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ORIGINAL ARTICLE

Linkage disequilibrium and association with methamphetamine dependence/psychosis of μ-opioid receptor gene polymorphisms

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Several studies indicate that the μ-opioid receptor plays a role in addiction not only to opiate drugs but also to alcohol and non-opiate addictive drugs. Our studies aim to reveal the associations between gene polymorphisms and methamphetamine (MAP) dependence/psychosis. We newly identified several polymorphisms and four substantial linkage disequilibrium (LD) blocks in the μ-opioid receptor (*OPRM1*) gene. We found significant differences in both genotype and allele frequencies of the single-nucleotide polymorphism (SNP) IVS2+G691C between control ($n=232$) and MAP-dependent/psychotic patients ($n=128$). There was also a significant association between IVS2+G691C and patients with transient psychosis. These results suggest that the *OPRM1* gene variations may be a factor in development and prognosis of MAP psychosis.
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Keywords: single-nucleotide polymorphism; human μ-opioid receptor; methamphetamine; dependence; psychosis

Introduction

Several studies indicate that the μ-opioid receptor plays a role in addiction not only to opiate drugs but also to non-opiate drugs of abuse.[1–5] Positive or negative regulation of the expression and/or function of the μ-opioid receptor may be involved in the mechanisms of drug dependence on both opiate and non-opiate drugs of abuse.[4,6,7] Several single-nucleotide polymorphisms (SNPs) that cause amino-acid substitutions (non-synonymous), synonymous SNPs and SNPs in the non-coding regions and introns in the μ-opioid receptor (*OPRM1*) gene have been reported in African-American and Caucasian populations.[7–9] Various studies of the association between frequencies of polymorphisms in the *OPRM1* gene and drug dependence on opioids, alcohol or other substances in African-American, Caucasian, Hispanic, Han Chinese and Swedish populations have been reported.[10–18] One of these SNPs, A118G, which alters the receptor function,[10] was associated with risk for drug abuse,[14,15] although contradictory data were also reported.[12,13,18]

Psychostimulants including methamphetamine (MAP) exert their reinforcing effects by modulating monoaminergic transmission, of which dopamine is supposed to be the most crucial.[19] The increase in dopamine release through dopamine transporters owing to MAP is primarily responsible for the induction of its reinforcing and psychogenic effects. Opioid receptor agonists are known to affect dopamine neurotransmission[20] and to attenuate MAP-induced enhance-

ment of dopamine neurotransmission.[21,22] Naloxone, an opioid receptor antagonist, potentiated the long-lasting dopamine depletion produced by MAP.[23] Furthermore, μ-opioid receptor-knockout mice display reduced reward from psychostimulants.[24] Other recent studies on animals also indicate complex interactions between the dopamine and opioid systems.[25–29] Therefore, it is possible that variations in the opioid receptor function could produce individual differences in the vulnerability to MAP dependence and/or psychosis. In a previous study, we found a significant association between the SNP A118G and the latency of MAP psychosis, although we did not find any significant association between the vulnerability to MAP dependence/psychosis and polymorphisms in exon 1 and its flanking regions of the *OPRM1* gene in Japanese subjects.[30] Recently, we identified the 3′-end of the major *OPRM1* transcript, MOR-1 mRNA.[31] In the present study, we extended the association studies to the downstream regions beyond the polyadenylation signal in the *OPRM1* gene to identify linkage disequilibrium (LD) blocks, and examined associations between polymorphisms in the *OPRM1* gene and MAP dependence and/or psychosis in Japanese.

Materials and methods

Subjects

One-hundred twenty-eight unrelated patients with MAP dependence/psychosis (99 males and 29 females, 35.9±1.0 years of age) meeting ICD-10-DCR criteria (F15.2 and F15.5) were used as case subjects; they were outpatients or inpatients of psychiatric hospitals. The patients had intravenously injected ($n=95$) or inhaled ($n=33$) MAP. All patients were diagnosed by two trained psychiatrists, and diagnoses were carried out on the basis of both interviews and all available information included in the hospital records. Patients were excluded if they had a clinical diagnosis of schizophrenia, another psychotic disorder or an organic mental syndrome. The 232 control subjects 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, and all subjects provided written informed consent for the use of their DNA samples for this research.

Control samples were used to find *OPRM1* gene polymorphisms in the Japanese population. We first analyzed 44 controls to find polymorphisms and then employed 188 additional samples for intron 2 (total 232), 135 for intron 3 (total 179), and 53 (total 97) and 135 (total 179) for the 3′untranslated region (UTR), respectively, to estimate allelic frequencies.

For the association study, we used 179 controls (139 males and 40 females, 34.6±1.5 years of age) for the IVS3+6113, IVS3+8761, IVS3+G5953A and IVS3+A6151G polymorphic sites, 213 controls (166 males and 47 females, 34.8±1.6 years of age) for the A118G polymorphic site (in our previous study[30]) and 232 controls (181 males and 51 females, 35.2±1.8 years of age) for the IVS2+G691C polymorphic site. Gender, age and geographical distribution were matched without any known bias from a larger pool of control subjects.

Patient subgroups

We divided patients into two or three categories. The clinical characteristics of the subgroups were as follows:

(a) *Latency of psychosis from the first MAP intake*: The course of MAP psychosis varied among patients, and some patients showed psychosis sooner after the first MAP intake, as reported previously.[32,33] The median latency was 3 years. Therefore, patients were divided into two categories based on the latency of the psychotic state after the first MAP intake: less than 3 years ($n=54$, average = 0.83 years) or more than 3 years ($n=53$, average = 9.98 years).

(b) *Duration of psychosis after the last MAP intake*: Some patients showed continued psychotic symptoms despite MAP abstinence, as reported previously.[32,33] Liability for the duration of psychosis may be determined, at least partly, by genetic variation. We previously reported that genetic variation in the dopamine transporter affects the prognosis of the psychotic state.[32,33] Therefore, patients were divided into two categories: transient and prolonged psychosis after MAP abstinence. Patients with transient psychosis showed a reduction of psychotic symptoms within 1 month after the discontinuance of MAP consumption and the beginning of neuroleptic treatment, and prolonged psychosis continued for more than 1 month even after the discontinuance of MAP consumption and the beginning of neuroleptic treatment. In this study, 72 patients had the transient type and 43 had the prolonged type of MAP psychosis.

(c) *Spontaneous relapse*: It has been well documented that once MAP psychosis has developed, patients in the remission phase are liable to spontaneous relapse without MAP reconsumption.[32,33] It is postulated that a sensitization phenomenon induced by repeated consumption of MAP develops in the brains of MAP psychosis patients and is the neural basis for enhanced susceptibility to relapse.[34] Therefore, the patients were divided into two groups according to the presence ($n=86$) or absence ($n=42$) of spontaneous relapse.

(d) *Multiple-drug abuse*: We divided MAP-dependent/psychotic patients into three groups based on drug use, MAP only ($n=36$), MAP plus easily accessible legal substances (e.g., alcohol, inhalants, hypnotics; $n=49$) or MAP plus illegal substances that are relatively difficult to access in Japan (e.g., cocaine, heroin; $n=43$). Note that MAP is easier to obtain in Japan than other illegal substances (e.g., cocaine, cannabis, heroin). The total number of patients in each category was not always 128 because not all data were available for several patients.

Genotyping

Genomic DNAs were extracted from peripheral blood samples by using the standard phenol extraction protocol. Northern blot analysis has revealed that the single predominant transcript of the *OPRM1* gene is the approximately 14 kb long MOR-1 mRNA in the human brain.[35] We analyzed all exons of the MOR-1 mRNA and 5′ and 3′ intronic regions of each exon. We also analyzed the parts of the intron-containing nucleotide repeats, which were found in the database for genomic contig sequence (Genbank Accession No. NT-025741). We independently screened the amino-acid coding regions of the *OPRM1* gene for sequence variations: exon 1 (290 bp) (in a previous study[30]), exons 2–3 (353 and 521 bp), exon 4 (39 bp) and a part (from the stop codon to 2951 bp downstream from the stop codon) of the 3′UTR in exon 4 (Figure 1). The neighboring intron sequences of each exon, intron 2 (773 bp) and some parts of intron 3 were also screened for sequence variations. In order to screen for all possible polymorphisms in the *OPRM1* gene, we first analyzed all regions of all PCR products of genomic DNA from 44 control subjects by using an automated DNA sequencer. Then, the regions that included frequent polymorphisms (allelic frequency >5%) and all exons were analyzed by an automated DNA sequencer using the genomic DNAs of the remaining control subjects. The rest of the 3′UTR of the exon 4 (10 682 bp) and the 197 bp 3′-flanking region (total 10 879 bp) of the gene were similarly analyzed with DNA samples of 12 control subjects and 36 primers. Primers were designed on the basis of the reference genomic contig sequence in the National Center of Biotechnology Information (Genbank Accession No. NT-025741). The LD blocks of the polymorphisms were estimated using HaploBlockFinder Version 0.7 (http://cgi.uc.edu/cgi-bin/kzhang/haploBlockFinder.cgi). D' and r^2 were calculated by using the appropriate formula in the Excel program.[36]

Statistical analysis

The χ^2 test was used for statistical analysis. The statistical significance level was set at $\alpha = 0.05$, and Bonferroni corrections were conducted on association analyses. The Hardy–Weinberg (HW) equilibrium was determined by using the χ^2 test. In the analysis of LD and estimation of haplotype frequencies, genotypic data from 179 control subjects were analyzed by using the Arlequin program available from http://anthro.unige.ch/arlequin.[37] The effect size and power calculations were performed by using the G*Power program (set α value as 0.05) available from http://www.psycho.uni-duesseldorf.de/aap/projects/gpower/.[38]

Results

Analysis of OPRM1 *gene sequence variation*

We analyzed exons 2–3 of the MOR-1 mRNA, parts of the introns and parts of exon 4 of the *OPRM1* gene using genomic DNA samples of Japanese control subjects. We found 19 SNPs (Table 1(1)), one dinucleotide polymorphism (DNP), IVS3+6113 $(GT)_{11-15}$ and one polynucleotide polymorphism (PNP), a 32-base pair repeat IVS3+8761 (GAC ATA TAT CAT AAT ATA TAT TAT CAT ATT AT)$_{2-17}$ (Table 1(2)). None of the polymorphisms deviated from the HW expectations. There was no significant difference in allelic frequencies of polymorphisms between subjects from the Setouchi and Kanto regions, where the majority of our present subjects were born and are living ($P>0.4$; χ^2 tests). The allelic frequency of SNP IVS2+G691C was remarkably higher in Japanese control subjects (81.5%) than in African-American or Caucasian populations (42.5–53.3%).[9,11] We did not find any SNP in the amino-acid coding regions of the *OPRM1* gene in Japanese subjects, except the SNP A118G reported previously.[30]

OPRM1 *genotypes and LD*

LD was quantified by using Lewontin's standardized coefficient D' (Figure 1). We identified a block of LD at the *OPRM1* locus that extended from intron 3 to the 3′UTR. The SNPs (IVS3-G8804A, TAA+G886A, T1371C, G1670A, C2007T, A2109G, G2287A and G2395C), in intron 3 and the 3′UTR, which spanned an 11-kbp sequence, were observed to be in a complete LD relationship (all possible $D'=1.000$). The remaining SNPs within the region, except TAA+A2109G (TAA+1260, 1709, 2274), showed a high LD, suggesting that these SNPs were also in the LD block. To determine the extent of the LD block in the 3′ downstream region, we analyzed sequence variations up to 14 kbp downstream from the stop codon (TAA) with DNA samples of 12 control subjects. We found 42 polymorphisms in this region, and of them, 27 were in absolute LD (all possible $D'=1.000$ and $r^2=1.000$, data not shown).

One representative SNP (TAA+A2109G) was selected from the polymorphisms in absolute LD. Seven SNPs in the rest of the *OPRM1* gene (the 5′-flanking region to the SNP IVS3-G8804A) showed over 5% allelic frequency. Thus, we selected these eight SNPs to calculate D' values. LD analyses showed that LD relationships with significant D' values were observed for all possible combinations of SNPs, except for IVS3+G5953A and IVS3+G8497T (Table 2). LD relationships with significant r^2 values were also observed for the combinations of TAA+A2109G with IVS3+A6151G or IVS3+A8449G. The matrix of D' values revealed a contiguous block of high values ($D'>0.97$) for four sequential markers, IVS3+A6151G to TAA+A2109G. Two regions, from A118G to IVS3+G5807A and from IVS2+G691C to IVS3+G5953A, were also in LD blocks with high D' values ($D'>0.7$). Interestingly, in spite of the diversity of the number of repeats, there were also significant LDs ($P<0.05$ by the Arlequin program) between the DNP and PNP in intron 3 and the eight SNPs (Figure 1), as in the case of the DNP in intron 1.[26] These results suggest at least three recombination positions between A118G and IVS2+G691, IVS3+G5807A and IVS3+G5953A, and IVS3+G5953A and IVS3+A6151G. The following LD blocks were found in the *OPRM1* gene by haplotype analysis using the HaploBlockFinder Version 0.7 program: (A) the region containing SNP A118G (around exon 1), (B) the region containing

Table 1 Polymorphisms found in the *OPRM1* gene of control subjects: (1) list of SNPs and (2) DNP and PNP

Position	*SNP name*	*Allelic frequency (%)*	*Sample size*	*Reported allelic frequency (%)*	*rs number*	*References*
(1) List of SNPs						
Exon 1	A118G	45.3	213	7.5–25.8	rs 1799971	10–12,15,26
Intron 2	IVS2+G31A	2.6	232	4.2	rs 9479757	15
	IVS2+G518A	<1.5	232			
	IVS2+G691C	81.9	232	42.5–53.3	rs 2075572	9,11
Intron 3	IVS3+G5807A	44.4	179		rs 3798683	
	IVS3+G5953A	13.1	179		rs 599548	
	IVS3+A6151G	92.5	179		rs 598682	
	IVS3+A8449G	9.2	179		rs 9384179	
	IVS3+C8497T	31.3	179		rs 9371774	
	IVS3−G8804A	9.5	179		rs 609148	
3′UTR	TAA+G886A	10.8	97			
	TAA+T1306C	<1.5	97			
	TAA+T1371C	10.8	97			
	TAA+G1670A	11.4	44			
	TAA+G1709A	5.7	44			
	TAA+C2007T	11.4	44			
	TAA+A2109G	8.4	179			
	TAA+A2274G	11.7	179			
	TAA+G2287A	90.8	179			
	TAA+G2395C	8.4	179			

(2) DNP and PNP	*Number of repeats*	*11*	*12*	*13*	*14*	*15*
IVS3+6113 (GT)*n*	Control ($2n=358$) (%)	35 (9.8)	1 (0.3)	284 (79.3)	33 (9.2)	5 (1.4)

	Number of repeats	*2*	*—*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
IVS3+8761 (32 bp)*n*	Control ($2n=358$) (%)	34 (9.5)	—	1 (0.3)	12 (3.4)	100 (27.9)	33 (9.2)	118 (33.0)	18 (5.0)	9 (2.5)	6 (1.7)	25 (7.0)	2 (0.6)

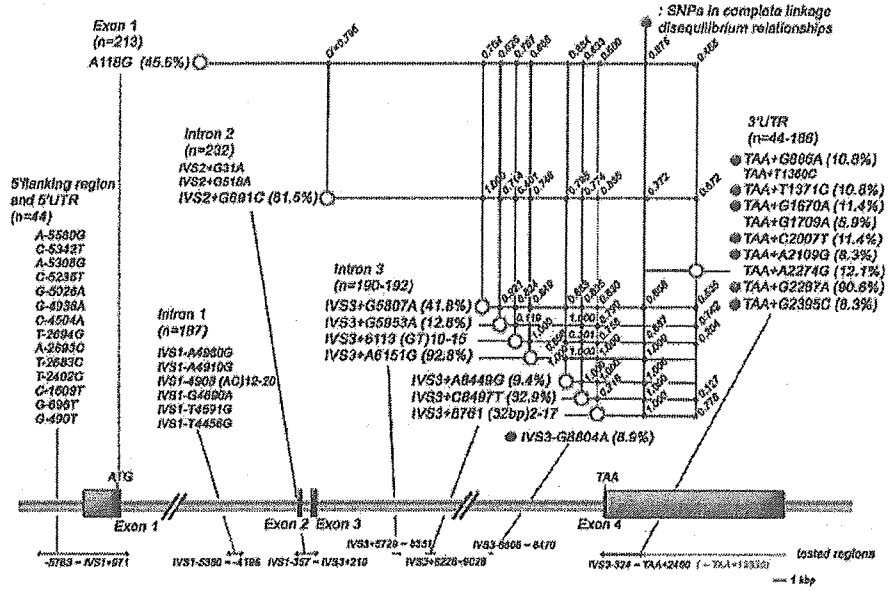

Figure 1 Allelic frequencies and LD relationships of polymorphisms in the *OPRM1* genes of Japanese control subjects. Tested regions (the 5′-flanking region, exon 1 and part of intron 1 were examined in a previous study[30]) are indicated under the scheme of the *OPRM1* gene, and allelic frequencies are shown in the brackets after each polymorphism. The numbers near the small black dots at the crossing of lines represent *D′* values for each combination of polymorphisms. Polymorphisms in the region from TAA + 2400 to TAA + 13 830 (gray bar in tested regions) are not shown because of the small number of subjects ($n = 12$).

Table 2 LD among SNPs in *OPRM1* gene of control subjects

Locus		A118G	IVS2+ G691C	IVS3+ G5807A	IVS3+ G5953A	IVS3+ A6151G	IVS3+ A8449G	IVS3+ C8497T	TAA+ A2109G	TAA+ A2274G	TAA+ G2287A
		D′									
A118G			0.795	0.704	0.626	0.866	0.884	0.633	0.875	0.458	0.890
IVS2+G691C		0.125		1.000	0.714	0.746	0.795	0.775	0.772	0.672	0.795
IVS3+G5807A		0.453	0.134		0.921	0.849	0.883	0.895	0.868	0.535	0.883
IVS3+G5953A		0.052	0.341	0.102		0.023	0.009	1.000	0.034	0.742	0.009
IVS3+A6151G	r^2	0.054	0.201	0.047	0.000		1.000	1.000	1.000	1.000	1.000
IVS3+A8449G		0.069	0.284	0.063	0.000	0.001		1.000	1.000	1.000	0.967
IVS3+C8497T		0.159	0.062	0.291	0.069	0.037	0.046		1.000	0.763	1.000
TAA+A2109G		0.061	0.241	0.055	0.001	0.892	0.901	0.042		1.000	1.000
TAA+A2274G		0.024	0.266	0.030	0.484	0.011	0.014	0.035	0.014		1.000
TAA+G2287A		0.070	0.284	0.063	0.000	0.880	0.934	0.046	0.989	0.014	

Light gray shading indicates a significant LD with a high *D′* (>0.7) and dark gray shading indicates that both *D′* and r^2 values are high.

IVS2 + C691G and IVS3 + G5807A (at least 6.4 kb from intron 2 to about 5.9 kb 3′ downstream from the exon 3–intron 3 junction), (C) the region containing IVS3 + G5953A (around 6 kb 3′ downstream from the exon 3–intron 3 junction) and (D) the region containing other SNPs (at least 23.4 from 6.1 kb 3′ downstream from the exon 3–intron 3 junction to the 3′-end of exon 4).

Relationship between OPRM1 *gene polymorphisms and MAP dependence/psychosis*

We selected four representative SNPs (A118G, IVS2 + G691C, IVS3 + G5953A and IVS3 + A6151G) from the estimated LD blocks by using HaploBlockFinder to study the association between polymorphisms in the *OPRM1* gene and MAP dependence/psychosis. We found significant differences in both genotype and allele frequency at IVS2 + G691C (χ^2 test, $P = 0.012$ and 0.011, respectively) between the controls and all patients with MAP dependence/psychosis (Table 3). These significances were also shown after the Bonferroni correction (corrected significance level was $\alpha = 0.0125$). The calculated statistical powers for the SNPs A118G, IVS2 + G691C, IVS3 + G5953A and IVS3 + A6151G were 0.67, 1.00, 0.41 and 0.93, respectively. Although we further estimated the haplotypes and calculated *D′* values of these

four SNPs, there was no significant difference ($P=0.30$) in haplotypes between the control and MAP-dependent/psychotic subjects (Table 4).

We also studied the association between polymorphisms in the *OPRM1* gene and the clinical parameters (latency of psychosis, psychosis prognosis, spontaneous relapse and multiple-substance abuse). The significant associations are presented in Table 5. Based on the latency of psychosis, three SNPs (A118G, IVS2+G691C and IVS3+A6151G) showed significant differences in genotype frequencies (χ^2 test, $P=0.040$, 0.0085 and 0.027, respectively) between the control and MAP-dependent subjects whose psychosis appeared less than 3 years from the first MAP intake. There was also a significant difference ($P=0.039$) in the SNP IVS2+G691C between the control and MAP subjects whose psychosis appeared more than 3 years after the first MAP intake, but this significance level was lower than that for the short latency group. In the prognosis of psychosis subgroups, only one SNP, IVS2+G691C, showed a significant difference in genotype frequency ($P=0.0034$) between the control and MAP-dependent subjects whose psychosis continued for less than 1 month after the last MAP intake. The association between SNP IVS2+G691C and the transient course of MAP psychosis was still statistically significant after the Bonferroni correction (corrected significance level was $\alpha=0.00625$). Similarly, in the spontaneous relapse subgroups, only the SNP IVS2+G691C showed a significant difference in genotype frequency ($P=0.014$) between the control and MAP subjects with spontaneous relapse. In the multiple-drug abuse subgroups, SNP IVS2+G691C again showed a significant difference in genotype frequency ($P=0.0059$) between the control and MAP-dependent/psychotic subjects who did not use any other drug. Most analyses of subcategories showed enough statistical power to detect a small size effect, although some showed weaker power (A118G for MAP-dependent/psychotic subjects who did not use any other drug, (0.31) IVS3+G5953A for transient prognosis of psychosis (0.29) or for no spontaneous relapse, (0.13) and IVS3+A6151G for later appearance of psychosis (0.16)).

Discussion

Recently, LD mapping has proved to be a powerful tool for identifying genes underlying complex clinical traits.[39,40] The identification of at least four LD blocks in the *OPRM1* gene in the present study may be useful in future association studies of polymorphisms in the *OPRM1* gene with other clinical traits. The incidence of recombination may be high in the *OPRM1* gene relative to other genes owing to its telomeric position on chromosome 6 (6q24-q25). However, the present study indicated that a high degree of LD still exists within the *OPRM1* gene. This preservation of LD was

Table 3 Allelic and genotypic frequencies of SNPs in control and MAP-dependent/psychotic subjects

Locus	Control subjects				MAP-dependent/psychotic subjects				
	Genotypic data		Allelic data		Genotypic data			Allelic data	
	Number	Allelic frequency (%)	Number		Number	Allelic frequency (%)	P-value	Number	P-value
A118G	A 67 (0.31) A/G 99 (0.46) G 47 (0.22) Total 213	45.3	A 233 (0.55) G 193 (0.45) Total 426		A 49 (0.38) A/G 54 (0.43) G 25 (0.19) Total 128	40.6	$P=0.43$	A 152 (0.59) G 104 (0.41) Total 256	$P=0.23$
IVS2+G691C	G 6 (0.03) G/C 72 (0.31) C 154 (0.66) Total 232	81.9	G 84 (0.18) C 380 (0.82) 464		G 12 (0.09) G/C 43 (0.33) C 73 (0.57) Total 128	73.8	$P=0.012$	G 67 (0.26) C 189 (0.74) Total 256	$P=0.011$
IVS3+G5953A	G 135 (0.75) A/G 41 (0.23) A 3 (0.02) Total 179	13.1	G 311 (0.87) A 47 (0.13) Total 358		G 96 (0.75) A/G 28 (0.22) A 4 (0.03) Total 128	14.1	$P=0.70$	G 220 (0.86) A 36 (0.14) Total 256	$P=0.74$
IVS3+A6151G	A 2 (0.01) A/G 23 (0.13) G 154 (0.86) Total 179	92.5	A 27 (0.08) G 331 (0.92) Total 358		A 1 (0.01) A/G 26 (0.20) G 101 (0.79) Total 128	89.1	$P=0.21$	A 28 (0.11) G 228 (0.89) Total 256	$P=0.15$

Table 4 Haplotype analysis in *OPRM1* gene of control and MAP-dependent/psychotic subjects

	Control Frequency (%)	MAP dependence/psychosis Frequency (%)	Haplotype			
			A118G	*IVS2+G691C*	*IVS3+G5953A*	*IVS3+A6151G*
1	0.2	0.0	A	C	A	A
2	2.4	0.5	A	C	A	G
3	1.4	0.0	A	C	G	A
4	32.9	33.6	A	C	G	G
5	0.5	0.0	A	G	A	A
6	8.0	11.5	A	G	A	G
7	5.1	10.9	A	G	G	A
8	2.9	2.8	A	G	G	G
9	0.6	1.5	G	C	A	G
10	44.1	38.2	G	C	G	G
11	1.5	0.5	G	G	A	G
12	0.4	0.0	G	G	G	A
13	0.0	0.4	G	G	G	G

exemplified in the 3′ region of the gene. We found absolute LD among polymorphisms in the region spanning nearly 31 kb from intron 3 to beyond the end of the 3′UTR (TAA + 13.6 kb). This indicates that recombination has been remarkably rare within the block. This long and absolute LD block suggests that a set of many polymorphisms in the block may affect the expression level of the *OPRM1* gene.

We found a significant association between the SNP IVS2 + G691C in intron 2 and MAP dependence/psychosis even after the Bonferroni correction in the present study. This result provides pharmacogenetic evidence for cross-talk between the opioid and dopamine systems, as has been suggested by other studies.[20,27–29,41,42] On the other hand, the SNP A118G was not significantly associated with MAP dependence/psychosis, as shown in our previous report.[30] These results, taken together with the fact that the SNP A118G was found only in the coding regions of the *OPRM1* gene in Japanese subjects, could suggest that polymorphisms in the non-coding regions rather than in the coding regions of the *OPRM1* gene affect MAP dependence/psychosis.

Use of MAP induces a strong psychological dependence, and repeated use frequently results in psychotic states, symptoms of which are similar to those seen in schizophrenia of the paranoid type.[32,34] Interestingly, the SNP IVS2 + G691C was significantly associated with the transient course of MAP psychosis even after the Bonferroni correction. We consider that the SNPs A118G, IVS2 + G691C and IVS3 + A6151G tend to be associated with the short latency of MAP psychosis. Similarly, the SNP IVS2 + G691C tended to be associated with the spontaneous relapse of MAP psychosis. Although these differences were not significant after the Bonferroni correction, we think that these tendencies reflect the existence of an association between *OPRM1* polymorphisms and MAP psychosis. It has also been reported that the Bonferroni correction could be conservative for SNPs in LD.[43] Taken together, these significant differences and tendencies toward differences suggest that these SNPs might be related to the severity of MAP psychosis. In particular, the SNP IVS2 + G691C might be related to a rapid induction of psychosis. Furthermore, the SNP IVS2 + C691G had a tendency to be associated with single-drug abusers of MAP rather than multiple-drug abusers in the present study. This result is consistent with previous reports that the association was not observed in individuals with alcohol and drug dependence who were multiple-drug abusers.[9,11] There are reports that increased frequency of MAP use facilitated the appearance of MAP psychosis.[44,45] However, the information available on our subjects was not sufficient to confirm the relation between the frequency of MAP use and MAP psychosis. Although the statistical power for most analyses of subcategories of MAP psychosis was sufficient to detect an association, there is some possibility that a Type 1 error, the false rejection of the null hypothesis, exists in analyses of some subcategories with weaker powers. Further study with a larger number of subjects is required to determine the detailed association of these SNPs with the subcategories. Although it is unclear whether or not MAP dependence susceptibility has an association with the polymorphisms of the *OPRM1* gene found in our present analysis using MAP dependent/psychotic subjects, the present results of subcategory analyses suggest that the opioid system may be involved in vulnerability to MAP psychosis.

The mechanisms underlying the relationship between the SNP IVS2 + G691C and vulnerability to MAP dependence/psychosis have not yet been clarified. It has been revealed that the sequence variation in the amino-acid non-coding region of the murine μ-opioid receptor (*Oprm1*) gene caused changes in the expression level of mRNA and the analgesic effect of morphine among different strains of mice.[46,47] Furthermore, scatter plot analysis for comparison of the *OPRM1/Oprm1* genes showed highly conserved regions between these species.[7,31] One of the possible mechanisms is that the SNP IVS2 + G691C, and/or other polymorphisms in the LD block, might be located on the sites of DNA-

Table 5 Association between polymorphisms and clinical parameters of MAP psychosis

Locus		*Control subjects*		*MAP dependent/psychotic*							
				Group 1			*Group 2*			*Group 3*	
		Number	*Frequency (%)*	*Number*	*Frequency (%)*		*Number*	*Frequency (%)*		*Number*	*Frequency (%)*
(1) Latency of psychosis				*<3 years*			*⩾3 years*				
A118G	A	67 (0.31)		25 (0.46)			20 (0.38)				
	A/G	99 (0.46)	45.3	24 (0.44)	31.5	$P=0.0404$	25 (0.47)	38.7	$P=0.4691$		
	G	47 (0.22)		5 (0.09)			8 (0.15)				
	Total	213		54			53				
IVS2+G691C	G	6 (0.03)		6 (0.11)			5 (0.09)				
	G/C	72 (0.31)	81.9	20 (0.37)	70.4	$P=0.0085$	19 (0.36)	72.6	$P=0.0394$		
	C	154 (0.66)		28 (0.52)			29 (0.55)				
	Total	232		54			53				
IVS3+A6151G	A	2 (0.01)		0 (0.00)			1 (0.019)				
	G/A	23 (0.13)	92.5	15 (0.28)	86.1	$P=0.0269$	7 (0.132)	91.5	$P=0.9060$		
	G	154 (0.86)		39 (0.72)			45 (0.849)				
	Total	179		54			53				
(2) Prognosis of psychosis				*Transient*			*Prolonged*				
IVS2+G691C	G	6 (0.03)		8 (0.11)			3 (0.07)				
	G/C	72 (0.31)	81.9	27 (0.38)	70.1	$P=0.0034$	13 (0.30)	77.9	$P=0.3303$		
	C	154 (0.66)		37 (0.51)			27 (0.63)				
	Total	232		72			43				
(3) Spontaneous relapse				*Non-existent*			*Existent*				
IVS2+G691C	G	6 (0.03)		6 (0.07)			5 (0.12)				
	G/C	72 (0.31)	81.9	30 (0.35)	75.6	$P=0.1250$	14 (0.33)	71.4	$P=0.0143$		
	C	154 (0.66)		50 (0.58)			23 (0.55)				
	Total	232		86			42		42		
(4) Poly-drug abuse				*None*			*Easily accessible drug*			*Drug difficult to obtain*	
IVS2+G691C	G	6 (0.03)		5 (0.14)			3 (0.06)			1 (0.02)	
	G/C	72 (0.31)	81.9	11 (0.31)	70.8	$P=0.0059$	15 (0.31)	78.6	$P=0.4401$	18 (0.42)	76.7 $P=0.3801$
	C	154 (0.66)		20 (0.56)			31 (0.63)			24 (0.56)	
	Total	232		36			49			43	

binding proteins and/or other modulators, and thus can change the expression level of the *OPRM1* gene. A comparison of expression levels of the *OPRM1* gene in individuals with the different forms of the variants may help to elucidate the roles of the variants.

Twenty-thousand MAP abusers in Japan have been arrested annually in the last 30 years. The number of MAP abusers in Japan has recently been stable, although the number was larger at the end of the Second World War.[45,48] In Japanese MAP abusers, single-drug abuse is the most common form, because MAP is relatively easy to obtain among illegal substances, whereas other illegal substances (e.g., cocaine, cannabis, heroin) are quite difficult to obtain. In Japan, the number of MAP abusers arrested was 100 times more than abusers of cocaine or cannabis. MAP abuse is not limited to specific Japanese populations. For example, high school students have recently started to use MAP casually to lose weight or just for fun.[45] Further, more than 80% of MAP abusers show MAP psychosis, and MAP may be one of the causes of the recent increase in crime. Thus, MAP abuse is a very serious and noticeable social problem in Japan. The present findings may lead to a better understanding of genetic vulnerability to MAP abuse and might provide suggestions to solve this problem.

The cross-talk between the opioid and dopamine systems underscores the role of the μ-opioid receptor in dependence on non-opiate drugs. The present findings suggest that the sequence variation of the *OPRM1* gene may be useful as a genetic marker to predict the development and prognosis of MAP psychosis.

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Duality of interest

None declared.

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No association was found between a functional SNP in ZDHHC8 and schizophrenia in a Japanese case–control population

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Abstract

ZDHHC8 is a new and attractive candidate for a schizophrenia-susceptibility factor. First, several lines of linkage studies showed that 22q11, on which ZDHHC8 is located, is a "hot" region. Second, fine linkage disequilibrium mapping revealed a significant association around ZDHHC8. Moreover, a very recent study reported that one single nucleotide polymorphism (SNP: rs175174) in ZDHHC8 might affect the splicing process, the ZDHHC8 knock-out mice showed the gender-specific phenotype, and the transmission disequilibrium test (TDT) using this SNP also showed significant association with human female schizophrenia. Thus, we attempted a replication study of this SNP using relatively large Japanese case–control samples (561 schizophrenics and 529 controls). No association was found between schizophrenia and controls even after dividing samples by gender. Because our sample size provided quite high power, ZDHHC8 may not play a major role in Japanese schizophrenia. And our results did not support the gender-specific effect of this SNP.

Keywords: Chromosome 22q11; Gender difference; Candidate gene

The 22q11 region (OMIM: #600850 SCZD4) is associated with increased risk for schizophrenia [2]. Two independent meta-analyses of linkage studies showed the linkage around 22q11 [1,5], although one negative result was also reported [8]. This chromosome region contains at least three genes, COMT [12], PRODH2 and DGCR6 [7], implicated as susceptibility genes for schizophrenia.

Recently, ZDHHC8 was reported as a new and attractive candidate gene on 22q11 from the evidence of a genetic association study and animal study [6,9]. In the initial genetic association study, Liu et al. showed that three single nucleotide polymorphisms (SNPs) in ZDHHC8 were associated with schizophrenia. One of these SNPs (rs175174), which was located in intron 4 of ZDHHC8, showed the most highly significant *P* value [6]. This intronic SNP seemed to modify ZDHHC8 expression by causing imperfect splicing, intron retention and reduced enzyme activity. In addition, *Zdhhc8* knockout mice had a gender-dependent dimorphic deficit in prepulse inhibition similar to schizophrenia and reactivity to the psychomimetic *N*-methyl-D-aspartate (NMDA) receptor blocker dizocilpine. In the light of these findings, the transmission disequilibrium test (TDT) divided samples according to gender differences, revealing that human female schizophrenia was significantly associated with this SNP [9]. Thus, we here provide a replication study of rs175174 in ZDHHC8 using Japanese case–control samples.

A total of 561 patients with schizophrenia (259 female; mean age ± standard deviation (S.D.) 49.6 ± 16.4 years: 302

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male; 47.0 ± 14.9) and 529 controls (270 female; 39.7 ± 15.4 years: 259 male; 34.9 ± 12.4 years) were genotyped for association analysis of rs175174. Moreover, in additional linkage disequilibrium (LD) mapping around this SNP, 95 schizophrenic patients (50 female and 45 male) and 96 controls (44 female and 52 male), part of each sample used in association analysis, were genotyped for three SNPs. The general characterization of these subjects and a description of their psychiatric assessment according to identical criteria were published elsewhere [13]. After explaining the study to all subjects, written informed consent was obtained from each. This study was approved by the Ethics Committee of the Nagoya University Graduate School of Medicine and Fujita Health University.

Genomic DNA was extracted from peripheral blood of all subjects. For rapid genotyping of SNPs, rs175174 and additional three SNPs for LD mapping (rs175169, rs175175 and rs2292570), polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) assays were developed. The information of PCR primers is available on request. The PCR reactions of all SNPs were carried out in a 10 μl volume containing 10 ng genomic DNA, 0.4 M of each primer, 200 μM of dNTP, 1× PCR Gold Buffer, 1.5 mM $MgCl_2$ and 0.25 U of Amplitaq Gold™ (Applied Biosystems Japan Ltd., Tokyo, Japan), using the GeneAmp™ PCR system 9700 (Applied Biosystems Japan Ltd.). PCR cycling conditions consisted of an initial denaturation step at 95 °C for 9 min, followed by 45 cycles of 95 °C for 15 s, 56 °C for 20 s, 72 °C for 30 s, and ending with a final extension step at 72 °C for 7 min. PCR product was digested using appropriate restriction enzymes according to the manufacture's recommendation (New England Biolabs, England, UK) (Table 1). DNA fragments were resolved by electrophoresis in a 6% acrylamide gel stained with ethidium bromide.

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by χ^2 test. Marker-trait association analysis was also evaluated by χ^2 test (SPSS 10.0J, SPSS Japan Inc., Japan). To evaluate pairwise LD matrices among SNPs (by D' and r^2), we used the software HAPLOVIEW version 2.05 (developed in Mark Daly's lab., URL; http://www.broad.mit.edu/personal/jcbarret/haploview/index.php). This software also defined "LD blocks" by reasonable criteria based on 95% confidential bounds on D' values [4]. Power calculation was performed using a statistical program prepared by Ohashi et al. [10]. The significance level for all statistical tests was 0.05.

In view of the gender differences in gene effects, we included analyses of samples divided according to the gender. Both in cases and controls, genotype frequencies of total, female and male samples were not significantly different from HWE.

In association analysis, we could not find associations of rs175174 with schizophrenia in either male or female (Table 2).

Next, to test whether rs175174 is representative for ZDHHC8 or not, we performed LD mapping using three additional SNPs around ZDHHC8 (Fig. 1). LD matrices between each pair of SNPs showed strong LD both in cases and controls (Table 1). Even after dividing samples according to the gender, all LD patterns showed the same trends (data not shown). These findings may suggest that the LD pattern of ZDHHC8 is a block-like pattern and that rs175174 is the "representative SNP" of this gene.

The power based on genotype relative risk (GRR) was calculated to evaluate the non-significant results due to type II error. When we set the GRR at 1.28, 1.42 and 1.40 in all, female and male samples, respectively (multiplicative model), our sample size provided powers of more than 80%.

We could not replicate an original positive association using TDT of ZDHHC8 with schizophrenia by the present case–control association analysis among Japanese. Nor could we replicate the gender-specific effect of the risk SNP. In this association analysis, our sample sizes provide enough power to deny the hypothesis. We also performed the fine LD mapping of Japanese samples and showed that the LD pattern of ZDHHC8 was the same block-like pattern as one of the samples from the United States and South Africa. The results provide evidence that not only rs175174 but also ZDHHC8 would not be a susceptibility factor for schizophrenia in either Japanese females or males. The discrepancy between Japanese and the samples from the United States and South Africa may derive from ethnic differences.

A couple of limitation should be addressed to discuss the present results. Initially, the mean age of controls is much younger than that of patients in the present study. This means that a number of young controls, although not more than five subjects given a lifetime morbidity risk of 0.8–1.0%, may go on to develop schizophrenia. This confounding factor might weaken the power of the present study. Another limitation

Table 1
SNPs in LD mapping and pairwise LD matrices

SNP ID	D'				Restriction enzyme
	rs175169	rs175174	rs175175	rs2292570	
rs175169		0.97 (0.78)	1.0 (0.29)	1.0 (0.67)	*Bsl*I
rs175174	0.97 (0.80)		1.0 (0.36)	1.0 (0.58)	*Bse*RI
rs175175	1.0 (0.26)	1.0 (0.31)		1.0 (0.21)	*Alw*NI
rs2292570	0.93 (0.76)	0.97 (0.70)	1.0 (0.23)		*Tsp*RI

Upper diagonal figures are D' (r^2) of controls and lower diagonal figures are D' (r^2) of schizophrenia.

Table 2
Association analysis of rs175174

Samples	Number	G/G	G/A	A/A	*P* value (genotype)	MAF[a]	*P* value (allele)
Total							
SCZ	561	238	245	78		0.357	
CON	529	205	259	65	0.213	0.368	0.618
Female							
SCZ	259	114	106	39		0.355	
CON	270	112	130	28	0.133	0.344	0.714
Male							
SCZ	302	124	139	39		0.359	
CON	259	93	129	37	0.457	0.392	0.260

SCZ: schizophrenia; CON: control.
[a] Minor allele frequency.

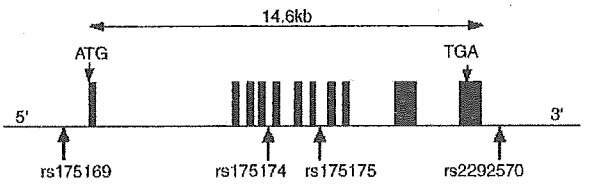

Fig. 1. Genomic structure of ZDHHC8 and SNPs used in association analysis and LD mapping. Vertical bars represent exons of ZDHHC8, and each number under arrows represents SNP ID.

which must be exercised is that the other candidates related to the neurodevelopmental and neuroprotective effect of ZDHHC8 would be in locus heterogeneity [11]. For example, ZDHHC8 encodes a putative transmembrane palmitoyltransferase modulating numerous classes of neuronal proteins including proteins important for neuronal development, neurotransmitter receptors such as NMDA [3]. Thus, the combined effect between ZDHHC8 and the other genes might be a stronger predisposing factor. Further genetic analysis including related candidate genes would definitely be required for a conclusive result.

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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

Table 1
Fatty acid amide hydrolase (FAAH) genotype distributions and allele frequency in patients with methamphetamine (METH) dependence/psychosis

Group	N	Genotype				Allele		
		Pro/Pro (%)	Pro/Thr (%)	Thr/Thr (%)	P-value	Pro (%)	Thr (%)	P-value
Control-1	200	139 (69.5)	58 (29.0)	3 (1.5)		336 (84.0)	64 (16.0)	
METH dependence/psychosis	153	105 (68.6)	43 (28.1)	5 (3.3)	0.57	253 (82.7)	53 (17.3)	0.68
Age of first use								
<20 years	76	49 (64.5)	25 (32.9)	2 (2.6)		123 (81.0)	29 (19.1)	
≥20 years	77	56 (72.7)	18 (23.4)	3 (3.9)	0.45	130 (84.4)	24 (15.6)	0.45
Multi-substance abuse								
No	48	30 (62.5)	17 (35.5)	1 (2.1)		77 (80.2)	19 (19.8)	
Yes	105	75 (71.4)	26 (24.8)	4 (3.8)	0.49	176 (83.8)	34 (16.2)	0.52
Latency of psychosis								
<3 years	60	43 (71.7)	16 (26.7)	1 (1.7)		102 (85.0)	18 (15.0)	
≥3 years	81	54 (66.7)	24 (29.6)	3 (3.7)	0.75	132 (81.5)	30 (18.5)	0.52
Prognosis of psychosis								
Transient	85	53 (62.4)	29 (34.1)	3 (3.5)		135 (79.4)	35 (20.6)	
Prolonged	56	43 (76.8)	12 (21.4)	1 (1.8)	0.19	98 (87.5)	14 (12.5)	0.11
Spontaneous relapse of pyschotic symptoms								
No	76	47 (61.8)	26 (34.2)	3 (3.9)		120 (78.9)	32 (21.1)	
Yes	65	50 (76.9)	14 (21.5)	1 (1.5)	0.16	114 (87.7)	16 (12.3)	0.06

Numbers in parentheses indicate percentages. Statistical analysis was performed by a chi-square test of Fisher's exact test.

Table 2
FAAH genotype distributions and allele frequency in schizophrenia patients

Group	N	Genotype				Allele		
		Pro/Pro (%)	Pro/Thr (%)	Thr/Thr (%)	P-value	Pro (%)	Thr (%)	P-value
Control-2	337	233 (69.1)	99 (29.4)	5 (1.5)		565 (84.0)	109 (16.0)	
Schizophrenia	260	180 (69.2)	75 (28.8)	5 (1.9)	0.91	435 (83.7)	85 (16.3)	0.94
Paranoid type	127	90 (70.9)	34 (26.8)	3 (2.4)	0.63	214 (84.3)	40 (15.7)	0.92
Hebephrenic type	127	83 (65.4)	43 (33.9)	1 (0.0)	0.61	209 (82.3)	45 (17.7)	0.62

Numbers in parentheses indicate percentages. Statistical analysis was performed by a chi-square test of Fisher's exact test.

consumption, latency to onset of psychosis, prognosis, spontaneous relapse, and multi-substance abuse status but found no significant association with any clinical feature. The discrepancy between the previous and present findings may result from differences of substance class. The present study analyzed methamphetamine abusers, however, Spine et al. analyzed "street-drug users". Although, they did not specify the kinds of drugs in their paper, marijuana use has been epidemic in the US, followed by cocaine and morphine. It is possible that the majority of patients examined in the previous study abuse marijuana and that is why the "street-drug use" of the previous study was associated with a mutant allele of the FAAH gene. This hypothesis should be addressed in future study. Alternatively, the ethnicity of the subjects must be considered. Our subjects were Japanese, and theirs were Caucasian. Japanese controls showed the 129Thr allele at 14.6–16.0%, compared to Caucasians at 28.3–30.6%. Homozygosity of the mutant allele was observed in only 1.5% of Japanese subjects. The rarity of the mutant homozygote in our Japanese population may result in a lack of genetic risk of the FAAH gene for Japanese "street-drug users".

No significant association with the Pro129Thr nonsynonymous polymorphism of the FAAH gene with schizophrenia was revealed. This is consistent with Sipe's study of a Caucasian population. Previously, we reported that a triplet repeat polymorphism of the CNR1 gene, which encodes the human CB1 cannabinoid receptor, was significantly associated with patients with schizophrenia, especially the hebephrenic subtype [36]. Several clinical studies have shown that exogenous cannabinoid ligands could precipitate schizophrenia, worsen prognosis, and induce relapse. Our present and previous genetic findings of the endocannabinoid system indicated that variants of cannabinoid receptors, rather than an altered endogenous agonist produced by the FAAH variant, may be important in the etiology of schizophrenia.

The power analysis showed that the present sample size had a power of 0.89 and 0.98 to detect a small effect size ($w = 0.12$) at an alpha value of 0.05 to detect significant allelic associations between Control-1 and total methamphetamine patients and between Conrol-2 and total schizophrenic patients, respectively. The present total sample size can be therefore considered to be large enough statistically. However, the

statistical power deteriorated in the analysis with regard to the subgroups of patients, and our results must be verified with a larger sample to conclude.

In conclusion, the cannabinoid pathway may be implicated in drug abuse, addiction, and also the pathophysiology of schizophrenia, but the Pro129Thr nonsynonymous polymorphism of the FAAH gene is not significantly associated with either methamphetamine dependence/psychosis or schizophrenia, at least in a Japanese population.

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