GTT ACC TCA
	11F	GAT CTA AGC CTT TCC TCT GTG TTC	11R	GAC ACA GAA ACA CAT GCA AGC ACT

RESULTS

To identify polymorphisms in the *TPH2* gene, all coding exons (1 to 11) and exon-intron boundaries were analyzed using genomic DNA from 16 Japanese METH-dependent/psychotic subjects. Ten single nucleotide polymorphisms (SNPs) and two insertion / deletion polymorphisms were identified. One polymorphism, Exon11+(C3) 500(C2), was novel (Table **2**). Two SNPs, rs7305115 (Exon7+A131G) and rs4290270 (Exon9+A57T), were synonymous mutations and Eon2+C18A was a non-synonymous mutation. Three linkage disequilibrium (LD) regions were found, rs11178998 (Exon1-A42G) to rs41265611 (IVS1+60 (I/D)), rs11179003 (IVS4+C4821T) to rs10879348 (IVS6+G144A), and rs4760816 (IVS6+C6106T) to rs7305115 (Exon7+A131G), in the sense that all genotypic patterns in all 16 samples analyzed were the same. Each one of the SNPs was chosen and a total of nine SNPs were genotyped for further analysis. LD mapping was analyzed by using SNPs having minor allele frequencies of over 10% in both samples (Table **4**). LD was observed from rs17110566 (IVS6+G152A) to rs17110747 (Exon11+G654A) and from rs4290270 (Exon9+A57T) to rs41317114 (IVS11+G128C) (Fig. **1** and Table **3**).

Association analyses were performed on these nine polymorphic positions using 162 METH dependent/psychotic patients and 243 controls. Genotypic frequencies in these

Table 2. ***TPH2*** **Gene Variants Found in the Japanese Population**

Position[1)]	Location	rs Number[2)]	SNP Name	Variation	Function
30029	5' side	rs11178998	Exon1-A42G	A/G	
30241	Intron 1	rs41265611	IVS1+60(I/D)	TCT/del	
32694	Exon 2		Exon2+C18A[3)]	C/A	nonsynonymous (Ser41Tyr)
40601	Intron 4	rs11179003	IVS4+C4821T	C/T	
63953	Intron 6	rs10879348	IVS6+G144A	G/A	
63961	Intron 6	rs17110566	IVS6+G152A	G/A	
69915	Intron 6	rs4760816	IVS6+C6106T	C/T	
70176	Exon 7	rs7305115	Exon7+A131G	A/G	synonymous (Pro312Pro)
113549	Exon 9	rs4290270	Exon9+A57T	A/T	synonymous (Ala375Ala)
123114	Exon 11		Exon11+(C3)500(C2)	C3/C2	
123268	Exon 11	rs17110747	Exon11+G654A	G/A	
123663	3' side	rs41317114	IVS11+G128C	G/C	

[1)]Position: nucleotide position number in the NCBI nucleotide database under accession number AC090109. [2)] rs number: NCBI SNP database. [3)] This SNP was reported as C2755A [14].

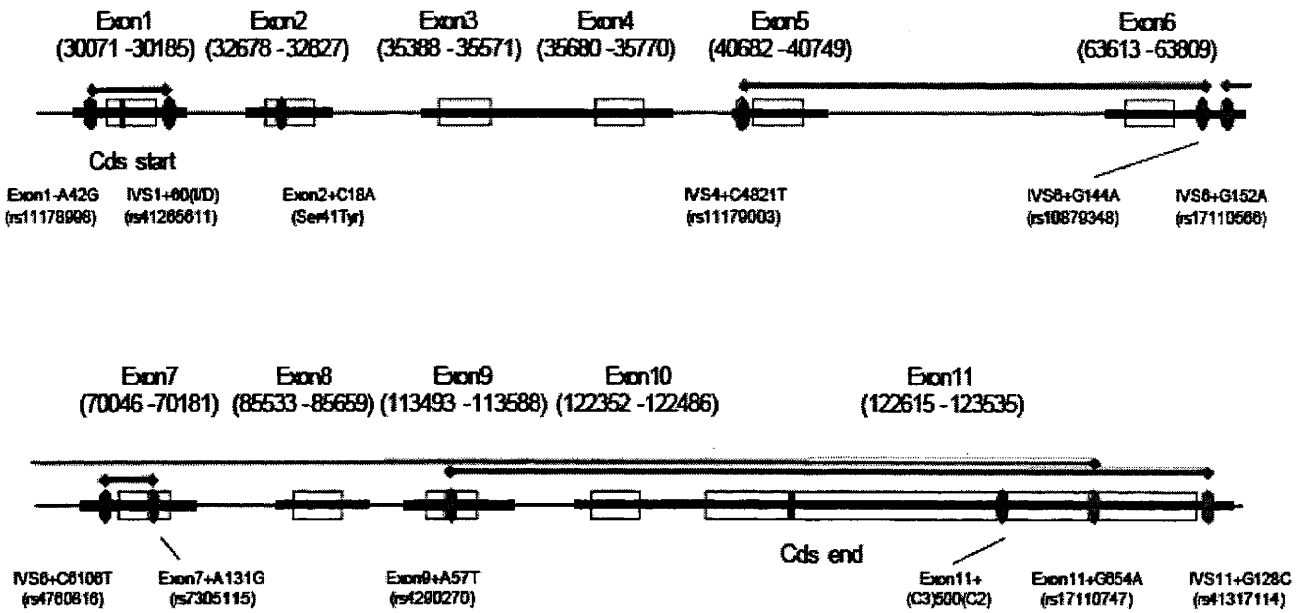

Fig. (1). Location and linkage disequilibrium mapping of the *TPH2* gene polymorphisms. All the coding exons and their regions were taken from the NCBI database under accession number AC090109. Red ovals indicate the polymorphic positions, solid black lines the analyzed regions, and solid red lines the LD block.

Table 3. Linkage Disequilibrium Mapping of the *TPH2* Gene

	rs17110566 (IVS6+G152A)	rs4760816 (IVS6+C6106T)	rs4290270 (Exon9+A57T)	rs17110747 (Exon11+G654A)	rs41317114 (IVS11+G128C)	
rs17110566		0.9392	0.6138	0.8581	0.0348	
rs4760816	0.9724		0.7301	0.9253	0.0092	
rs4290270	0.5262	0.5881		0.9284	0.6051	D'
rs17110747	0.8437	0.7885	0.9774		0.9399	
rs41317114	0.0111	0.2179	0.6284	0.9123		
			r^2			

D' and r^2 values for Control samples are shown in the upper right and lower left, respectively.

Table 4. Genotypic and Allelic Distribution of the *TPH2* gene SNPs in the METH Dependent/Psychotic Patients and the Control Groups

SNP	Group	Genotype (%)			P	Allele (%)		P
rs11178998 (Exon1-A42G)		A/A	A/G	G/G		A	G	
	METH	130 (80%)	29 (18%)	3 (2%)	0.102	289 (89%)	35 (11%)	0.617
	Control	197 (81%)	46 (19%)	0 (0%)		440 (91%)	46 (9%)	
Exon2+C18A		C/C	C/A	A/A		C	A	
	METH	146 (90%)	16 (10%)	0 (0%)	0.914	308 (95%)	16 (5%)	0.807
	Control	222 (91%)	21 (9%)	0 (0%)		465 (96%)	21 (4%)	
rs10879348 (IVS6+G144A)		G/G	G/A	A/A		G	A	
	METH	136 (84%)	26 (16%)	0 (0%)	0.975	298 (92%)	26 (8%)	0.920
	Control	206 (85%)	37 (15%)	0 (0%)		449 (92%)	37 (8%)	
rs17110566 (IVS6+G152A)		G/G	G/A	A/A		G	A	
	METH	123 (76%)	35 (22%)	4 (2%)	0.552	281 (87%)	43 (13%)	0.406
	Control	173 (71%)	64 (26%)	6 (2%)		410 (84%)	76 (16%)	
rs4760816 (IVS6+C6106T)		C/C	C/T	T/T		C	T	
	METH	28 (17%)	85 (52%)	49 (30%)	0.314	141 (44%)	183 (56%)	0.200
	Control	57 (23%)	121 (50%)	65 (27%)		235 (48%)	251 (52%)	
rs4290270 (Exon9+A57T)		A/A	A/T	T/T		A	T	
	METH	29 (18%)	80 (49%)	53 (33%)	0.840	138 (43%)	186 (57%)	0.777
	Control	49 (20%)	115 (47%)	79 (33%)		213 (44%)	273 (56%)	
Exon11+(C3)500(C2)		C3/C3	C3/C2	C2/C2		C3	C2	
	METH	159 (98%)	3 (2%)	0 (0%)	0.357	321 (99%)	3 (1%)	0.357
	Control	242 (100%)	1 (0%)	0 (0%)		485 (100%)	1 (0%)	
rs17110747 (Exon11+G654A)		G/G	G/A	A/A		G	A	
	METH	92 (57%)	63 (39%)	7 (4%)	0.956	247 (76%)	77 (24%)	0.888
	Control	136 (56%)	95 (39%)	12 (5%)		367 (76%)	119 (24%)	
rs41317114 (IVS11+G128C)		G/G	G/C	C/C		G	C	
	METH	119 (73%)	38 (23%)	5 (3%)	0.719	276 (85%)	48 (15%)	0.462
	Control	187 (77%)	50 (21%)	6 (2%)		424 (87%)	62 (13%)	

Table 5. Genotypic Distribution of the *TPH2* Gene SNPs in Clinically Subcategorized METH Subjects

SNP	Groups	Subgroup		N	Genotype			P
rs17110566 (IVS6+G152A)					G	G/A	A	
	Control			243	173	64	6	
	METH	Latency of Psychosis	<3 years	64	53	10	1	0.172
			≧3 years	67	47	18	2	0.966
		Prognosis of Psychosis	Transient (<1 month)	87	67	17	3	0.421
			Prolonged (≧1 month)	52	38	13	1	0.951
		Spontaneous Relapse	Not present	101	78	21	2	0.517
			Present	56	42	12	2	0.694
rs4760816 (IVS6+C6106T)					C	C/T	T	
	Control			243	57	121	65	
	METH	Latency of Psychosis	<3 years	64	13	35	16	0.771
			≧3 years	67	9	35	23	0.165
		Prognosis of Psychosis	Transient (<1 month)	87	15	39	33	0.125
			Prolonged (≧1 month)	52	7	34	11	0.107
		Spontaneous Relapse	Not present	101	19	51	31	0.577
			Present	56	8	30	18	0.306
rs4290270 (Exon9+A57T)					A	A/T	T	
	Control			243	49	115	79	
	METH	Latency of Psychosis	<3 years	64	8	35	21	0.338
			≧3 years	67	13	32	22	0.990
		Prognosis of Psychosis	Transient (<1 month)	87	16	37	34	0.541
			Prolonged (≧1 month)	52	6	34	12	0.058
		Spontaneous Relapse	Not present	101	17	52	32	0.712
			Present	56	10	27	19	0.923
rs17110747 (Exon11+G654A)					G	G/A	A	
	Control			243	136	95	12	
	METH	Latency of Psychosis	<3 years	64	35	28	1	0.438
			≧3 years	67	37	26	4	0.947
		Prognosis of Psychosis	Transient (<1 month)	87	52	31	4	0.827
			Prolonged (≧1 month)	52	26	25	1	0.366
		Spontaneous Relapse	Not present	101	57	41	3	0.712
			Present	56	32	21	3	0.970
rs41317114 (IVS11+G128C)					G	G/C	C	
	Control			243	187	50	6	
	METH	Latency of Psychosis	<3 years	64	49	15	0	0.411
			≧3 years	67	48	16	3	0.552
		Prognosis of Psychosis	Transient (<1 month)	87	65	19	3	0.852
			Prolonged (≧1 month)	52	38	13	1	0.767
		Spontaneous Relapse	Not present	101	77	21	3	0.966
			Present	56	38	17	1	0.282

N: Number of samples.
P: Significance values between the METH subjects and the controls.

SNPs were within the Hardy-Weinberg expectations. No significant differences were found in the allelic or genotypic frequencies of these SNPs between the METH dependent/psychotic patients and the controls (Table **4**). Since the minor allele frequency of the Exon11+(C3)500(C2) SNP was less than 1% in controls, this SNP was excluded from the haplotype analysis. No significant difference (P=0.448) was observed in a differentiation test between all pairs of samples based on haplotype frequencies by the Arlequin program.

Subcategory analyses were conducted on the clinical parameters (latency of psychosis, prognosis psychosis, and spontaneous relapse). SNPs having minor allele frequencies of over 10% in both samples were used for this analysis: rs17110566 (IVS6+G152A), rs4760816 (IVS6+C6106T), rs4290270 (Exon9+A57T), rs17110747 (Exon11+G654A), and IVS11+G129C. No significant associations with clinical parameters were observed (Table **5**).

DISCUSSION

We analyzed the *TPH2* gene polymorphisms in a Japanese population and found ten SNPs and two insertion/deletion variants, among which one variant was novel. However, we failed to identify any variants or haplotypes in the *TPH2* gene examined in this study which were associated with METH dependence/psychosis.

Exon2+C18A is a nonsynonymous SNP and the corresponding amino acid is changed from Ser to Tyr at peptide position 41 (S41Y). This SNP was reported as C2755A by Lin and colleagues in a Han Chinese population [14]. They transfected plasmids containing full-lengthTPH2 protein–encoding sequences with two alternative alleles into SH-SY5Y cells and found that the amount of serotonin in SH-SY5Y cells expressing the 41Y allele was about 36% lower than in cells expressing the 41S allele. Despite the strong scientific rationale for studying polymorphisms in the *TPH2* gene in METH dependence/psychosis, we could not identify any variants or haplotypes associated with the phenotype. These results were comparable to those for cocaine use. Both cocaine and METH increase extracellular dopamine in the brain, and increased dopamine in the nucleus accumbens is thought to underlie the reinforcing effects of drugs of abuse [5, 33]. The association of cocaine dependence in subjects of African descent with TPH2 SNPs was analyzed by Dahl and colleagues, but they failed to identify any SNPs that were associated with the cocaine-dependent phenotype [34]. The disparity between these results and the previously reported results for heroin addiction [29] suggest that the *TPH2* gene has little effect in psychostimulants with the characteristics of indirect dopaminergic agonists.

Our results indicate that the *TPH2* gene variations may not be vulnerability factors in METH dependence/psychosis, and indeed that they are likely to make a small or no contribution to the development of METH dependence/psychosis.

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