use of the cre-lox P recombination system. Conditional BDNF mutant mice were hyperactive after exposure to stressors, and had higher levels of anxiety when evaluated in a light/dark exploration test (Rios et al., 2001). These mutant mice also had mature onset obesity characterized by a dramatic increase in body weight, increased linear growth, and elevated serum levels of leptin, insulin, glucose, and cholesterol. These findings suggest that BDNF performs an essential maintenance function in the regulation of anxiety-related behavior and food intake through central mediators in both the basal and fasting state (Rios et al., 2001).

3. Human studies

3.1. Human serum

Based on the role BDNF plays in eating behavior, it is important to assess the potential contribution of BDNF to the pathophysiology of eating disorders. Recently, we found that serum levels of BDNF in female patients with AN or BN were significantly decreased compared with those of age-matched normal control subjects, and we also found a significant positive correlation between the Bulimic Investigatory Test, Edinburgh (BITE) symptom scale (the severity of symptoms of bulimia), and the Hamilton Depression Rating Scale (HDRS; the severity of depression) in patients (Nakazato et al., 2003). Recently, decreased levels of serum BDNF in patients with AN were also reported in Italian population (Monteleone et al., 2004), consistent with our data (Nakazato et al., 2003). In addition, we reported that the serum levels of BDNF in drug-naive patients with a major depressive disorder were significantly decreased compared with normal controls, and that the serum levels of BDNF were negatively correlated with the HDRS scores in patients (Shimizu et al., 2003). These findings are interesting, since depressive symptoms have a high degree of comorbility with eating disorders (Becker et al., 1999; Kaye et al., 2000; Walsh and Devlin, 1998; Klein and Walsh, 2004; Hsu, 2004). Thus, it should be noted that alteration in eating disorders is greater than that in major depressive disorders, although reduction in serum BDNF may not be a selective for eating disorders. Taken together, it seems that BDNF plays a role in the pathophysiology of eating disorders, although replicated studies using large sample should be conducted to confirm this.

3.2. Genetic studies

Genetic epidemiological studies have provided evidence for a strong genetic contribution to the pathogenesis of eating disorders (Becker et al., 1999; Hinney et al., 2000; Kaye et al., 2000; Kendler, 2001; Walsh and Devlin, 1998; Klein and Walsh, 2004). The human BDNF gene is located on chromosome 11p14, and consists of 11 exons. The 196G/A (val66met) gene polymorphism, which converts a valine (val) to methionine (met) at codon 66 in the 5′ pro-region of the human BDNF gene, encodes a precursor peptide (pro-BDNF; Fig. 1; Lu, 2003b; Egan et al., 2003). Whereas BDNF 196G/A (val66met) gene polymorphism does not affect the function of a mature BDNF protein, it has been shown to dramatically alter the intracellular trafficking and packaging of pro-BDNF, and, thus, the regulated secretion of the mature BDNF protein (Egan et al., 2003). At cellular levels, marked deficits were observed in the intracellular distribution, processing, and secretion of met-BDNF, suggesting that pro-BDNF may play a critical role in synaptic targeting and activity-dependent secretion at synapses (Egan et al., 2003; Lu, 2003b). Remarkably, healthy human subjects with the met allele exhibit impaired hippocampal activity and memory function (Egan et al., 2003). Furthermore, it is also reported that met-BDNF carriers exhibited relatively diminished hippocampal engagement in comparison with valine homozygotes during both encoding and retrieval processes, implicating a specific genetic mechanism for substantial normal variation in human declarative memory (Hariri et al., 2003). These findings suggest that a genetically driven variation in BDNF secretion may significantly impact human hippocampal function and memory (Egan et al., 2003; Lu, 2003b).

A strong association of AN with the restricting type and the BDNF 196G/A (val66met) polymorphism has been demonstrated, suggesting that the met allele may be a susceptibility factor to eating disorders, mainly to the restricting type of AN (Ribases et al., 2003). We also found an association between BDNF 196G/A gene polymorphism and eating disorders (Koizumi et al., 2004). Remarkably, the G/A heterogeneity in patients with the restricting type of AN and patients with the binging/purging type of BN was higher than that of normal controls, although it is currently unknown how this G/A heterogeneity may contribute to susceptibility to eating disorders (Koizumi et al., 2004). Recently, it has been demonstrated that, in cell cortical neurons, met-BDNF could alter the intracellular distribution and activity-dependent secretion of val-BDNF, indicating that components of the regulated secretary machinery interact specifically with a signal in the pro-BDNF (Chen et al., 2004). Thus, it is likely that perturbations in BDNF trafficking may be implicated in the pathogenesis of eating disorders although it is currently unclear how the val-met substitution of pro-BDNF is implicated in the pathophysiology of eating disorders.

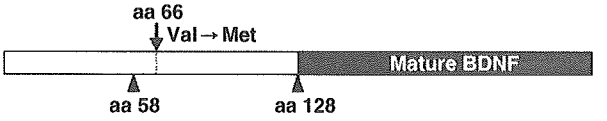

Fig. 1. Pro-BDNF. Arrowheads indicate known protease cleavage sites involved in processing of the mature BDNF form, as well as of the secreted pro-BDNF. Arrow indicates the position of the BDNF 196G/A (val66met) polymorphism.

It has been reported recently that the BDNF 196G/A gene polymorphism is strongly associated to all subtypes (AN, restricting-type AN, binge-eating/purging AN and BN) of eating disorders in six different centers from five European countries (France, Germany, Italy, Spain and UK; Ribases et al., 2004). Taken together, these findings suggest that the BDNF 196G/A (val66met) gene polymorphism might be associated with a susceptibility to eating disorders. Another single nucleotide polymorphism (−270C/T) located in the promoter region (exon 5) of the BDNF gene was associated with BN in the Florencian in Italy and German samples although the joint analysis of all centers did not reveal a positive association between this SNP and ED (Ribases et al., 2004). In contrast, we did not find a positive association between this SNP (−270C/T) and eating disorders in female Japanese samples (Koizumi et al., submitted for publication). Furthermore, it has been reported recently that the 196G/A and −270C/T polymorphisms on the BDNF gene are not associated with AN (118 patients) or BN (80 patients) in the German population (Friedel et al., 2005). To confirm the role of the BDNF gene in pathogenesis of eating disorders larger samples need to be assessed.

Interestingly, we reported recently that the frequency of healthy subjects who carried the A (met) allele was significantly higher in Japanese (41.1%) than in Italians (29.7%) or Americans (18.0%), suggesting an ethnic difference in BDNF 196 G/A (val66met) polymorphism (Shimizu et al., 2004). It is currently unknown whether Japanese have a higher incidence of eating disorders than Italians or Americans. Furthermore, it is doubtful that Japanese have poorer hippocampal and memory function than Europeans and Americans. Future detailed research is needed to determine whether the hippocampal and memory functions of the Japanese are poorer than those of Europeans and Americans.

3.3. Personality traits

A certain type of personality or temperament has been found to be at risk for developing neuropsychiatric diseases, including eating disorders (Becker et al., 1999; Kaye et al., 2000; Fairburn and Harrison, 2003; Barbarich et al., 2003). The mortality rate for AN is approximately 5.6% per decade, and suicide is the second most common cause of death after complications of eating disorders (Sullivan, 1995). It is also reported that women with eating disorders have specific personality traits associated with a suicidal risk, independently of the presence of current depressive symptoms (Youssef et al., 2004). Recently, we reported that female subjects with the met allele of BDNF val66met polymorphism had high scores in reward dependence on the Temperament and Character Inventory, and high scores in extraversion on the revised NEO Personality Inventory (NEO-PI) compared with female subjects with the val allele, suggesting an association between reward dependence (or extraversion) personality traits and the BDNF genotype in female Japanese subjects (Itoh et al., 2004). Furthermore, it has also been reported that this polymorphism is associated with NEO-PI domain neuroticism, a risk factor for depression in Caucasian subjects (Sen et al., 2003). These findings suggest that the BDNF 196G/A (val66met) gene polymorphism could be associated with personality traits which might be implicated in the development of eating disorders.

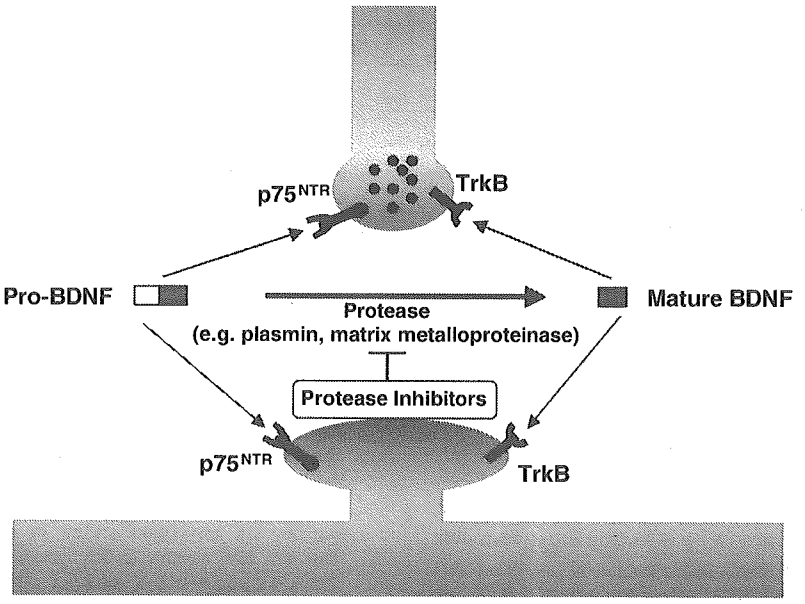

Fig. 2. Extrasynaptic cleavage of pro-BDNF to mature BDNF. Pro-BDNF preferentially binds $p75^{NTR}$. Pro-BDNF is cleaved by proteases (e.g., plasmin, matrix metalloproteinase) at synapses, converting mature BDNF. Mature BDNF preferentially binds TrkB receptor. [With a modification of Lu (2003b)].

4. Concluding remarks

It is well known that disturbances in neuronal systems have been found to play a role in the modulation of feeding, mood, and impulse control. These neuronal systems include neuropeptides [corticotropin-releasing hormone (CRH), opioids, neuropeptide-Y (NPY), peptide YY (PYY), vasopressin, oxytocin, cholecystokinin (CCK), leptin, and ghrelin] and monoamines (5-HT, dopamine, norepinephrine) (Barbarich et al., 2003). Recent studies with positron emission tomography (PET) demonstrated that altered activity of 5-HT neuronal pathway persists after recovery from eating disorders (Frank et al., 2002), and that brain 5-HT_{1A} receptor binding is increased in several cortical areas in patients with BN during their state of impulsive binge eating (Tiihonen et al., 2004). These findings suggest the possibility that these psychobiological alterations might contribute to a vulnerability to the development of eating disorders.

As described above, abnormal eating behaviors in heterozygous BDNF (±) knock-out mice were alleviated by administration of the antidepressants (first choice for the pharmacologic treatment of eating disorders), which can increase the BDNF levels in the brain. Therefore, new agents which can increase the BDNF levels may exert therapeutic activity in patients with eating disorders. As another approach, BDNF gene therapy (Fernandez-Espejo, 2004) would be ideal for delivering therapeutic molecules to site-specific regions of the central nervous system, further supporting the potential feasibility of BDNF therapy as a compliment to other therapies which are employed to alleviate abnormal eating behaviors in patients with eating disorders.

The discovery that pro-BDNF preferentially activates the p75 neurotrophin receptor ($p75^{NTR}$), whereas mature BDNF preferentially binds the TrkB receptors, suggests that the pro-region of BDNF may be critical for $p75^{NTR}$ signaling (Fig. 2; Chao and Bothwell, 2002; Lu, 2003a,b; Barde, 2004) It has also been shown that pro-BDNF is avidly processed by several proteases, including plasmin and specific matrix metalloproteinases (Fig. 2; Lee et al., 2001). Very recently, it has been demonstrated that tissue plasminogen activator (tPA), by activating the extracellular protease plasmin, converts the pro-BDNF to the mature BDNF, and that this conversion is critical for late-phase long-term potentiation in mouse hippocampus (Pang et al., 2004). Thus, it is likely that, as an endogenous extracellular enzyme system, tPA/plasmin is capable of converting proBDNF to mature BDNF. Within the BDNF gene, 196G/A (val66met) gene polymorphism seems to be a good candidate for a causative variant, either by affecting the processing of the mature form or by affecting the interaction of the secreted and the extracellulary processed pre-pro form with $p75^{NTR}$, thus modulating apoptotic signaling through the alternate pathway. Further studies on the genetic analysis of receptors ($p75^{NTR}$ and TrkB) in eating disorders are needed. In the future, we hope to gain a more complete understanding of the signal transduction pathway via the $p75^{NTR}$ and TrkB receptors, in order to provide new perspectives on treating eating disorders.

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ORIGINAL ARTICLE

Haplotype association between $GABA_A$ receptor γ2 subunit gene (GABRG2) and methamphetamine use disorder

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ABSTRACT

Psychostimulant use disorder and schizophrenia have a substantial genetic basis. Evidence from human and animal studies on the involvement of the γ-aminobutyric acid (GABA) system in methamphetamine (METH) use disorder and schizophrenia is mounting. As we tested for the association of the human $GABA_A$ receptor gamma 2 subunit gene (GABRG2) with each diagnostic group, we used a case–control design with a set of 178 subjects with METH use disorder, 288 schizophrenics and 288 controls. First, we screened 96 controls and identified six SNPs in GABRG2, three of whom we newly reported. Next, we selected two SNPs, 315C>T and 1128+99C>A, as representatives of the linkage disequilibrium blocks for further case–control association analysis. Although no associations were found in either allelic or genotypic frequencies, we detected a haplotypic association in GABRG2 with METH use disorder, but not with schizophrenia. This finding partly replicates a recent case–control study of GABRG2 in METH use disorder, and thus indicates that GABRG2 may be one of the susceptibility genes of METH use disorder.

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Keywords: $GABA_A$ γ2 subunit gene; methamphetamine; substance use disorder; polymorphism; haplotype; schizophrenia

INTRODUCTION

In recent years there has been a pronounced increase in use of psychostimulants involving methamphetamine (METH).[1] Lifetime prevalence of psychostimulant use in some developed countries is found in 1–3% of the adult population,[2] and psychostimulant use in any form may lead to abuse or dependence with physiological, psychological and behavioral component.[3] Findings from family and twin studies suggest that the genetic contribution is important for the development of psychostimulant use disorders. Heritability estimates from a population-based twin study for METH use disorder are substantial,[4,5] for example, 66% for psychostimulant abuse.[6]

The dopamine system is a prime candidate for genetic influence on drug abuse, particularly METH abuse, because it is thought to be involved in the reward and reinforcing mechanism in the meso-cortico-limbic system in the nucleus accumbens.[7] Moreover, the primary site of biological activity of METH is the dopamine transporter in this system.

Instead, a role for the γ-aminobutyric acid (GABA) system in drug abuse is also suggested in accumulating evidence. First, the irreversible GABA-transaminase inhibitor, γ-vinyl GABA, attenuates such increase of the dopamine release in the nucleus accumbens following acute administration of METH.[8] Second, QTL mapping for acute alcohol withdrawal severity suggests that a polymorphism in the $GABA_A$ receptor γ2 subunit gene in mice is genetically correlated with this phenotype.[9] A third line of evidence involves several case–control association studies, suggesting that the human $GABA_A$ receptor γ2 subunit gene (GABRG2) is marginally associated with METH use disorder,[10] and is also associated with alcoholism comorbid with antisocial personality disorder,[11] although there are conflicting results.[12,13] Therefore, it is possible that GABRG2 affects vulnerability to substance use disorder, including METH use disorder.

On the other hand, a number of post-mortem studies have reported an altered GABA neurotransmission in schizophrenia. These studies reported that release and uptake of GABA at synaptic terminals were reduced in schizophrenic cortex[14–16] and that the activity of glutamic acid decarboxylase (GAD), the synthesizing enzyme for GABA, GAD mRNA expression, and the density of GABAergic interneurons, were reduced in the prefrontal cortex (PFC) of schizophrenics.[17–20] Although there was reportedly no significant change in overall mRNA levels for $GABA_A$ receptor subunits,[17] expression of the alternately spliced short isoform of $GABA_A$ receptor γ2 subunit, γ2S, was markedly reduced in the PFC of schizophrenics.[21] The relative over-representation of the γ2L subunit, which possesses an additional phosphorylation site within the eight amino acids inserted, should result in a functionally less active form of the receptor,[22,23] and this defective GABAergic system may be involved in the development of schizophrenia. The evidence of linkage analysis from multiple genome scans of schizophrenia within 5q31–34 where GABRG2 locates also support the involvement of this gene in the development of schizophrenia.[24–27]

Here, we explored the possible contributions of GABRG2 in both METH use disorder and schizophrenia. We systemically searched all exons and the intronic branch sites of GABRG2 for polymorphisms, and examined haplotype-based case–control association analysis with both METH use disorder and schizophrenia.

RESULTS

Our screening of 96 controls in all exons and the flanking intronic splice sites of GABRG2 revealed six SNPs, which were designated 'Asn79Ser', '315C>T', '588T>C', '922+20G>A', '1129−1482A>C', and '1230C>T'. Minor allele frequencies and a schematic graph of these SNPs are presented in Table 1 and Figure 1, respectively. Of all identified SNPs, 315C>T, 588T>C (rs211037) and 922+20G>A have been reported elsewhere.

To evaluate the linkage disequilibrium (LD) in the 96 screened samples using several widely used measures (D', Δ_2 and *P*-value), we genotyped five SNPs in GABRG2 (two SNPs (315C>T, 588T>C) of identified SNPs, two SNPs (rs2268583, rs2284780) from the dbSNP database, and one SNP (1128+99C>A) reported as *Bam*HI RFLP previously[11]). These SNPs were selected because they showed sufficient heterozygosity (a frequency of minor allele>0.1) to detect a small effect of a susceptibility gene presumed to underlie complex disorders, and they were distributed almost evenly on the entire exonic regions of the gene (Figure 1).

Table 1 SNPs in GABRG2 and minor allele frequencies

SNP	*SNP position*	*Minor allele frequency*	*Reference*
107+740C>T	Intron 1	0.302	rs2268583
Asn79Ser	Exon 2	0.005	
315C>T	Exon 3	0.300	
588T>C	Exon 5	0.480	rs211037
922+20G>A	Intron 7	0.020	
923−466C>T	Intron 7	0.480	rs2284780
1128+99C>A	Intron 8	0.480	*Bam*HI C>A
1129−1482A>T	Intron 8	0.236	
1230C>T	Exon 9	0.005	

Estimation of LD between each pairwise SNP is presented in Table 2. These results show that the first three and the last two consecutive SNPs were in complete or nearly complete LD with each other. Therefore, we selected two SNPs (315C>T and 1128+99C>A) as representatives of these nearly complete LD regions for further case–control association analysis.

In addition to screened 96 samples, we genotyped 178 subjects with METH use disorder, 288 schizophrenics, and 288 controls in all. Two representative SNPs were in moderate LD with each other in METH use disorder ($D'=0.72$), schizophrenia ($D'=0.51$) and control subjects ($D'=0.61$). Genotypic and allelic frequencies of two SNPs in each population are summarized in Table 3. The genotypic distributions of each SNP did not significantly deviate from the Hardy–Weinberg equilibrium in either METH use disorder, schizophrenia or control subjects ($P=0.98$, 0.84 and 0.70 at 315C>T and $P=0.15$, 0.62 and 0.06 at 1128+99C>A, respectively). The distributions of each SNP did not differ significantly between each diagnostic group and controls in both allele and genotype frequencies (Table 3).

The distributions of haplotypic frequencies estimated using the expectation-maximization algorithm implemented in the Arlequin 2.0 significantly differed between METH use disorder and control subjects ($P=0.044$). In contrast, there was no significant difference in haplotypic distributions between schizophrenic and control subjects ($P=0.356$, Table 4). From examining at-risk haplotypes predisposed to METH use disorder, only two haplotypes, T–C and T–A (defined by 315C>T−1128+99C>A), were found to confer the significant susceptibility to this disorder. By applying the Bonferroni correction, this finding becomes nonsignificant for haplotype T–A (corrected $P=0.120$) and remains

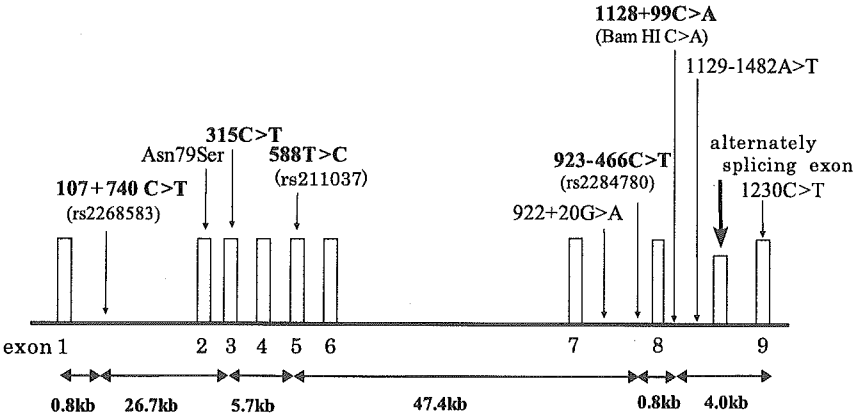

Figure 1 Schematic presentation of identified and reported GABRG2 SNPs. Solid box represents exons. The SNPs in bold type were used to evaluate LD structure.

Table 2 Pairwise linkage disequilibrium in controls

				D' Δ^2		
		rs2268583	*315C>T*	*588T>C*	*rs2284780*	*1128+99C>A*
P-value	rs2268583		1.000 0.976	0.926 0.457	0.376 0.058	0.376 0.058
	315C>T	$<1.0\times10^{-5}$		0.962 0.482	0.608 0.141	0.608 0.141
	588T>C	$<1.0\times10^{-5}$	$<1.0\times10^{-5}$		0.643 0.315	0.643 0.315
	rs2284780	0.002	0.0002	$<1.0\times10^{-5}$		1.000 1.000
	1128+99C>A	0.002	0.0002	$<1.0\times10^{-5}$	$<1.0\times10^{-5}$	

Table 3 Genotypic and allelic distributions of the GABRG2 SNPs in patients with METH use disorder and schizophrenia *vs* controls

SNP	*Sample*	*n*	*Genotype*			*Rarer allele*	*P-value*	
			CC	*CT*	*TT*	*T*	*Genotype*	*Allele*
315C>T	METH	178	87 (49%)	75 (42%)	16 (9%)	107 (30%)	0.374	0.174
	SCZ	288	151 (52%)	116 (40%)	21 (7%)	158 (27%)	0.818	0.594
	Control	288	157 (55%)	113 (39%)	18 (6%)	149 (26%)		
			CC	*CA*	*AA*	*A*	*Genotype*	*Allele*
1128+99C>A	METH	178	56 (31%)	79 (44%)	43 (24%)	165 (46%)	0.603	0.281
	SCZ	288	64 (22%)	139 (48%)	85 (30%)	309 (54%)	0.317	0.238
	Control	288	80 (28%)	128 (44%)	80 (28%)	288 (50%)		

Table 4 Haplotypic distributions of the GABRG2 gene in patients with METH use disorder and schizophrenia *vs* controls

Sample	*Haplotypes (315C>T–1128+99C>A)*				*P-value*
	C–C	*C–A*	*T–C*	*T–A*	
METH	0.275	0.425	0.262	0.039	0.044
SCZ	0.261	0.464	0.202	0.072	0.356
Control	0.314	0.428	0.186	0.072	

significant for haplotype T–C (corrected $P=0.028$). The presumed at-risk haplotype T–C has an estimated frequency of 18.6% among controls and 26.2% among METH use disorder subjects. The estimated odds ratio of haplotype T–C was 1.55 (95% CI (1.13–2.13)).

DISCUSSION

Our results provide supportive evidence for a haplotypic association in GABRG2 with METH use disorder, but not with schizophrenia. This association suggests that the susceptibility variant for METH use disorder may lie within the region in positive LD with the at-risk haplotype reconstructed in this study. The patterns of LD were shown to be two block like, the first block represented by 315C>T (covering rs2268583 at intron 1 to rs211037 at exon 5), and the second block represented by 1128+99C>A (covering rs2284780 at intron 7 to 1128+99C>A at intron 8). Since we found no association between each representative SNP and METH use disorder in either allelic or genotypic frequencies, the possibility arises that susceptibility variant can be located outside of these block-like regions. The second block includes the splicing regulatory elements surrounding the spliced exon, which bind to the polypyrimidine tract binding protein, the splicing regulator.[28–30] Actually, we screened this regulatory region thoroughly through direct sequencing of the 96 samples, however, could not find any variant in these elements. Other splicing regulatory elements that bind to another splicing regulator Nova-1 were located in intron 8, about 3.5 kb downstream of 1128+99C>A.[31,32] If the second block does not cover the latter splicing regulatory elements, these regions can be a susceptible candidate. Recently, a significant association was reported between rs4480617 at the 5′-UTR of GABRG2 and METH use disorder in females.[10] Therefore, this SNP or other variants in the promoter region also can be another candidate. Given that the sample size of 96 used to identify SNPs in this study provides more than 80% power to detect SNPs with about 1% minor allele frequency,[33] we are almost unlikely to overlook common nonsynonymous SNPs predisposed to METH use disorder.

As has been widely discussed, a spurious association can arise because of confounding such as population stratification and clinical heterogeneity, given the problems of reliability due to no use of structured interviews. However, our data are partly in agreement with a recent report[10] that found the significant association between GABRG2 and METH use disorder in females. This provides further corroboration that our haplotypic association with METH uses disorder is not spurious, although potential sources of bias such as ascertainment bias still remain possible. For example, subjects suffering from not only METH use disorder but also METH-induced psychosis are more likely to seek medical care and thus to be ascertained. Such 'spurious comorbidity'[34] of psychosis may account for the apparent association in this study. In the present study, we did not stratify the METH use disorder sample according to the comorbidity of METH-induced psychosis because the sample size was too small for reliable analysis. Although the precise prevalence of the comorbid METH-induced psychosis remains unknown, the data in the late 1940s and early 1950s in Japan indicating that about 10% of METH users had METH-induced psychosis[35] would suggest that comorbid METH-induced psychosis is over-represented in our clinically ascertained sample with METH use disorder.

As no association exists between GABRG2 and schizophrenia in our sample, association between GABRG2 and METH use disorder would not likely be attributable to spurious comorbid METH induced-psychosis, which may share the pathophysiology of susceptibility with schizophrenia, the so-called sensitization phenomena.[35] On the contrary, the comorbid polysubstance-related disorder over-represented in our sample with METH use disorder can account for the apparent association in this study. Indeed, previous findings suggesting nonspecific substance dependence vulnerability[5] supported the existence of such a 'misattributed' association in our study. In addition to concurrent comorbidity, we also cannot deny the possibility of spurious comorbid bias caused by the past comorbid diseases because of not examining the past history of any mental diseases systematically. METH use subjects in our study included a large number of patients who experienced first psychotic symptoms after METH use for a relatively short duration and participants in the special program designed for drug use disorder, in which they could not participate if they suffered from other psychiatric problems. The low levels of comorbidity in METH use subjects may reflect such biased ascertainment.

There is indeed a neuroscientific framework to link GABRG2 and METH use disorder. First, several lines of investigation[7] implicate the mesolimbic dopamine system in psychostimulant-induced motor activity. Furthermore, it was shown in a pharmacological study[36] that a GABAergic system in PFC modulated the motor response to psychostimulants by inhibiting PFC pyramidal neurons. Second, a tentative association was found for a GABRG2 SNP and the frontally located event-related potential (ERP) complex N100/P200 after auditory stimuli.[37] Thus, the prefrontal activation difference may reflect the differential GABRG2 activities derived from variants of the gene. Accordingly, GABRG2 activities in PFC could affect the modulation of mesolimbic reward circuitries, which might be associated with vulnerability of METH use disorder.

Overall our results indicate that GABRG2 may play a role in the risk of METH use disorder development in this population. Analysis of the promoter region or the splicing regulatory elements in intron 8 in a future study would be a logical next step in searching for a susceptible variant of GABRG2 in METH use disorder. However, it remains uncertain whether the associated phenotype may reflect the vulnerability of METH-specific abuse or nonspecific substance abuse.

METHODS

Subjects

All patients in this study were unrelated and recruited from three medical institutes participating the Japanese Genetics Initiative for Drug Abuse (JGAIDA).[38] They were diagnosed according to DSM-IV criteria by the consensus of at least two experienced psychiatrists on the basis of unstructured interviews and review of the medical records prior to genotyping.

The number of the patients with METH uses disorder, comprised of 164 METH-dependent subjects, and 14 METH abuse subjects, and schizophrenia were 178 (144 males and 34 females) and 288 (140 males and 148 females), respectively. The ages of each patient group were 18–69 years old (mean7 SD; 36.77 12.0) and 15–75 (39.67 14.0), respectively. No patient with schizophrenia had severe physical complications or other Axis-I disorders according to DSM-IV when enrolled in this study, because seven schizophrenic subjects with METH use disorder were excluded based on the criteria that restricted a comorbid diagnosis of any psychotic disorder other than METH-induced psychosis. Among the subjects with METH use disorder, 150 (124 males and 25 females) have a comorbid diagnosis of METH-induced psychosis, three of anorexia nervosa, one of obsessive-compulsive disorder, and one of major depressive disorder. Additionally, 119 subjects with METH use disorder have abuse or dependence on drugs other than METH. The past history of any mental illness was not examined. The ages of METH-induced psychotic subgroup were 19–69 years old (37.77 12.3). No patient with METH use disorder had any severe physical complications when enrolled in this study. The 288 unrelated healthy volunteers (152 males and 136 females), aged 19–65 years (33.67 13.0), were comprised of hospital staff members and medical students at Fujita Health University. All healthy controls were also psychiatrically screened based on unstructured interviews. After complete description of the study to each subject, written informed consent was obtained. This study was approved by the ethics committee of each JGAIDA institute.

SNP Identification

Genomic DNA was isolated from whole blood using PUREGNER (Gentra system, Minneapolis, MN 55447, USA). For denaturing high-performance liquid chromatography (DHPLC) analysis, we designed specific primer sets amplifying all GABRG2 exons and the flanking intronic splice sites, based on GenBank sequence (NM000816 and NT023133) (primer sequences are available on request).

Polymerase chain reaction (PCR) was performed in a 10-μl volume containing 10 ng sample DNA, 0.4 M of each primer, 200 μM each dNTP, 1 × PCR Gold Buffer, 1.5 mM $MgCl_2$ and 0.25 U of Amplitaq Goldt (Applied Biosystems Japan Ltd, Tokyo, Japan), using GeneAmpt PCR system 9700 (Applied Biosystems Japan Ltd). PCR cycling conditions consisted of an initial denaturation step at 951C for 9 min, followed by 45 cycles of 951C for 15 s, 601C for 20 s, 721C for 30 s, and ending with a final extension step at 721C for 7 min.

To screen for nucleotide variants, the obtained PCR products from all screened samples were analyzed by DHPLC with the WAVEt system (Transgenomics Japan Ltd, Tokyo, Japan). The PCR products showing variant chromatograms were amplified again and then sequenced with an ABI PRISMt 3100 Genetic Analyzer (Applied Biosystems Japan Ltd). Furthermore, to screen for any kinds of nucleotide variants in the splicing regulatory elements surrounding the spliced exon, we performed direct sequencing of the 96 controls. The conditions for DHPLC analysis and direct sequencing were reported previously.[39]

SNP Genotyping

To confirm the sequencing result and to genotype the variants in additional samples, the DHPLC analysis using the primer extension methods were developed for genotyping 588T>C by modifying the method of Hoogendoorn *et al*,[40] as reported previously.[39] All the remaining SNPs examined were genotyped using PCR-restriction fragment length polymorphism (PCR-RFLP) methods. Of four RFLP sites selected, the *Bam*HI restriction site in the eighth exon was genotyped as described by Loh *et al*,[12] while for the rest of the three SNPs, PCR-RFLP methods were developed (detailed information on experimental procedures is available upon request).

Statistical Analysis

Tests for Hardy–Weinberg equilibrium, the calculation of LD measures such as D′, Δ_2 and *P*-value and the estimation of haplotypic frequencies were carried out using Arlequin software 2.0.[41] The haplotypic frequencies between each patient group and controls were also compared using Arlequin software 2.0. The genotypic and allelic frequencies among each patient group and control group were compared with an exact test, using SPSS (version 10). A two-tailed level of 5% was chosen for the type I error rate. We have not corrected for multiple testing so as to avoid false negative findings.

Following Ohashi and Tokunaga,[40] we estimated the power of association analysis for our sample size of 178 subjects with METH use disorder, 288 schizophrenics and 288 controls under multiplicative model of inheritance, assuming a population susceptibility allele frequency of 0.30 at 315C>T and 0.48 at 1128+99C>A, the value in our screened samples. Setting the type I error rate at 5% and Genotype relative risk at more than 1.4 and 1.5, we obtained more than 80% power for direct association analysis of METH use disorder and schizophrenia, respectively.

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DUALITY OF INTEREST

None declared.

ABBREVIATIONS

METH	*methamphetamine*
GABA	*γ-aminobutyric acid*
GABRG2	*The human $GABA_A$ receptor gamma 2 subunit gene*
GAD	*glutamic acid decarboxylase*
PFC	*prefrontal cortex*
LD	*linkage disequilibrium*
DHPLC	*denaturing high-performance liquid chromatography*
PCR-RFLP	*polymerase chain reaction-restriction fragment length polymorphism*

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Letter to the Editor

Further Analysis of Microsatellite Marker in the *BDNF* Gene

To the Editor:

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin superfamily, which includes growth factors that promote cell survival, differentiation, and cell death. The human *BDNF* gene located on chromosome 11p14 is encoded by a gene of approximately 67 kb and consists of 13 exons (AF 411339). The dinucleotide (AC or GT) repeat in intron 12 was originally reported by Proschel et al. [1992]. There are a number of reports regarding the dinucleotide repeat, indicating both positive and negative associations in several psychiatric diseases such as schizophrenia [Sasaki et al., 1997; Hawi et al., 1998; Wassink et al., 1999], bipolar disorder [Neves-Pereira et al., 2002], obsessive-compulsive disorder [Hall et al., 2003], and childhood onset mood disorders [Strauss et al., 2004]. All of these studies considered it as a simple CA (or GT) repeat. Here, we first report that this repeat consists of three different and continuous dinucleotide repeats [-$(GC)_{n1}$-$(AC)_{n2}$-$(AG)_{n3}$-], not a simple CA (or GT) repeat.

This study was performed after obtaining approval from the ethics committees of Chiba University Graduate School of Medicine, and all subjects provided written informed consent for the use of their DNA samples for this research. Analysis of dinucleotide repeat of the *BDNF* gene was performed using a direct sequencing (see Supplemental Materials).

TABLE I. Allele Frequencies and Heterozygosities of Dinucleotide Repeats in 222 Japanese Female Subjects

$(GC)_{n1}$	
n1 = 6	5 (1.1%)
5	341 (76.8%)
4	58 (13.1%)
0	40 (9.0%)
Hetrozygosity	37.8%
$(AC)_{n2}$	
n2 = 13	8 (1.8%)
12	195 (43.9%)
11	181 (40.8%)
10	8 (1.8%)
9	47 (10.6%)
8	5 (1.1%)
Hetrozygosity	63.1%
$(AG)_{n3}$	
n3 = 3	282 (63.5%)
2	162 (36.5%)
Hetrozygosity	47.7%

It was originally considered as a simple CA (or GT) repeat [Proschel et al., 1992], but we found that this repeat consists of three different and continuous dinucleotide repeats [-$(GC)_{n1}$-$(AC)_{n2}$-$(AG)_{n3}$-] (Table I). The numbers of repeats (n1, n2, and n3) vary among (6, 5, 4, 0), (13, 12, 11, 10, 9, 8) and (3, 2), respectively. Their heterozygosities in control Japanese subjects were 0.378 at $(GC)_{n1}$, 0.631 at $(AC)_{n2}$, and 0.477 at $(AG)_{n3}$.

As mentioned above, there are a number of reports regarding the microsatellite marker in the human *BDNF* gene. Therefore, reanalysis of this microsatellite marker should be needed to confirm an association between dinucleotide repeat (CA or GT repeat) and psychiatric disorders although the functional relevance of the microsatellite marker is currently unknown.

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Research Articles

A Functional Glutathione S-Transferase P1 Gene Polymorphism Is Associated With Methamphetamine-Induced Psychosis in Japanese Population

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Several lines of evidence suggest that oxidative stress plays a role in the mechanisms of action of methamphetamine (MAP) in the human brain. Given the role of glutathione *S*-transferases (GSTs) in the protection against oxidative stress, genes encoding the GSTs have been considered as candidates for association studies of MAP abuse. This study was undertaken to investigate the role of the functional polymorphism of *GSTP1* gene exon 5 (Ile105Val) in the pathogenesis of MAP abuse. Genotyping for *GSTP1* gene polymorphism exon 5 (Ile105Val) in 189 MAP abusers and 199 normal controls was performed by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP). Association between *GSTP1* gene polymorphism and clinical features (prognosis of psychosis (transient-type and prolonged-type), spontaneous relapse (positive and negative), and poly-substance abuse) of MAP abusers was evaluated. Significant differences in the frequency of both alleles ($P = 0.026$, odds ratio: 1.70, 95% confidence intervals (CI) 1.06–2.72) and genotypes ($P = 0.029$) between MAP abusers and controls were detected. In particular, a significant difference in both genotype frequency ($P = 0.013$) and allele frequency ($P = 0.014$, odds ratio: 1.84, 95% CI 1.13–2.97) between MAP abusers with psychosis (transient-type and prolonged-type) and controls was detected. Our findings suggest that the polymorphism (Ile105Val) on exon 5 of the *GSTP1* gene may contribute to a vulnerability to psychosis associated with MAP abuse in Japanese population.

KEY WORDS: **methamphetamine; psychosis; drug abuse; genetic factor; polymorphism**

INTRODUCTION

Abuse of methamphetamine (MAP) is a growing problem worldwide. Some lines of evidence suggest that both environmental and genetic factors might contribute to drug abuse vulnerability [Merikangas et al., 1998; Kendler et al., 2000; Rawson et al., 2002; Uhl et al., 2002; Cami and Farre, 2003]. It is well known that MAP induces a strong psychological dependence, and that repeated further consumption of MAP results in psychotic states, the symptoms of which resemble those of the paranoid type of schizophrenia [Sato et al., 1983, 1992].

Positron emission tomography (PET) imaging studies of the brains of MAP abusers have demonstrated that the density of dopamine (DA) transporters is significantly decreased in the caudate/putamen of MAP abusers [Sekine et al., 2001; Volkow et al., 2001]. Such findings suggest that the long-term use of MAP leads to the damage of dopaminergic neurons in the human brain. It has been shown that MAP-induced neurotoxicity in the brain requires the involvement of striatum DA and

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also involves mechanisms associated with oxidative stress, further suggesting that oxidative stress in dopaminergic pathways might be implicated in MAP-induced neurotoxicity [Cadet et al., 2003]. There are a number of papers demonstrating the neuroprotective effects of glutathione or its related compounds on MAP- or DA-induced neurotoxicity [Choi et al., 2002; Shimizu et al., 2002; Fukami et al., 2004; Hashimoto et al., 2004]. In addition, it is also well known that dopaminergic pathways in the mesocorticolimbic systems can play an important role in drug reward [Kalivas, 2002]. Therefore, polymorphisms in genes that regulate dopaminergic pathways may contribute to interindividual differences as regards a vulnerability to drug abuse [Koob and Le Moal, 1997].

The glutathione *S*-transferases (GSTs: EC 2.5.1.18) belong to a family of multifunctional enzymes that catalyze the conjugation of reduced glutathione with electrophilic groups of a wide variety of compounds including carcinogens, environmental contamination, and products of the oxidative process [Mannervik, 1985; Smythies and Galzigna, 1998; Hayes and Strange, 2000]. Because of their important role in the cellular defense against oxidative stress, GSTs are of interest in the context of association studies of MAP abuse. The genes encoding three classes of GSTs, i.e., GSTM (mu, chromosome 1p13.3), GSTP (pi, chromosome 11q13), and GSTT1 (theta, chromosome 22q11.2), are known to be polymorphic [Watson et al., 1998; Stucker et al., 2002; De Roos et al., 2003; Kelada et al., 2003; Wang et al., 2003]. Recently, we reported an association between *GSTM1* gene deletion and female MAP abusers, suggesting that *GSTM1* gene deletion may contribute to a vulnerability to MAP abuse in Japanese subjects [Koizumi et al., 2004]. Furthermore, it has been reported that genetic polymorphisms of GSTP1 exon 5 (rs947894, Ile105Val (A > G)) and exon 6 (rs1799811, Ala114Val (C > T)) have functional relevance to the GST gene product resulting in reduced GST enzyme activity (~30%) [Board et al., 1989; Zimniak et al., 1994; Ali-Osman et al., 1997; Watson et al., 1998]. Taken together, such findings appear to suggest that individuals with these variant GSTP1 genotypes which result in reduced GSTP1 enzymatic activity may be at greater risk of MAP abuse. In order to verify the potential role of the *GSTP1* gene in the pathogenesis of MAP abuse, we analyzed a polymorphism of the *GSTP1* gene in subjects with a diagnosed MAP-related disorder.

METHODS

This study was performed after obtaining the approval of the ethics committees of each affiliated institute of the Japanese Genetics Initiative for Drug Abuse (JGIDA). All subjects provided written informed consent for the use of their DNA samples for this study. The subjects were 189 patients (149 males and 40 females, age: 36.9 ± 11.9 years (mean ± SD), age range: 18–69 years) with MAP dependence and a psychotic disorder meeting the ICD-10-DCR criteria (F15.2 and F15.5) who were outpatients or inpatients of psychiatric hospitals of the JGIDA (Table I). The control subjects were 199 age-, gender-, and geographical origin-matched normal controls (157 males and 42 females, age: 37.2 ± 10.5 years (mean ± SD), age range: 19–73 years), the majority of whom were with no past history and no family history of drug dependence or psychotic disorders. Diagnoses were made by two trained psychiatrists by interview and available information including hospital records. Patients were excluded if they had a clinical diagnosis of schizophrenia, another psychotic disorder, or an organic mental syndrome as reported previously [Ujike et al., 2003]. All subjects were Japanese, born and living in restricted areas of Japan including northern Kyushu, Setouchi, Tyukyou, Toukai, and Kantou.

The patients were divided into subgroups by some characteristic clinical features (Table I). The patients were divided by poly-substance abuse status, 55 patients abuse MAP only in their lifetime, and 116 patients abuse some other drugs besides MAP in present or past. Organic solvent was most frequently abused besides MAP, followed by marijuana. Cocaine and heroine were rarely abused in the present study. Prognosis of MAP psychosis was various among patients, and some patients showed continuous psychotic symptoms even after MAP discontinuance as previously reported [Sato et al., 1983, 1992]. Therefore, patients were divided into two categories of prognosis, transient-type and prolonged-type, based on duration of psychotic state after MAP discontinuance. Thus, patients with transient-type whose psychotic symptoms improves within 1 month after discontinuance of MAP consumption and beginning of treatment with antipsychotic drugs, and those with prolonged-type whose psychosis continues for more than 1 month even after discontinuance of MAP consumption and beginning of treatment. In this study, patients with transient- and prolonged-types of MAP psychosis were 94 and 65, respectively (Table I). It has been well documented that once MAP psychosis has developed, patients in remission state becomes reliable to spontaneous relapse without re-consumption of MAP [Sato et al., 1983, 1992]. It is postulated that sensitization phenomenon induced by repeated consumption of MAP should be developed in the brain of MAP psychosis patients which result in neural basis for enhanced susceptibility to relapse. Therefore, the patients were divided into two groups according to presence or absence of spontaneous relapse. The patients with and without spontaneous relapse were 62 and 111, respectively (Table I).

Two polymorphisms on exon 5 and exon 6 of the *GSTP1* gene have previously been reported. We analyzed exon 5 (rs947894, Ile105Val) of the *GSTP1* gene in this study, since no minor

TABLE I. Characteristics of Control Subjects and MAP Abusers

Variable	Controls	Abusers	*P*-value
Sex, M/F	157/42	149/40	0.989[a]
Age, mean ± SD, years	37.2 ± 10.5 (19–73)	36.9 ± 11.9 (18–69)	0.813[b]
Prognosis of psychosis			
Transient type		94	
Prolonged type		65	
Spontaneous relapse			
Positive		62	
Negative		111	
Poly-substance abuse			
No		55	
Yes		116	

[a]The comparison between two groups was performed using the χ^2 test.
[b]The comparison between two groups was performed using the *t*-test.

allele frequency of the polymorphism of exon 6 (rs1799811, Ala114Val) was detected among Japanese normal subjects [Ishii et al., 1999]. Genotyping for this gene was performed by PCR-RFLP analysis. The polymorphic site in exon 5 (Ile105-Val) was amplified as reported previously [Wang et al., 2003]. The primers of exon 5 of the *GSTP1* gene were GSTP1-5F (5′-GTAGTTTGCCCAAGGTCAAG-3′) and GSTP1-5R (5′-AGC-CACCTGAGGGGTAAG-3′). After performing PCR, a 433 bp DNA fragment was amplified for GSTP1 exon 5, followed by 2 hr digestion with BsmA I (New England Biolabs, Inc., Beverly, MA). The fragments were separated on 2% agarose gel stained with ethidium bromide. The wild-type (A/A), heterozygous genotype (A/G), and mutant genotype (G/G) yielded two bands (328 and 105 bp), four bands (328, 222, 106, and 105 bp), and three bands (222, 106, and 105 bp), respectively.

The differences between groups were evaluated by Fisher's exact test. The odds ratio and 95% confidence intervals (CI) between two variables were calculated as an estimate of risk. Differences were considered significant at $P < 0.05$.

RESULTS

The frequencies of allele and genotypes in MAP abusers and controls are shown in Table II. The genotype distribution in both MAP abusers and controls was in the Hardy–Weinberg equilibrium. The differences in both genotype frequency ($P = 0.029$) and allele frequency ($P = 0.026$) between MAP abusers and controls were found to be significant (Table II). The frequency of carrying the G allele in MAP abusers was significantly higher ($P = 0.026$, odds ratio: 1.70, 95% CI 1.06–2.72) than that of controls.

Next, we examined the association between the clinical features of MAP abusers (i.e., prognosis of psychosis, spontaneous relapse, and poly-substance abuse) and normal controls. A significant difference in both genotype frequency ($P = 0.013$) and allele frequency ($P = 0.014$, odds ratio: 1.84, 95% CI 1.13–2.97) between MAP abusers with psychosis (transient-type and prolonged-type) and controls was detected (Table II). There was a significant difference in genotype frequency ($P = 0.045$) between MAP abusers with transient-type psychosis and controls, and was a trend toward difference in allele frequency ($P = 0.052$, odds ratio: 1.75, 95% CI 1.01–3.06) between MAP abusers with transient-type psychosis and controls. There was also a significant difference in both genotype frequency ($P = 0.028$) and allele frequency ($P = 0.039$, odds ratio: 1.96, 95% CI 1.07–3.59) between MAP abusers with prolonged-type psychosis and controls. Furthermore, a significant difference in terms of both genotype frequency ($P = 0.009$) and allele frequency ($P = 0.009$, odds ratio: 2.00, 95% CI 1.19–3.35) between MAP abusers with negative spontaneous relapse and controls was detected (Table II). Moreover, there was a trend toward difference in both genotype frequency ($P = 0.052$) and allele frequency ($P = 0.053$, odds ratio: 1.70, 95% CI 1.00–2.88) between MAP abusers with poly-substance abuse and controls (Table II).

DISCUSSION

Our findings suggest that a functional polymorphism (Ile105Val) on exon 5 of the *GSTP1* gene may contribute to MAP abuse vulnerability in Japanese subjects. Since a polymorphism (Ile105Val) on exon 5 has been shown to be of functional significance in terms of enzyme activity [Zimniak et al., 1994; Watson et al., 1998], individuals with the G allele (valine) would be expected to have decreased GST detoxification. Based on the role of GSTs in the antioxidant system preventing MAP-induced neurotoxicity, variant *GSTP1* genes might lead to an excess of metabolic products (e.g., DA-quinone) of the oxidative process induced by the administration of MAP, and may furthermore lead to MAP-induced neurotoxicity in the brain, including damage of the dopaminergic neurons, as compared to the products associated with the A allele (isoleucine) of *GSTP1* gene. We also found that the frequency of the G allele in MAP abusers with psychosis (transient-type and prolonged-type) was significantly higher than that of controls, suggesting that this *GSTP1* gene polymorphism may be associated with MAP-induced psychosis in Japanese subjects. Thus, it appears to be the case that the GSTP1 polymorphism (Ile105Val) on exon 5 may contribute to a susceptibility to MAP-induced psychosis among Japanese subjects. In contrast, we found an association between GSTP1 polymorphism (Ile105Val) and negative spontaneous relapse, whereas no association between this polymorphism and positive spontaneous relapse was detected. Taken together, it seems that GSTP1 polymorphism (Ile105Val) may be implicated in MAP-induced psychosis, but not spontaneous relapse, although further studies using a large sample are necessary.

It has been suggested that DA-quinones synthesized by auto-oxidation of DA might play a role in MAP-induced neurotoxicity in the brain, and that glutathione and GST might play a role in the detoxification against DA-quinone induced neurotoxicity [Smythies and Galzigna, 1998; LaVoie and Hastings, 1999; Whitehead et al., 2001; Shimizu et al., 2002; Asanuma et al., 2003]. Thus, DA auto-oxidation results in the formation of DA-quinones, which readily participate in nucleophilic addition reactions with sulfhydryl groups on free cysteine, glutathione, or cysteine found in proteins including DA transporters [Smythies and Galzigna, 1998;

TABLE II. Genotype and Allele Frequencies of the GSTP1 Exon 5 Gene Polymorphism in Controls and MAP Abusers

Ile105Val (A > G)		Genotype				Allele		
rs947894	n	AA	AG	GG	*P*	A	G	*P*
Control	199	167 (83.9%)	32 (16.1%)	0 (0%)		366 (92.0%)	32 (8.0%)	
Abuser	189	144 (76.2%)	41 (21.7%)	4 (2.1%)	0.029*	329 (87.0%)	49 (13.0%)	0.026*
Prognosis of psychosis	159	119 (74.8%)	36 (22.6%)	4 (2.5%)	0.013*	274 (86.2%)	44 (13.8%)	0.014*
Transient	94	71 (75.5%)	21 (22.3%)	2 (2.1%)	0.045*	163 (86.7%)	25 (13.3%)	0.052
Prolonged	65	48 (73.8%)	15 (23.1%)	2 (3.1%)	0.028*	111 (85.4%)	19 (14.6%)	0.039*
Spontaneous relapse								
Positive	62	50 (80.6%)	11 (17.7%)	1 (1.6%)	0.255	111 (89.5%)	13 (10.5%)	0.463
Negative	111	81(73.0%)	27 (24.3%)	3 (2.7%)	0.009**	189 (85.1%)	33 (14.9%)	0.009**
Poly-substance abuse								
No	55	44 (80.0%)	9 (16.4%)	2 (3.6%)	0.065	97 (88.2%)	13 (11.8 %)	0.254
Yes	116	87 (75.0%)	28 (24.1%)	1 (0.9%)	0.052	202 (87.1%)	30 (12.9%)	0.053

*$P < 0.05$.
**$P < 0.01$ as compared to control (Fisher's exact test).

Whitehead et al., 2001]. Based on the known role of GSTs in the process of antioxidant defense, we considered the possibility that MAP abusers with the G allele of GSTP1 polymorphism were more susceptible to MAP-induced psychosis or to a spontaneous relapse of MAP abuse. In this study, we found significant differences in the distribution of genotype and allele frequencies between MAP abusers with psychosis and controls. Furthermore, we found a significant difference between MAP abusers with negative spontaneous relapse and controls. Taken together, it is likely that the polymorphism (Ile105Val) on exon 5 of the *GSTP1* gene could be a risk factor for the development of MAP-induced psychosis in Japanese subjects.

It is reported that the frequency (18%) of the G allele in Asians such as Taiwanese is lower than that in African-American (42%) and European-American (33%) [Watson et al., 1998]. The frequency (8%; our study) of the G allele in Japanese control subjects is significantly ($\chi^2 = 13.3$, $P = 0.0003$) lower than that (18%) of Taiwanese, suggesting the ethnic difference between Asians and European- and African-Americans for the polymorphism (Ile105Val) on exon 5 of GSTP1. Therefore, it may be of interest to examine the association between the *GSTP1* gene polymorphism and methamphetamine abusers in European- and African-Americans. If replication studies are confirmed, the polymorphism (exon 5 Ile105Val) of GSTP1 would be only the known specific mechanism by which genetic variation leads to a risk for the development of MAP-induced psychosis. Interestingly, our recent PET study demonstrated that the antioxidant *N*-acetyl-L-cysteine (a precursor for glutathione synthesis) could attenuate significantly the reduction of DA transporter in monkey striatum after repeated administration of MAP [Hashimoto et al., 2004]. In addition, we reported that *N*-acetyl-L-cysteine attenuated hyperlocomotion, development of sensitization, and neurotoxicity after administration of MAP [Fukami et al., 2004], suggesting that *N*-acetyl-L-cysteine would be a suitable drug for treatment of MAP abuse. As described in "Introduction," it is likely that endogenous antioxidant glutathione plays a role in the behavioral changes and neurotoxicity associated with MAP abuse. Taken together, our findings may shed light on some of the neurobiological mechanisms and pathways that lead to the development of MAP abuse, and could thereby facilitate the development of novel treatments and prevention strategies for MAP abuse.

In conclusion, our findings indicate that a polymorphism (exon 5 Ile105Val) of the *GSTP1* gene may contribute to a vulnerability to MAP abuse among Japanese subjects. Furthermore, it is likely that this polymorphism (exon 5 Ile105Val) of the *GSTP1* gene could be a risk factor for the development of MAP-induced psychosis in Japanese subjects.

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A nonsynonymous polymorphism in the human fatty acid amide hydrolase gene did not associate with either methamphetamine dependence or schizophrenia

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Abstract

Genetic contributions to the etiology of substance abuse and dependence are topics of major interest. Acute and chronic cannabis use can produce drug-induced psychosis resembling schizophrenia and worsen positive symptoms of schizophrenia. The endocannabinoid system is one of the most important neural signaling pathways implicated in substance abuse and dependence. The fatty acid amide hydrolase (FAAH) is a primary catabolic enzyme of endocannabinoids. To clarify a possible involvement of FAAH in the etiology of methamphetamine dependence/psychosis or schizophrenia, we examined the genetic association of a nonsynonymous polymorphism of the FAAH gene (Pro129Thr) by a case-control study. We found no significant association in allele and genotype frequencies of the polymorphism with either disorder. Because the Pro129Thr polymorphism reduces enzyme instability, it is unlikely that dysfunction of FAAH and enhanced endocannabinoid system induce susceptibility to either methamphetamine dependence/psychosis or schizophrenia.

Keywords: The fatty acid amide hydrolase (FAAH); Methamphetamine dependence/psychosis; Schizophrenia; Nonsynonymous polymorphism; The Pro 129Thr polymorphism

In 1988, the existence of a cannabinoid receptor in the brain was found, and its gene was cloned two years later [7,19]. To date, at least two different cannabinoid receptors, CB1, CB2, and putative endogenous agonists, including anandamide and 2-arachidonylglycerol, have been identified [8]. CB1 receptors are the only cannabinoid receptors that have been found in the central nervous system (CNS). A number of studies

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have indicated that brain CB1 receptors mediate the behavioral and neurochemical properties of cannabis, e.g., marijuana, including the rewarding effect, tolerance, and physical dependence [9,24,30]. CB1 receptors and endogenous agonists are involved not only in cannabinoid dependence but also dependence on other classes of drugs, such as alcohol, morphine, and cocaine [11,22,23,38]. Interaction between cannabinoids and amphetamines, including methamphetamine, has also been reported. Thus, the cannabinoid receptor antagonist AM251 inhibited methamphetamine self-administration by reduction of methamphetamine withdrawal [37]. Another antagonist, SR141716A, potentiated the stimulating effects of amphetamine [18]. The CB1 agonist WIN 55, 212-2 or delta 9-tetrahydrocannabinol, a main ingredient of cannabis, induced cross-sensitization to amphetamine [14,21]. These findings implicate the endocannabinoid system as one of the most important signaling pathways in drug abuse and dependence.

In the 1980s, fatty acid amide hydrolase (FAAH), the integral membrane cannabinoid enzyme, was identified [27]. FAAH is widely expressed in neuronal cells in the CNS, predominantly in the neocortex, hippocampal formation, amygdala, and cerebellum [10,31]. FAAH serves as a primary catabolic regulator of the endogenous cannabinoid ligand anandamide and related fatty acid amide-signaling molecules [15,16]. It has been reported that the metabolic activity of FAAH plays important roles in the CNS by ensuring rapid termination of specific signaling processes of the cannabinoid system [3–5]. Mice lacking FAAH (FAAH −/− mice) are severely impaired in their ability to degrade anandamide. As a consequence, the brain level of anandamide in FAAH −/− mice is increased 15-fold, and they exhibit CB1 receptor-dependent behavioral responses, including less sensitivity to several pain stimuli, hypomotility, hypothermia, analgesia, and catalepsy [2]. Mutation of the FAAH gene may induce dysregulation of the endogenous cannabinoid system, and result in alternation in brain addiction/reward pathways. Recently, Sipe et al. [28] reported that the presence of a polymorphism of the FAAH gene that converts a conserved proline residue to threonine at the 129 position (Pro129Thr) is a risk factor for problem substance abuse and dependence in a Caucasian population. We tried to determine if a genetic association of the Pro129Thr nonsynonymous polymorphism of the FAAH gene in patients with methamphetamine dependence in a Japanese population.

Furthermore, acute and chronic cannabis exposure can precipitate a psychotic state, with hallucinations and delusions resembling schizophrenia. Cannabis consumption also worsens positive symptoms of schizophrenia, and could result in a poor outcome and liability to relapse [12,13,20,29,32]. Two cohort studies showed cannabis use in adolescence increased greatly the risk of schizophrenia in adulthood [1,39]. In addition, recent studies showed an increased density of CB1 receptors in the prefrontal cortex and an increased level of anandamide in the CSF in schizophrenia [6,17,40]. These findings led to a cannabinoid hypothesis of schizophrenia [34]. Therefore, we examined a possible association between Pro129Thr polymorphism of the FAAH gene and schizophrenia.

The subjects were 153 patients with methamphetamine dependence (124 males and 29 females; mean age, 37.8 years; S.D. 12.1 years) meeting the ICD-10-DCR criteria (F15.2), who were outpatients or inpatients in psychiatric hospitals of the Japanese Genetics Initiative for Drug Abuse (JGIDA), and 200 age-, gender-, and geographical origin-matched normal controls (designated Control-1, 162 males and 38 females; mean age, 37.3 years; S.D. 12.1 years), who were mostly medical staff members without a past individual or family history of drug dependence or psychotic disorders. One hundred and forty-one of patients also suffered from methamphetamine psychosis (F15.5, 118 males and 23 females; mean age, 38.5 years; S.D. 12.1 years). The patients with methamphetamine dependence and/or psychosis were divided into several subgroups by clinical features according to age at first consumption, latency of psychosis, prognosis, and multi-substance abuse status. Seventy-six patients (49.7%) had consumed methamphetamine before the age 20 years, and 77 patients (50.3%) had first consumed methamphetamine after they were 20 years old. The latency of psychosis was less than 3 years after the first methamphetamine consumption in 60 patients (42.6%) and 3 or more years in 81 patients (57.4%). Forty-eight patients (31.4%) had abused only methamphetamine during their lifetime, and 105 patients (68.6%) had abused drugs other than methamphetamine in the past or present. Besides methamphetamine, organic solvents were the most frequently abused drugs, followed by marijuana. Cocaine and heroin were rarely abused. The prognosis of methamphetamine psychosis varied among patients, and some patients showed continuous psychotic symptoms even after methamphetamine discontinuance, as previously reported [25,26]. Therefore, patients were divided into two categories of psychosis, the transient type and the prolonged type, based on the duration of the psychotic state after methamphetamine discontinuance, as described in our previous study [36]. Patients with the transient type showed remission of psychotic symptoms within one month after the discontinuance of methamphetamine consumption and beginning of treatment with neuroleptics, and those with the prolonged type had psychosis that continued for more than one month even after this discontinuance of methamphetamine consumption and beginning of neuroleptic treatment. In this study, 85 patients (60.3%) were the transient type and 56 patients (39.7%) were the prolonged type. It has been well-documented that once methamphetamine psychosis has developed, patients in the remission state become liable to spontaneous relapse without reconsumption of methamphetamine [25,26,35]. Such enhanced liability to relapse may result from a sensitization phenomenon developed during methamphetamine abuse, and may be affected by genetic traits [33]. Therefore, the patients were divided into two groups according to the presence or absence of spontaneous relapse. The number of patients with a history of

spontaneous relapse was 65 (46.1%) and those without were 76 (53.9%).

To determine the relationship between the Pro129Th polymorphism and schizophrenia, we examined 260 patients (151 males and 109 females; mean age, 44.5 years; S.D. 13.1 years) fulfilling the ICD-10 diagnostic criteria for schizophrenia. Assessment for diagnosis and subtype of schizophrenia was performed by trained psychiatrists on the basis of all available information, including hospital notes. One hundred and twenty-seven patients (68 males and 59 females; mean age, 39.1 years; S.D. 13.1 years) were diagnosed with the paranoid type, 127 (82 males and 45 females; mean age, 39.6 years; S.D. 13.9 years) with the hebephrenic type, 2 with the catatonic type, and 4 residual type of schizophrenia. Age-, gender-, and geographical origin-matched control subjects for the schizophrenia patients were recruited (designated Control-2, 194 males and 143 females; mean age, 47.2 years; S.D. 11.8 years). Subjects with a positive personal or familial history of major psychiatric disorders were excluded from the control group. This study was performed after obtaining approval from the ethics committees of each institute of JGIDA, and all subjects provided written informed consent for the use of their DNA samples in this research.

The genomic DNA was extracted from peripheral leukocytes using the standard phenol/$CHCl_3$ method. A Pro129Thr polymorphism of the FAAH gene was amplified by polymerase chain reaction (PCR), with 3% dimethyl sulfoxide and 0.75 units of Taq DNA polymerase in a total volume of 15 μl reaction mixture using the following primer sets: 5′-ATG TTG CTG GTT ACC CCT CTC C-3′ and 5′-TCA CAG GGA CGC CAT AGA GCT G-3′. Initial denaturation was performed for 5 min at 95 °C. Then, 35 cycles were performed (30 s of denaturing at 95 °C, 30 s of annealing at the appropriate temperature, and 30 s of extension at 72 °C), followed by a final extension at 72 °C for 5 min. The PCR products were then digested with EcoO109I and analyzed on 3.0% agarose gels.

Deviation of the genotype counts from Hardy–Weinberg equilibrium was tested using a chi-square goodness-of-fit test. The statistical significance of differences in the genotype distribution and allele frequency between patients and controls was assessed by a chi-square test or Fisher's exact test at a significance level of 0.05. All genotyping was performed in a blinded fashion, with the control and case samples mixed randomly. Allele frequencies were calculated using allele-counting methods.

The genotype distribution and allele frequencies of Pro129Thr polymorphism of the FAAH gene for patients with methamphetamine dependence/psychosis or schizophrenia, Control-1, and Control-2 are shown in Tables 1 and 2. The genotype distribution of all patients and controls subjects did not deviate significantly from Hardy–Weinberg equilibrium at the polymorphic locus. No significant differences were found in the frequency of the genotype or allele of the Pro129Thr polymorphism between patients with methamphetamine dependence and Control-1 (genotype, $\chi^2 = 1.22$, d.f. = 2, $P = 0.57$; allele, $\chi^2 = 0.22$, d.f. = 1, $P = 0.68$). No significant differences were found in the frequency of the genotype or allele between subcategories of methamphetamine-dependent patients whose age at first methamphetamine consumption was less 20 years or more than 20 years (genotype, $\chi^2 = 1.80$, d.f. = 2, $P = 0.45$; allele, $\chi^2 = 0.65$, d.f = 1, $P = 0.45$). Nor was there a significant difference between patients with and without multiple substance abuse (genotype, $\chi^2 = 2.01$, d.f. = 2, $P = 0.49$; allele, $\chi^2 = 0.60$, d.f = 1, $P = 0.52$), or patients whose latency of methamphetamine-induced psychosis was less and more than 3 years (genotype, $\chi^2 = 0.66$, d.f. = 2, $P = 0.75$; allele, $\chi^2 = 0.51$, d.f = 1, $P = 0.52$), or between patients with transient and prolonged psychosis (genotype, $\chi^2 = 3.26$, d.f. = 2, $P = 0.19$; allele, $\chi^2 = 3.08$, d.f = 1, $P = 0.11$), or patients with and without spontaneous relapse of psychotic symptoms (genotype, $\chi^2 = 4.56$, d.f. = 2, $P = 0.16$; allele, $\chi^2 = 4.36$, d.f = 1, $P = 0.06$).

No significant differences were found in the frequency of the genotype or allele of the Pro129Thr polymorphism between schizophrenia patients and Control-2 (genotype, $\chi^2 = 0.18$, d.f. = 2, $P = 0.91$; allele, $\chi^2 = 0.01$, d.f. = 1, $P = 0.94$). With regard to the subcategories of schizophrenia, no significant differences were found in the frequency of the genotype or allele between patients with paranoid type schizophrenia and Control-2 (genotype, $\chi^2 = 0.67$, d.f. = 2, $P = 0.63$; allele, $\chi^2 = 0.02$, d.f = 1, $P = 0.92$), or patients with hebephrenic type schizophrenia and Control-2 (genotype, $\chi^2 = 1.15$, d.f. = 2, $P = 0.61$; allele, $\chi^2 = 0.32$, d.f. = 1, $P = 0.62$).

FAAH is a primary and rapid catabolizer of endocannabinoids, such as anandamide and 2-arachidonoyl glycerol, and FAAH knockout mice show a robust increase in brain anandamide. Therefore, homozygosity of a mutant allele of the gene may induce hyperactivity of cannabinoid signaling in the brain. The present study showed no significant association between methamphetamine dependence/psychosis and the Pro129Thr nonsynonymous polymorphism of the FAAH gene. Sipe et al. [28] reported that the Pro129Thr polymorphism is strongly associated with street-drug use and problem drug/alcohol use, especially with illegal drug use by Caucasians. They found that the odds ratios of the mutant 129Thr/Thr homozygote for risk of problem drug/alcohol use and street-drug use in individuals were 4.5 and 2.2, respectively [28]. The Pro129Thr mutation did not significantly impact the catalytic properties of FAAH, but it was found to produce a significantly greater sensitivity to proteolytic degradation, and may have direct effects on the regulation of the FAAH proteins [28]. These findings suggest that dysfunction of FAAH and enhanced endocannabinoid level due to genetic mutation may constitute a risk factor for problem drug use. However, our data indicated that dysfunction of FAAH did not affect the risk of methamphetamine dependence/psychosis in a Japanese population. We also examined a possible association between clinical features of methamphetamine dependence/psychosis, such as age at first methamphetamine