Table 1
Distribution of the polymorphisms in the *RGS4* gene

SNP marker	Controls			Minor allele frequency		Schizophrenics			Minor allele frequency	Statistics Genotype	Statistics Allele	
										p value	X^2	*p* value
rs10917670	aa	ag	gg			aa	ag	gg				
(*n*=1815)	20.2%	49.3%	30.5%	0.455	(*n*=1805)	21.7%	48.0%	30.8%	0.449	0.594	0.30	0.584(0.924)
rs951436	gg	gt	tt			gg	gt	tt				
(*n*=1918)	21.9%	48.6%	29.5%	0.468	(*n*=1918)	21.3%	51.0%	27.6%	0.462	0.554	0.33	0.566(0.909)
rs951439	gg	ga	aa			gg	ga	aa				
(*n*=1904)	30.1%	50.8%	19.1%	0.427	(*n*=1911)	32.5%	50.0%	17.9%	0.445	0.112	2.47	0.116(0.270)
rs2661319	gg	ga	aa			gg	ga	aa				
(*n*=1911)	14.0%	45.3%	40.7%	0.381	(*n*=1915)	13.8%	48.5%	37.7%	0.367	0.212	1.63	0.202(0.429)

Genotype-based association was tested with Cochran-Armitage test for trend.
Allele-based association was tested: nominal *p* values (and corrected *p* values for multiple testing (with permutation test)) were shown.

2.3. Statistical analysis

The Hardy–Weinberg equilibrium, linkage disequilibrium and allelic/haplotype frequencies, as well as an association between SNP or haplotype and schizophrenia, were determined with the Haploview software program (http://www.broad.mit.edu/mpg/haploview/). Permutation tests were also performed to calculate corrected *p* values for multiple testing by the Haploview software. Genotype-based association was tested with Cochran–Armitage test for trend. Statistical significance was accepted at $p<0.05$.

3. Results

Genotypic distributions of the four SNPs among the subject groups are shown in Table 1. Distributions of all four SNPs did not differ from the Hardy–Weinberg equilibrium. No association with schizophrenia was detected with the rs10917670 ($p=0.58(0.92)$), rs951436 ($p=0.57(0.91)$), rs951439 ($p=0.12(0.27)$), and rs2661319 ($p=0.20(0.43)$). These four SNPs were in linkage disequilibrium each other ($D'=0.84$–0.97, $r^2=0.35$–0.72) (Table 2). ATAA and GGGG were the major haplotypes of the gene, as observed in other ethnic groups. The frequencies of these haplotypes did not differ significantly between the schizophrenia and control groups (Table 3).

Table 2
Linkage disequilibrium between SNPs in Japanese population

	rs10917670	rs951436	rs951439	rs2661319
rs10917670		0.85	0.87	0.84
rs951436	0.52		0.96	0.97
rs951439	0.72	0.62		0.96
rs2661319	0.35	0.65	0.42	

Values at upper right show *D*' and those at lower left do R^2.

4. Discussion

Talkowski et al. (2006) reported a meta-analysis of *RGS4* polymorphisms in schizophrenia based on genotype data from 13,807 individuals. Although significant associations with individual SNPs/haplotypes were not observed, global analysis revealed significant transmission distortion, in particular, overtransmission of two common haplotypes that account for the vast majority of all haplotypes. In their meta-analysis of case-control associations, a modest association was detected when the two common haplotypes were combined and compared against all other haplotypes combined (frequency of two common haplotypes/frequency of rare haplotypes: cases, 0.834/0.166; control samples, 0.817/0.183; $p=0.09$). This association was markedly significant in a Chinese population (cases, 0.857/0.143; control samples, 0.518/0.482; $p=0.0001$) (Talkowski et al., 2006), although another Chinese case-control study did not show this association (Guo et al., 2006). In the present study, the frequencies of these haplotypes were similar between the two groups (cases, 0.742/0.258; control samples, 0.747/0.253).

Talkowski et al. (2006) reported that separate analyses of 3486 cases and 3755 controls samples detected a

Table 3
Haplotype distribution of the RGS4 gene

Haplotype	Frequeicy in the controls	Frequency in the schizophrenics	X^2	*p* value
ATAA	0.396	0.410	1.39	0.239(0.782)
GGGG	0.346	0.337	0.62	0.431(0.970)
GTGA	0.089	0.090	0.01	0.911(1.00)
GGGA	0.083	0.089	1.18	0.277(0.847)
GTAA	0.025	0.024	0.20	0.654(1.000)
AGGG	0.027	0.019	5.83	0.016(0.066)
ATGA	0.016	0.011	3.22	0.073(0.313)

Nominal *p* values and corrected *p* values for multiple testing (in parenthesis) were shown.

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significant association with rs951436 (p=0.01). In a Chinese population, significant association of the G allele of rs951436 was reported (OR=1.54, p=0.002) (Talkowski et al., 2006), although another study did not report this association (OR=1.19, p=0.11) (Guo et al., 2006). We calculated the power of our study on the basis of the genotype data. With a genetic relative risk of 1.15, and assuming an α value of 0.05 and a risk allele frequency of 0.4, the sample size of the present study had a power of 0.92. Therefore, the present study could validly conclude that an association between SNP 4 and schizophrenia is not likely, even in East Asian populations.

The *RGS4* gene is small, spanning approximately 7 kb, and the four SNPs that have been reported to be associated with schizophrenia are included in one haplotype block with two major haplotypes in the Caucasian and East Asian populations (http://www.hapmap.org/cgi-perl/gbrowse/hapmap_B35/). The haplotype block extends from the 5′ flanking region to the 5′ midregion of the *RGS4* gene. Subjects of the meta-analysis of Talkowski et al. (2006) numbered 13,807, and subjects in our study numbered 3821. Neither study indicates a contribution of the haplotype block to the genetic susceptibility to schizophrenia. Results of the present study clearly exclude the 5′ genomic region of the *RGS4* gene in the susceptibility to schizophrenia. However, this does not exclude the possibility of genetic variation(s) in the 3′ region of the gene. Further analysis, particularly resequencing of this region, is necessary.

5. Author disclosure

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Author Ishiguro designed the study, ran the experiment and wrote the manuscript. Author Horiuchi and Koga prepared the sample analyzed. Author Inada, Iwata, Ozaki, Ujike, Muratake, Someya, managed the sample collection. Author Arinami undertook the statistical analysis and supervised this study.

No author has conflict of interest.

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No association between the glutamate decarboxylase 67 gene (GAD1) and schizophrenia in the Japanese population

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Abstract

Postmortem studies regarding schizophrenia revealed altered expression of genes related to γ-amino butyric acid neurotransmission system. One of the most consistent findings is the reduced level of 67 kDa glutamic acid decarboxylase isoform (GAD_{67}). Moreover, several studies reported positive associations between the GAD_{67} gene (GAD1) and schizophrenia. These reasons, motivated us to carry out replication study regarding association between GAD1 (fourteen tagging SNPs) and schizophrenia in Japanese population (562 schizophrenic patients and 470 controls). However we couldn't confirm significant association that had been previously reported. Considering size of our sample and strategy that corresponds well with the approaches used in gene-based association analysis, our conclusion is that GAD1 does not play a major role in schizophrenia in Japanese population.

Keywords: Schizophrenia; GAD1; Linkage disequilibrium; GABA; Association study

1. Introduction

Abnormalities in the γ-amino butyric acid (GABA) neurotransmission system are thought to be involved in the pathophysiology of schizophrenia. Several postmortem studies showed altered expression of genes related to GABA in schizophrenic patients. One of the most consistent findings is related to reduced level of 67 kDa glutamic acid decarboxylase isoform (GAD_{67}), key enzyme in GABA synthesis (Lewis et al., 2005).

The GAD_{67} is encoded by the glutamic acid decarboxylase 1 gene (GAD1) located on 2q31, and two studies reported positive associations between GAD1 and schizophrenia (Straub et al., 2003; Addington et al., 2005). Interestingly, those studies showed positive association of: 1) childhood onset schizophrenia (COS) and 2) cortical gray matter volume loss with schizophrenia (Addington et al., 2005). Moreover, a

Abbreviations: γ-amino butyric acid, (GABA); 67 kDa isoform of glutamic acid decarboxylase, (GAD_{67}); Glutamic acid decarboxylase 1 gene, (GAD1); Childhood onset schizophrenia, (COS); Adult onset schizophrenia, (AOS); Single nucleotide polymorphism, (SNP); Linkage disequilibrium, (LD); Minor allele frequency, (MAF); Genotype relative risk, (GRR); Age at onset, (AAO).

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significant association between GAD1 and schizophrenia in two independent adult onset schizophrenia (AOS) samples has been reported as well, suggesting biological continuity between COS and AOS (Straub et al., 2003).

In contrast, three other studies reported no association between GAD1 and schizophrenia, although, due to sample size, possibility of type II error cannot be excluded (De Luca et al., 2004; Lundorf et al., 2005; Zhang et al., 2005).

It is widely accepted that there are limitations in interpreting the results of simple replication studies that examine the same or a smaller number of single nucleotide polymorphisms (SNPs) as in the original study. Aforementioned can be explained by the differences in allele frequency or variation of linkage disequilibrium (LD) structure (population dependence). To overcome these limitations, a gene-based approach rather than an SNP-based or haplotype-based approach is currently recommended (Neale and Sham, 2004). In such studies it is important to: (1) include both gene and gene flanking regions in testing for association, and (2) select genetic variants which adequately reflect the LD background in the targeted population (e.g. tagging SNPs).

Applying this gene-based association concept, we tested the association between GAD1 tagging SNPs and schizophrenia using relatively large samples in the Japanese population.

2. Materials and methods

2.1. Subjects

The sample used in this study was comprised of 562 schizophrenia patients (301 males and 261 females; range=15–82 years old, median=44 years old, mean±SD=44.9±15.2 years) and 470 healthy controls (269 males and 201 females; range=19–95 years old, median=34 years old, mean±SD=37.5±14.6 years). All subjects were unrelated and with Japanese ethnicity. Subject attributes and psychiatric assessment were identical to those described elsewhere (Ikeda et al., 2005).

Subsequent to study description, written informed consent was asked from each subject. This study was approved by the Ethics Committee at Fujita Health University and Nagoya University.

2.2. SNP selection

We first included marginal and significant SNPs (Markers 4, 5, and 6) reported by others (Addington et al., 2005) and a potent functional SNP (Marker 3; located 2246 base pairs (bp) upstream from the initial exon and Marker 12; located in 3′UTR) (Table 1). Next, we consulted the HapMap database (release#21, population: Japanese in Tokyo (JPT), minor allele frequency (MAF): more than 0.01). In this step, we determined the boundaries of the GAD1 gene that covered 5′-flanking regions including 5000 bp from the initial exon and 1700 bp downstream (3′) from the last exon (GenBank accession No. NM_000817: Supplementary Figure 1). Then eleven 'tagging SNPs' were selected with the criterion of an r^2 threshold greater than 0.8 in 'pair-wise tagging only' mode using the 'Tagger' program (de Bakker et al., 2005), implement of HAPLOVIEW software (Barrett et al., 2005). These tagging SNPs were used in the subsequent association analysis (since Markers 4 and 6 were listed in the HapMap data, they were force-included with these 'tagging SNPs'). Overall, 14 tagging SNPs were examined.

2.3. SNP genotyping

All SNPs were genotyped by TaqMan assay (Applied Biosystems Japan Ltd, Tokyo). Detailed information, including reaction conditions, can be seen in another paper (Ikeda et al., 2005).

2.4. Statistical analysis

Marker-trait association was evaluated by the χ^2 test (allele and genotype-wise analyses). For haplotype-wise analysis, LD blocks were initially defined in accordance with Gabriel's criteria, and haplotype frequencies were estimated in each LD block with the Expectation–Maximization algorithm. Log likelihood ratio tests were performed for global *P*-values (COCAPHASE 2.403 program, Dudbridge, 2003). Power calculation was performed with a web-based statistical program, Genetic Power Calculator (Purcell et al., 2003). Power was estimated under multiplicative model of inheritance, assuming the disease prevalence to be 1% and the population susceptibility allele frequencies to be the values in observed in control samples.

3. Results

All genotype frequencies of these SNPs were in Hardy–Weinberg equilibrium. The LD matrices of the 14 tagging SNPs we tested are shown in Supplementary Figure 2. No association was found between cases and controls in allele-, genotype- or haplotype-wise analyses (Table 1).

Table 1
Association analysis of tagging SNPs in GAD1

SNP ID[a]		Positions[b]	Blocks[c]	Genotype distribution[d]								MAFs[e]			*P*-value		
				N		M/M		M/m		m/m							
				SCZ	CON	SCZ	CON	SCZ	CON	SCZ	CON	SCZ	CON		Genotype	Allele	Haplotype
Marker 1	rs1978340	1	I	561	466	371	306	175	148	15	12	.18	.18	(.31)	.978	.915	.549
Marker 2	rs3762561	625	I	561	469	368	292	165	158	28	19	.20	.21	(0)	.297	.500	
Marker 3	rs12185692	704		561	466	269	237	244	194	48	35	.30	.28	(.37)	.610	.328	
Marker 4	rs3749034*	3353	II	561	466	244	211	265	216	52	39	.33	.32	(.16)	.795	.517	.194
Marker 5	rs2270335*	4574	II	561	466	245	212	265	214	51	40	.33	.32	(0)	.836	.574	
Marker 6	rs2241165*	8257	II	561	466	244	212	265	215	52	39	.33	.31	(.19)	.768	.484	
Marker 7	rs3828275	12618		561	467	266	224	247	200	48	43	.31	.31	(.49)	.894	.980	
Marker 8	rs2241164	17437		562	470	189	142	271	243	102	85	.43	.44	(.31)	.459	.444	
Marker 9	rs769407	23586	III	561	468	314	261	215	179	32	28	.25	.25	(.24)	.982	.900	.438
Marker10	rs3791851	28552	III	561	467	298	238	226	191	37	38	.27	.29	(.25)	.582	.350	
Marker 11	rs3791850	37978	III	561	468	431	370	119	93	11	5	.13	.11	(.18)	.429	.275	
Marker 12	rs769395	46681	III	561	466	287	233	237	193	37	40	.28	.29	(.27)	.484	.431	
Marker 13	rs16858996	48980		561	468	370	295	176	153	15	20	.18	.21	(.11)	.302	.197	
Marker 14	rs17701824	49124		561	468	266	221	231	204	64	43	.32	.31	(.43)	.458	.622	

Numbers in parenthesis represent MAFs in Caucasians (cited from HapMap database and Applied Biosystems database).

[a] IDs with asterisk represent significant or marginally significant SNPs in Addington's report.

[b] Based on Accession No. NT005403.16.

[c] Determined by HAPLOVIEW.

[d] *N* = number, M = major allele, m = minor allele, SCZ = schizophrenia, CON = control.

[e] MAFs = minor allele frequencies.

Power analyses showed that the power was more than 80% when genotype relative risk (GRR) was set at 1.3–1.5 under a multiplicative model of inheritance.

4. Discussion

In this study, we were unable to confirm an association between GAD1 and schizophrenia in the Japanese population.

Addington et al. (2005), using an *in silico* approach, reported that positive SNPs in the 5′ region (Marker 4 and 5 in our study) of GAD1 possibly have various effects on gene expression. They speculated that these SNPs may alter the expression level of GAD1. Their findings supported previous postmortem studies showing down-regulation of GAD67 in schizophrenics. This data suggests that the 5′-flanking region of this gene might harbor schizophrenia-susceptibility factor. However, our results do not support the positive association (Markers 4 and 5) in the 5′-flanking region of GAD1 previously reported by Addington, even though we examined the other three SNPs in the 5′-flanking region (Markers 1, 2 and 3) in addition to Addington's two SNPs. Our sample size also showed quite high power. From the viewpoint of the common disease-common variant hypothesis (Chakravarti, 1999), our data didn't provide the evidence that GAD1 have a major role in schizophrenia in the Japanese population.

We also included an explorative analysis of gender effect and age at onset (AAO: $N=310$), for the following reasons: 1) Straub et al. (2003) and Addington et al. (2005) reported that relations were significantly greater or stronger in females, and 2) this analysis allowed us to consider the relation between the negative results of our AOS samples in Japanese and the positive COS samples in Caucasians. However, no associations were found in analysis subdivided by gender, or between AAO and GAD1 allele and haplotypes (evaluated by haplotype trend regression analysis; Liu and Muse, 2005: data not shown).

Although the strategy we used for the present association analysis corresponded well with that used in gene-based association analysis (Neale and Sham, 2004), several limitations must be outlined. First, in case of examination of possible association between GAD1 and schizophrenia, causal variants with extremely rare MAFs, allelic heterogeneity, or locus heterogeneity should be considered (Neale and Sham, 2004). In such situations, quite large sample sizes are needed for rare variants searching; i.e. more than 3500 cases and controls are required for 80% power when GRR is set at 1.5 and MAF is 1% (Purcell et al., 2003). Second, our control subjects were significantly younger than case subjects. Thus, our control samples may have included a number of individuals below the age of peak risk for schizophrenia-onset, and this confounding factor has potential for decreasing power of this study. Third, GAD1 may influence the function and morphology of the dorsolateral prefrontal cortex (Addington et al., 2005; Lewis et al., 2005). Therefore, endophenotypic approaches such as cognitive function, brain imaging and other phenotypes reflecting characteristics of GAD1 will be needed in the future (Gottesman and Gould, 2003).

In conclusion, our gene-based analysis of GAD1 showed no association between this gene and AOS in the Japanese population. Further studies using different population samples will be needed to conclude whether GAD1 is a race specific or rare phenotype specific susceptibility factor for AOS.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.schres.2006.12.020.

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Possible association of *β-arrestin 2* gene with methamphetamine use disorder, but not schizophrenia

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Recent investigations suggest that the AKT/glycogen synthase kinase 3 (GSK3) signaling cascade may be associated with the pathophysiology of schizophrenia and methamphetamine (METH) use disorder. One important molecule related to this cascade is β-arrestin 2 (ARRB2). We therefore conducted a genetic case–control association analysis of the gene for *ARRB2* with schizophrenia and METH use disorder in a Japanese population (547 people with schizophrenia, 177 with METH use disorder and 546 controls). A possible association of 'tag single nucleotide polymorphisms (SNPs)' was found in METH use disorder (rs1045280: $P_{genotype} = 0.0118$, $P_{allele} = 0.00351$; rs2036657: $P_{allele} = 0.0431$; rs4790694: $P_{genotype} = 0.0167$, $P_{allele} = 0.0202$), but no association was found with schizophrenia. We also evaluated the gene–gene interactions among ARRB2, AKT1, and GSK3B, which we previously reported for each of these diseases. However, no interaction was seen in our samples. This is the first association analysis of ARRB2, and our results indicate that ARRB2 may play a role in the pathophysiology of METH use disorder.

Keywords: AKT, GSK3, methamphetamine use disorder, schizophrenia, β-arrestin

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Dopamine D2 receptors are the main target of therapeutic agents for psychiatric diseases. So far, D2 receptors have been thought to inhibit cyclic AMP (cAMP) synthesis through interaction with Gαi/o and negatively regulate the activity of protein kinase A. However, several investigations showed that new intracellular proteins can facilitate some aspects of D2 receptor signaling, such as the AKT/glycogen synthase kinase 3 (GSK3) signaling cascade (Bonci & Hopf 2005). This AKT1–GSK3 signaling system has been discussed as a major target for lithium action, and it has been hypothesized that this system is involved in the pathophysiology of mood disorders (Gould & Manji 2005).

Recently, two *in vivo* studies using dopamine transporter knock-out (KO) mice or amphetamine administration showed that the AKT/GSK3 cascade partially mediates dopamine-associated behaviors (Beaulieu *et al.* 2004; Emamian *et al.* 2004). In terms of the relation between AKT1/GSK3 and schizophrenia, the convergent evidence from animal, postmortem and genetic studies has been reported (Emamian *et al.* 2004). Two genetic replication studies revealed significant associations of AKT1 with schizophrenia (Ikeda *et al.* 2004; Schwab *et al.* 2005), although our previous study of the GSK3β (*GSK3B*) gene did not support such an association in a Japanese population (Ikeda *et al.* 2005b).

Elsewhere, classical studies in Japan (Tatetsu *et al.* 1956) and UK (Connell 1958) showed that methamphetamine (METH) consumption induces psychosis at a high rate (92 and 100%, respectively). Because the symptomatology of METH-induced psychosis is similar to that of schizophrenia (especially paranoid type), METH-related disorders may also involve dopaminergic abnormalities. In support of this speculation, we reported an association between single nucleotide polymorphism (SNP) and haplotypes in AKT1 and METH use disorder (Ikeda *et al.* 2005c). AKT1 and GSK3β would therefore seem to be prime candidate genes for schizophrenia and METH use disorder, while the related molecules (or genes) of this cascade are also considered to be candidate factors for these disorders.

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A recent study showed that β-arrestin 2 (ARRB2) is an important mediator of the AKT/GSK3 cascade introducing dopamine-associated behaviors: ARRB2 KO mice exhibit significantly less pronounced locomotor activation than wild-type mice following the administration of amphetamine, and double mutation ARRB2/dopamine transporter KO mice display significantly lower locomotor activity than dopamine transporter KO mice (Beaulieu *et al.* 2005). This study also showed that ARRB2 interacts with AKT and protein phosphatase 2 A (PP2A) and that these signaling complexes are regulated by dopamine. According to this evidence, ARRB2 seems to be a promising candidate as a molecule related to the AKT/GSK3 signaling cascade and/or the pathophysiology of schizophrenia and METH use disorder.

We conducted this association analysis of the *ARRB2* gene (located on 17p13) with schizophrenia and METH use disorder in a Japanese population. We first evaluated the linkage disequilibrium (LD) structure of this gene and selected 'tag SNPs'. These 'tag SNPs' were then used to reflect the LD properties in the Japanese population in the following association analysis.

Materials and methods

Subjects

Five hundred and forty-seven patients with schizophrenia [283 males and 264 females: mean age ± standard deviation (SD) 45.6 ± 16.0 years] and 177 patients with METH use disorder (all patients were diagnosed as having METH dependence, 145 males and 32 females: mean age ± SD 36.8 ± 12.0 years) participated in this study. A total of 546 healthy controls (255 males and 291 females: mean age ± SD 37.0 ± 14.4 years) were recruited as control subjects. The subjects for the 'LD evaluation' were 96 controls who were also subjects in the association analysis. All subjects were unrelated each other and ethnically Japanese.

The patients were diagnosed according to DSM-IV or ICD-10DCR criteria with the consensus of at least two experienced psychiatrists on the basis of unstructured interviews and review of medical records. All healthy controls were also psychiatrically screened based on unstructured interviews.

Among the subjects with METH use disorder, 164 have a comorbid diagnosis of METH-induced psychosis, three of anorexia nervosa, one of obsessive-compulsive disorder and one of major depressive disorder. In addition, 129 subjects with METH use disorder or abuse have a dependence on drugs other than METH. Subjects with METH use disorder were excluded if they had a comorbid diagnosis of any psychotic disorder other than METH-induced psychosis. More detailed characterizations of these subjects were published elsewhere (Ikeda *et al.* 2005a; Nishiyama *et al.* 2005).

After description of the study, written informed consent was obtained from each subject. This study was approved by the Ethics Committee at Fujita Health University School of Medicine, Nagoya University Graduate School of Medicine and each participating institute of the Japanese Genetics Initiative for Drug Abuse (JGIDA).

SNP selection and LD evaluation of ARRB2

We first consulted the HapMap database (release#16c.1, June 2005, http://www.hapmap.org) and SNPBROWSER2.0 database (Applied Biosystems, Foster City, CA, USA) and selected five SNPs (SNP1: rs4790689, SNP2: rs1973555, SNP3: rs1045280, SNP4: rs2036657 and SNP5: rs4790694) with minor allele frequencies (MAFs) of more than 0.05 for LD evaluation (Supplemental Table S1).

Next, we genotyped these five SNPs using our own control samples to confirm the LD structure. In this step, we selected 'tag SNPs' with criteria on r^2 threshold greater than 0.8 in pairwise tagging mode using the TAGGER program (Paul de Bakker, http://www/broad.mit.edu/mpg/tagger/), an implement of the HAPLOVIEW software program (Barrett *et al.* 2005) for the following association analysis.

SNP genotyping of ARRB2

All SNPs were genotyped by TaqMan assay (Applied Biosystems). Further detailed information, including reaction conditions, can be seen in another paper (Ikeda *et al.* 2005a).

Statistical analysis

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by χ^2 test (SAS/Genetics, release 8.2, SAS Japan Inc., Tokyo, Japan).

Marker-trait association analysis was used to evaluate allelic and genotypic associations with χ^2 test or Fisher's exact test (SPSS 10.0 J, SPSS Japan Inc, Tokyo, Japan), and haplotypic association was investigated with log-likelihood ratio test (COCAPHASE 2.403). A more detailed description is given in our previous paper (Ikeda *et al.* 2005a).

We estimated the power of association for our sample size using GENETIC POWER CALCULATOR software (Purcell *et al.* 2003) with an α of 0.05 and a disease prevalence of 0.01.

To avoid false negative results, we did not correct for multiple testing, because Bonferroni correction is too conservative to apply to genetic association analysis (Nyholt 2001).

The significant level for all statistical tests was 0.05.

Results

For LD evaluation, five SNPs were genotyped for 96 controls. Three SNPs (SNP3–5) were selected as 'tag SNPs' by the TAGGER software (Supplemental Table S1). These genotypings of the three 'tag SNPs' were expanded for the following association analysis, and no genotype distributions in cases and controls showed deviation from HWE.

No association was found between schizophrenia and controls. Our sample size has a power of 0.80 to detect significant associations between each 'tag SNP' and schizophrenia, assuming a genotype relative risk (GRR) of 1.42–1.57 under a multiplicative model of inheritance. In contrast, all 'tag SNPs' (SNP3–5) were associated with METH use disorder in the allelic, haplotypic and/or genotypic analyses (Tables 1 and 2). Our METH use disorder samples are expected to yield power of 0.80 to detect significant association, assuming GRR of 1.58–1.75.

For further interpretation of these associations, we also included an explorative analysis of clinical subgroups and gender effects for the following reasons: our subjects with METH-induced psychosis were (1) the majority of our METH samples, this condition would be over-represented in our samples of METH use disorder, and (2) unmatched gender samples for METH use disorder (male = 145 and female = 32). Consequently, no common results among clinical subgroups or gender were obtained. However, SNP3 was associated with female METH use samples, and SNP5 was associated with total METH use disorder and METH-induced psychosis in male samples (Table 3). Global haplotypic analyses showed only trends for significance in male METH use disorder samples (global *P*-value = 0.0536) and male METH-induced psychosis samples (global *P*-value = 0.105) (Supplemental Table S2).

Discussion

A possible association of 'tag SNPs' in the *ARRB2* gene was obtained in the patients with METH use disorder but not with schizophrenia patients.

The results from the LD evaluation using the TAGGER program indicate that three 'tag SNPs' could represent the entire 5′-flanking regions and most 3′ regions. We speculate that an actual susceptibility variant of METH use disorder may exist within the LD region in these significant SNPs, because these 'tag SNPs' may be only markers with no functional effects (SNP3: synonymous substitution, SNP4 and SNP5: located in 3′ downstream of ARRB2). Further investigations including mutation scan and functional analysis will be required.

Our METH use disorder sample had several limitations, described in the *Results* section. In terms of clinical subgroups, because there was no high power association between ARRB2 and schizophrenia, the association between ARRB2 and total METH use disorder is perhaps not due to

Table 1: Association analyses of 'tag single nucleotide polymorphisms (SNPs)' in β-arrestin 2 (ARRB2) with schizophrenia and methamphetamine (METH) use disorder

			Genotype*			*P*-values†	
tag SNPs	Phenotype	Number	M/M	M/m	m/m	Genotype	Allele
SNP3 rs1045280 (T>C)	Schizophrenia	547	418	119	10	0.753	0.859
	METH use disorder	177	117	54	6	**0.0118**	**0.00351**
	Controls	546	417	122	7		
SNP4 rs2036657 (A>G)	Schizophrenia	547	430	109	8	0.871	0.596
	METH use disorder	177	129	44	4	0.117	**0.0431**
	Controls	546	436	103	7		
SNP5 rs4790694 (C>A)	Schizophrenia	547	460	85	2	0.703	0.382
	METH use disorder	177	138	39	0	**0.0167**	**0.0202**
	Controls	546	470	74	2		

*M, major allele; m, minor allele.

†Bold numbers represent significant *P*-values.

Table 2: Haplotypic analysis of three 'tag single nucleotide polymorphisms (SNPs)' (SNP3–5)

			Frequency (%)		
Phenotype	Global *P*-values	Marker haplotype	Case	Control	Individual *P*-values*
Schizophrenia	0.405	TAC	84.8	85.3	0.716
		CGA	6.03	5.19	0.400
METH use disorder	**0.0175**	TAC	80.2	85.4	**0.0194**
		CGA	8.60	5.20	**0.0210**

*Bold numbers represent significant *P*-values.

Table 3: Explorative analysis of clinical subgroups and gender in methamphetamine (METH) use disorder

			Genotype*			*P*-values†	
tag SNPs	Phenotype	Number	M/M	M/m	m/m	Genotype	Allele
SNP3	Male METH use disorder	145	99	40	6	0.224	0.152
	psychosis	138	96	38	4	0.523	0.340
	dependence	7	3	2	2	NA	NA
	Male control	255	187	64	4		
	Female METH use disorder	32	18	14	0	**0.0123**	**0.0112**
	psychosis	26	17	9	0	0.211	0.172
	dependence	6	1	5	0	NA	NA
	Female control	291	230	58	3		
SNP4	Male METH use disorder	145	108	33	4	0.466	0.375
	psychosis	138	104	31	3	0.695	0.558
	dependence	7	4	2	1	NA	NA
	Male control	255	197	55	3		
	Female METH use disorder	32	21	11	0	0.0652	0.0595
	psychosis	26	19	7	0	0.423	0.375
	dependence	6	2	4	0	NA	NA
	Female control	291	239	48	4		
SNP5	Male METH use disorder	145	113	32	0	**0.0174**	**0.0231**
	psychosis	138	108	30	0	**0.0224**	**0.0302**
	dependence	7	5	2	0	NA	NA
	Male control	255	223	31	1		
	Female METH use disorder	32	25	7	0	0.374	0.371
	psychosis	26	22	4	0	1	0.992
	dependence	6	3	3	0	NA	NA
	Female control	291	247	43	1		

*M, major allele; m, minor allele; NA, not analyzed.
†Bold numbers represent significant *P*-values.

spurious comorbid METH-induced psychosis, which may share the pathophysiology of susceptibility with schizophrenia (sensitization phenomena) (Ujike & Sato 2004). Thus, all significant SNPs might be closely associated with METH use disorder.

However, a recent investigation suggests that METH-induced psychosis has heavier family loading, probably heavier genetic loading than METH users without psychosis (Chen *et al.* 2005). The authors of that study suggested it is likely that dopaminergic abnormalities differ between METH used disorder with and without psychosis.

Furthermore, gender effects of METH use disorder and AKT/GSK signaling were also reported (Jang *et al.* 1997; Znamensky *et al.* 2003). We recognize that a larger sample will be required for the confirmation of clinical subgroups and a gender effect, because owing to the small sample size of METH subjects without psychosis and female METH subjects our explorative analysis mainly reflected the results from male subjects with psychosis. Therefore, our speculation is based mainly on the results of these subjects. Even taking such a conservative view of this explorative analysis, we consider that ARRB2 may be associated with METH-induced psychosis in males and that among the significant SNPs, SNP5 is the most plausible candidate variant. Our findings from comparing genotype frequencies of SNPs between METH-induced psychosis in males and in male controls would seem to indirectly support the possibility of a different genetic background for METH-induced psychosis (Chen *et al.* 2005). Chen *et al.* (2005) also reported significant differences in morbid risk, when the METH-induced psychosis subjects were divided into clinical subgroups of prolonged METH psychosis and brief METH psychosis. We could not perform association analyses between METH use disorders with and without psychosis in this study due to the small sample size and the differences in the inclusion criteria for prognosis of METH-induced psychosis (Morita *et al.* 2005). Meanwhile, the results from the haplotypic analyses, which showed no association with METH use disorder or METH-induced psychosis in male samples, may have been due to

type II error, because a power simulation showed that haplotypic analysis can decrease the statistical power of an association study [if an actual causal variant (or variant in absolute LD with that variant) is directly observed] (Bader 2001).

Recent *in vivo* studies suggest that the AKT/GSK3 signaling cascade is an attractive candidate factor for schizophrenia and METH use disorder (Beaulieu *et al.* 2004, 2005; Emamian *et al.* 2004). We have performed genetic association studies of AKT1 and GSK3B with schizophrenia (Ikeda *et al.* 2004, 2005b) and METH use disorder (Ikeda *et al.* 2005c) (for GSK3B, no association was obtained from subjects with METH use disorder; M. Ikeda, N. Ozaki, N. Iwata & JGIDA, unpublished observations). Summarizing these results, AKT1 (but not GSK3B and ARRB2) showed a significant association with schizophrenia, and AKT1 and ARRB2 (but not GSK3B) were associated for METH use disorder. To evaluate whether each risk SNP would be an independent risk factor or have interactions with each SNP in these genes, we analyzed the gene–gene interactions with the use of the Multifactor Dimensionality Reduction (MDR) method (Hahn *et al.* 2003). In this analysis, we considered two-locus interactions through four-locus interactions and analyzed six SNPs in AKT1, two SNPs in GSK3B and three SNPs in ARRB2. Three hundred and seventy-one schizophrenics, 161 METH use disorder subjects and 339 controls were tested, all of whom were identical in each study. However, no interactions were obtained with schizophrenia or METH use disorder (and METH-induced psychosis in males) (data not shown). Further studies will be required for conclusive results; however, these results indicate that the risk SNPs or haplotypes in AKT1 for schizophrenia, and those in AKT1 and ARRB2 for METH use disorder may attribute independently to these disorders.

In conclusion, our findings suggest that ARRB2 may play a role in the development of METH use disorder but not schizophrenia in the Japanese population. However, this positive finding is a preliminary one, given the several limitations of our sample and the fact that we did not perform genomic control to exclude population stratification. We must also consider the possibility that our findings merely demonstrated the pharmacogenetic fact that individuals metabolize drugs differently, and the results were not relevant to the genetics of psychoses but rather to their treatment. It will be necessary to validate or replicate our associations in other population samples.

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Supplementary material

Table S1. Pairwise linkage disequilibrium matrices and 'tag SNPs' in ARRB2.

Table S2. Explorative haplotypic analysis of clinical subgroups and gender in METH use disorder.

These materials are available as part of the online article from http://www.blackwell-synergy.com

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LETTERS TO THE EDITOR

Support for association of the *PPP3CC* gene with schizophrenia

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Calcineurin is a calcium-dependent protein phosphatase that plays an important role in cellular responses and calcium signal transduction.[1] Several studies have suggested that calcineurin is one of the key molecules in signal transduction in the brain and that dysfunction of calcineurin signaling could be linked to schizophrenia.[2,3] Calcineurin is a heteromeric protein complex consisting of a catalytic subunit (calcineurin A) and a regulatory subunit (calcineurin B).[1,4] *PPP3CC* encodes the calcineurin γ-catalytic subunit and is located on chromosome 8p21.3 within a few cM of markers reported to be linked to schizophrenia.[5–7,10] Gerber *et al.*[8] reported genetic associations of the *PPP3CC* gene with schizophrenia in populations from the United States and South Africa. However, only one replication study has been published, and these associations were not confirmed in 457 Japanese schizophrenic patients and 429 control subjects.[9] HapMap data indicated that a haplotype block spans almost the entire *PPP3CC* region in Japanese and European populations. The single nucleotide polymorphism (SNP) haplotype reported to be associated with schizophrenia by Gerber *et al.*[8] is located in the haplotype block. Therefore, we examined the associations in a large case–control study of 1645 schizophrenic patients and 1673 control subjects. This sample size has a power >0.98 to replicate the haplotypic association with the same magnitude as that reported by Gerber *et al.*,[8] assuming an α value $=0.05$, two-tailed, a haplotype relative risk of 1.23, and haplotype frequency of 0.26 or effect size of 0.1. The haplotype frequency in the Japanese population was reported by Kinoshita *et al.*[9]

All subjects were of Japanese descent and were recruited from the main island of Japan. The study included 1645 unrelated patients with schizophrenia (mean age $\pm$ s.d., 48.2 ± 14.5 years; 899 men and 745 women) diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV). Control subjects were 1673 mentally healthy unrelated subjects (age, 47.9 ± 14.3, 886 men and 787 women) without self-reported family histories of mental illness within second-degree relatives. The subjects studied by Kinoshita *et al.*[9] were not included in the present study. The present study was approved by the Ethics Committees of the University of Tsukuba, Niigata University, Fujita Health University, Nagoya University, Okayama University and Teikyo University and all participants provided written informed consent. To rule out population stratification between patients and controls in the present study, 35 SNPs that are not in linkage disequilibrium (LD) with each other were genotyped in all samples and analyzed with the STRUCTURE program 2.0.[5] No stratification was observed.

We genotyped five SNPs. SNP1 (rs10108011, CC21 in Gerber *et al.*[8]) and SNP2 (rs2461491, CCS3 in Gerber *et al.*[8]) were selected because Gerber *et al.*[8] reported nominally significant allelic association of these SNPs with schizophrenia. SNP4 (rs2449340), SNP3 (rs2461490) and SNP5 (rs1116085) were genotyped to distinguish common haplotypes with frequencies $\geqslant 5\%$ in the haplotype block based on HapMap data of the Japanese population. SNPs were genotyped with the TaqMan SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA) and ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).

Deviation from predicted Hardy–Weinberg frequency was examined by χ^2-test. Individual allelic and genotypic associations were examined by Fisher's exact test. LD between polymorphisms and haplotypic associations were evaluated with Haploview software version 3.32.[6] To deal with multiple testing, allelic associations were evaluated by permutation tests implemented in Haploview. The genotype distributions were evaluated by the Cochran–Armitage test without correction for multiple testing.

The genotype and allele distributions of the five SNPs in the patient group and control group are shown in Table 1. Distributions of these SNPs did not differ significantly from Hardy–Weinberg equilibrium. All five SNPs showed nominally significant allelic associations with schizophrenia and permutation tests revealed significant allelic associations of SNP1 ($P=0.012$), SNP3 ($P=0.005$) and SNP4 ($P=0.013$) with schizophrenia. The genotype distributions suggest that the minor allele of each SNP is likely to have an additive effect in the susceptibility to schizophrenia. These five SNPs are in LD; however, the LD is not complete. Therefore, these associations were not caused by a single SNP in the present study. As shown in Table 2, there were only two common haplotypes constructed from these SNPs. The most common haplotype in the control group was observed less frequently in the patient group ($P=0.034$) and the second most common

Table 1 Case-control comparisons of SNPs

Polymorphism (NCBI ID)	*Subjects*	n	*Genotype count (frequency)*			P[a]	*Allele count (frequency)*		P[b] (P[c])	*Odds ratio (95% CI)*	*HWE* P
			AA	AG	GG		A	G			
SNP1(CC21)	Affected	1639	828 (0.51)	644 (0.39)	167 (0.10)		2300 (0.70)	978 (0.30)	0.012	1.15	0.013
(rs10108011)	Controls	1665	897 (0.54)	636 (0.38)	132 (0.08)	0.013	2430 (0.73)	900 (0.27)	(0.030)	(1.03–1.28)	0.197
			GG	GA	AA		G	A			
SNP2(CCS3)	Affected	1645	568 (0.35)	781 (0.47)	296 (0.18)		1917 (0.58)	1373 (0.42)	0.076	1.09	0.335
(rs2461491)	Controls	1673	617 (0.37)	788 (0.47)	268 (0.16)	0.076	2022 (0.60)	1324 (0.40)	(0.149)	(0.99–1.21)	0.536
			CC	CG	GG		C	G			
SNP3	Affected	1636	690 (0.42)	731 (0.45)	215 (0.13)		2111 (0.65)	1161 (0.35)	0.005	1.16	0.330
(rs2461490)	Controls	1655	773 (0.47)	698 (0.42)	184 (0.11)	0.006	2244 (0.68)	1066 (0.32)	(0.015)	(1.05–1.28)	0.165
			GG	GT	TT		G	T			
SNP4	Affected	1639	719 (0.44)	716 (0.44)	204 (0.12)		2154 (0.66)	1124 (0.34)	0.013	1.14	0.216
(rs2449340)	Controls	1665	792 (0.48)	700 (0.42)	173 (0.10)	0.014	2284 (0.69)	1046 (0.31)	(0.038)	(1.03–1.26)	0.321
			GG	GA	AA		G	A			
SNP5	Affected	1628	491 (0.30)	786 (0.48)	351 (0.22)		1781 (0.54)	1501 (0.46)	0.070	1.09	0.440
(rs1116085)	Controls	1666	541 (0.32)	799 (0.48)	326 (0.20)	0.080	1868 (0.57)	1438 (0.43)	(0.196)	(0.99–1.21)	0.185

[a]The Cochran Armitage test.
[b]Fisher's exact test (two-sided).
[c]Permutation test (10 000 permutations).

Table 2 Haplotype comparisons between patients and control groups

Haplotype	*Frequency*		χ^2	*Individual* P *(uncorrected)*	*Global* P
	Patients	*Controls*			
SNP1–2–3–4–5					0.055
AGCGG	0.52	0.55	4.51	0.034	
GAGTA	0.26	0.24	3.09	0.079	
AAGTA	0.07	0.06	1.12	0.31	
AACGA	0.05	0.06	0.64	0.42	
AGCGA	0.05	0.05	1.36	0.24	
GACGA	0.02	0.01	0.41	0.52	
SNP1–2					0.023
AG	0.58	0.6	5.225	0.022	
GA[a]	0.29	0.27	4.390	0.036	
AA	0.13	0.13	0.002	0.96	

[a]The associated haplotype reported by Gerber *et al.*[8]

haplotype in the control group tended to be more frequently in the patient group ($P=0.079$). A global P-value for the 5 SNP haplotype was 0.055.

Gerber *et al.*[8] found associations of the G allele of SNP1 (CC21) and the A allele of SNP2 (CCS3) with schizophrenia by transmission disequilibrium test. In the present study, we found that the G allele of SNP1 and the A allele of SNP2 occurred more frequently in the patient group than in the control group ($P=0.003$, odds ratio (OR) = 1.15 for SNP1; $P=0.02$, one-sided, OR = 1.10 for SNP2). Gerber *et al.*[8] found that the most common haplotype was overtransmitted to patients in their US population. According to the data reported by Kinoshita *et al.*,[9] only two SNPs (SNP1 and SNP2 in the present study) are sufficient to distinguish the associated haplotype reported by Gerber *et al.*[8] from other haplotypes in the Japanese population. When haplotypes were constructed with SNP1 and SNP2, the haplotype that was the most common haplotype associated with schizophrenia in the US population was the second most common haplotype in our Japanese population. The second most common haplotype was more frequent in the patient group than in the control group ($P=0.036$, two-sided) in the present study (Table 2). Therefore, the present study replicates the allelic and haplotypic associations found in the US population described by Gerber *et al.*,[8] although the ORs of the haplotype were lower in the present study (OR = 1.12) than in the study by Gerber *et al.*[8] (OR = 1.23).

Kinoshita *et al.*[9] failed to replicate these associations. The OR of the G allele of SNP1 for schizophrenia

reported by Kinoshita *et al.*[9] was 1.11, which is similar to that (OR = 1.15) in the present study, although the ORs of the A allele of SNP2 and the second most common haplotype for schizophrenia were 1.0 in the Kinoshita *et al.*[9] study and 1.09 and 1.10 in the present study, respectively. Recently, Yamada *et al.*[7] reported a nominally significant association of SNP2 (CCS3, rs2461491) and a trend towards association of SNP1 (CC21, rs10108011) with schizophrenia in Japanese family based-association analysis.

The present study replicated the allelic and haplotypic associations of *PPP3CC* with schizophrenia. Thus, an association between genetic variations of *PPP3CC* and schizophrenia appears to exist in US and Japanese populations. However, the ORs of 1.10–1.15 observed in the present study indicate that the associations are weak and will be difficult to replicate without large sample sizes. Further studies are needed to evaluate wither alterations in calcineurin signaling contribute to the pathogenesis of schizophrenia.

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fMRI evidence for functional epistasis between COMT and RGS4

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COMT and RGS4 are promising candidate risk genes for schizophrenia[1] that impact on dopamine signaling[2–4] and on prefrontal function.[5,6] While, in general, convergent molecular pathways and neurophysiological effects implicate gene × gene interactions, there is growing evidence to support a specific direct interaction between risk alleles in COMT and RGS4. First, statistical epistasis between a number of putative schizophrenia risk genes, notably RGS4 and COMT, has been reported in risk for schizophrenia.[7] Second, a recent study in human postmortem dorsolateral prefrontal cortex demonstrated a significant correlation between both COMT val158met genotype and COMT enzyme activity and RGS4 mRNA levels, such that increased COMT enzyme activity (and Val allele load) predicted decreased RGS4 mRNA expression.[8] Using an fMRI working memory task in healthy subjects that robustly engages DLPFC in a manner sensitive to variation in both COMT and RGS4, we employed a moderated multiple regression approach to examine interactions between the COMT (rs4680 G/A(val158met)) and the RGS4 (rs951436 A/C ('Chowdari SNP4')),[9] SNPs most consistently associated with schizophrenia and with prefrontal activation. In line with earlier findings, we expected that the impact of genetic variation in RGS4 would be more pronounced on a COMT Val allele background, such that individuals with both RGS4 SNP4-A and COMT-Val would exhibit the most inefficient pattern of prefrontal cortical engagement (that is, greater prefrontal activation in the absence of performance differences).[10]

We studied 82 healthy Caucasian subjects using an N-back working memory task as described previously.[10] Subjects were genotyped for RGS4 and COMT[5,6] (COMT: 25 V/V, 43 V/M, 14 M/M; RGS4: 26 A/A, 36 A/C, 20 C/C). Since only two COMT Met/Met-RGS4 A/A subjects were available in this sample, V/M heterozygote and M/M homozygote subjects were combined. One-way analysis of variance (ANOVA) with five groups (COMT Val/Val, Met-carriers and RGS4 SNP4 A/A, A/C, C/C) revealed no significant between-group differences in performance (accuracy or reaction time), gender or age. Whole brain BOLD fMRI data were collected on a 3 T GE scanner (TE = 30 ms, TR = 2 s, flip angle = 90, FOV = 24 cm) while subjects performed an N-back working memory task (2-back and 0-back) as previously described.[10] A second level moderated multiple regression was used to map the main effects for

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ORIGINAL ARTICLE

Gap junction coding genes and schizophrenia: a genetic association study

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Abstract The aim of this study was to evaluate the association of genes that encode gap junction forming proteins and schizophrenia. Representative genetic candidates (Panx2 and Cx36) from two families of gap junction genes were selected for analysis. According to the present findings these genes represent both functional and positional candidates for schizophrenia. The sample was comprised of 381 schizophrenic patients, and the same number of matched controls was tested in this study in order to evaluate the possible influence of the aforementioned genes on the pathogenesis of schizophrenia. Four SNPs in the case of Panx2 and two SNPs in the case of Cx36 were selected for analysis. Allele-, genotype- and haplotype-wise association did not yield statistically significant results. These data do not suggest that Panx2 or Cx36 could increase the risk of schizophrenia in the Japanese population.

Keywords Gap junction · Panx2 · Cx36 · Schizophrenia · Genetic association · Case control · Gamma band oscillation

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Introduction

Schizophrenia is a severe mental disorder with a global morbid risk of approximately 1% and a strong genetic component in its etiology. The disease is characterized by abnormal perception, thought disturbances and impaired cognitive function (Sadock and Sadock 2005). One of the present views on the pathogenesis of schizophrenia is related to the disturbed connectivity of neuronal networks (Phillips and Silverstein 2003). The mechanism of the neuronal network depends on two types of synapses, namely, the usual chemical synapses and gap junctions (or electrical synapses) (Connors and Long 2004). A large body of evidence has shown that gap junctions have considerable influence on the stabilization and propagation of synchronized neuronal activity, especially in the beta (12–30 Hz) and gamma (30–80 Hz) frequency band of EEG (electroencephalogram) (LeBeau et al. 2003; Sohl et al. 2005). The aforementioned had been shown both in in vitro (Whittington et al. 1995; Szabadics et al. 2001; LeBeau et al. 2003; Mann et al. 2005; Traub et al. 2005) and in vivo studies, namely in experiments involving Cx36 (connexin 36) knock out mice (Hormuzdi et al. 2001; Buhl et al. 2003). Furthermore, gap junctions have the capability of bidirectional signal transmission and low-pass filter (LPF) characteristics that are markedly different from properties of chemical synapses. LPF as a term is used for describing the virtue of preferential transmission of sub-threshold potentials and low frequency stimuli that favor synchronous activity of neural networks (an extensive review is available at Sohl et al. 2005).

Two families of gap junction proteins have been described in humans: connexins and pannexins. The connexin family is divided into three subfamilies (alpha, beta and gamma) consisting of more than ten different proteins

(Rozental et al. 2000); however, Cx36 (subfamily gamma) is preferentially expressed in cell types of neural origin (Condorelli et al. 2000). Moreover, as has been mentioned before, several studies have indicated its role in the promotion of synchrony and power in a gamma frequency range of EEG (Hormuzdi et al. 2001; Buhl et al. 2003). The human pannexin (in a further text referred to as Panx) family consists of Panx1, Panx2 and Panx3. However, Panx2 is abundantly expressed in the brain and involved in forming gap junctions of principal cells or GABAergic interneurons (**g**amma **a**mino **b**utyric **a**cid) (Bruzzone et al. 2003).

Regarding schizophrenia, several lines of evidence have showed decreased power and synchrony in the gamma frequency band of EEG during cognitive tasks in comparison to the nonaffected group (Spencer et al. 2003, 2004; Hong et al. 2004; Waberski et al. 2004). In that regard, the reported deficit in gamma band oscillations might be contributed by impaired synchronization of pyramidal cells owing to nonadequate perisomatic inhibition by paralbumin—expressed GABAergic interneurons, (Lewis et al. 2005).

Therefore, regarding gene expression and the reported role in the generation of gamma band oscillations, genes that encode Cx36 and Panx2 can be seen as functional candidates for schizophrenia. Moreover, chromosomal locations of both Cx36 (15q14) and Panx2 (22q13) are reported as linkage regions for schizophrenia (Meyer et al. 2002; Kenneth and Lindon 2005). For this reason, the aforementioned genes are positional candidates as well.

Methods

Sample population

The sample used in this research consisted of 381 schizophrenia patients (229 males and 151 females; mean age 50 ± 15.1 years) and 381 healthy controls (160 males and 221 females; mean age 40 ± 14.6 years). For analysis of Cx36, the total sample size consisted of 762 subjects and had been genotyped. On the other hand, the genetic association between schizophrenia and Panx2 was tested with a subset of the original sample comprised of 384 subjects (case control ratio 1:1). All the subjects were unrelated and of Japanese ethnicity. Subjects included in the case cohort met the DSM-IV criteria for schizophrenia with the consensus of two psychiatrists on the basis of an unstructured diagnostic interview and review of their medical records. Prior to inclusion in the control cohort subjects were screened on the basis of brief diagnostic interviews by an experienced psychiatrist. Subsequent to the study description, written informed consent was requested from each subject. This study was approved by the Ethics Committee at Nagoya University.

SNP selection

In order to test for genetic association either between the positional or functional candidate and phenotype of interest, we implemented a gene-based approach. This method implies inclusion of both the gene region and gene flanking regions in the association study (Neale and Sham 2004). In other words, it is important to understand the gene as a functional unity of coding and regulatory regions. Later on, by taking advantage of the observed linkage disequilibrium in the region of interest, it was possible to scale down the number of single nucleotide polymorphisms (SNPs) to be included in the association analysis by rejecting the redundant SNPs and picking only haplotype tagging SNPs in accordance with observed linkage disequilibrium (LD).

SNPs were selected from the hapmap database (release #21; phase II; July 2006, population: Japanese in Tokyo) (HapMap Consortium 2005), and the tagging SNP strategy was based on two criteria: first to exclude redundant SNPs from genotyping by taking advantage of the observed linkage disequlibrium in order to achieve 95% haplotype coverage and second to exclude SNPs with minor allele frequencies less than 10% (because of substantial loss of power due to sample size). According to the aforementioned criteria, from initial SNPs, four tagging SNPs for Panx2 (rs17284210, rs3817816, rs4838859 and rs7292533) and two tagging SNPs for Cx36 were selected (rs3743123 and rs752876).

None of the polymorphisms that had been tested in this study were functional; however, we can speculate on the possible impact on gene expression. Moreover, we tried to achieve good coverage of the gene and gene-adjacent regions. The rationale for the latter lies in the fact that tagging SNPs may be used as a proxy for detecting functional polymorphisms (i.e., LD between tagging SNP and unknown functional polymorphisms).

Genotyping methods

Genomic DNA was extracted from peripheral blood. The restriction fragment length polymorphism (RFLP) method was performed for genotyping rs17284210, rs3817816 and rs7292533. On the other hand, the allelic discrimination assay (Applied Biosystem Japan Ltd., Tokyo) was carried out for genotyping rs4838859, rs3743123 and rs752876. For each 384-well plate used for the allelic discrimination assay, three nontemplate controls were included. Details regarding primer design, cycle condition and restriction enzymes used for RFLP assay, as well as data on the probe design for allelic discrimination assay, are available upon request.

Statistical methods

The LD blocks were defined in accordance with the four gamete rule (Wang et al. 2002). Haplotype frequencies were estimated by the expectation-maximization algorithm. The aforementioned (LD block definition and haplotype frequency estimation) are functions implemented in the Haploview software v3.32 that was used in this study (Barrett et al. 2005). Genotype deviations from the Hardy–Weinberg equilibrium, allelic and genotypic associations were tested by chi-squared statistics. Log likelihood ratio tests for haplotypic association between schizophrenia and SNPs that are in linkage disequilibrium were carried out by the software Unphased v2.403 (Dudbridge 2003) with a permutation test for the calculation of empirical significance levels for differences between haplotype frequencies in case and control cohorts. The experimental alpha level was set to 0.05.

Power analysis was performed in accordance with the general power calculation model for chi-squared statistics. In brief, power is determined with respect to the degree of freedom and predefined alpha level of the study, after assuming the effect size (in accordance with Cohen's criteria). For the power calculation, Sample Power software v 2.0 (SPSS inc.) was used.

Results

HapMap data revealed a discreet linkage disequilibrium pattern in the region of Panx2. In detail, pairwise LD analysis of Panx2 polymorphisms showed one LD block that comprises several polymorphisms. However, only rs4838859 and rs7292533 were sufficient for 95% haplotype coverage. In spite of the fact that the remaining two polymorphisms (rs17284210 and rs3817816) are neither in linkage disequilibrium nor within a separate LD block, we included them in the study design in order to achieve satisfactory gene coverage for association analysis. Concerning Cx36, pairwise LD analysis showed one LD block that spreads throughout the gene region. Two SNPs (rs3743123 and rs752876) were sufficient in order to achieve 95% of haplotype coverage. The LD pattern of our data was in accordance with the LD pattern predicted by the HapMap database data set (data not shown).

Deviation from Hardy–Weinberg equilibrium was not observed. Allele-, genotype- or haplotype-wise analysis did not provide sufficient evidence for association between two genes that encode gap junction proteins and schizophrenia (see Table 1). Regarding Panx2, in case effect size is set to medium (in accordance with Cohen's criteria), the sample that was characterized in our research had approximately 80% power for detecting genetic association. However, in case the size effect had been set to small, the calculated power was 50%. On the other hand, regarding the Cx36 sample, the power was estimated to be more than 80% even if the effect size was set to small.

Discussion

The common disease-common variants hypothesis postulates that linkage disequilibrium should be detected by the

Table 1 Genotype distribution

Gene	SNP[a]	Block[b]	Cohort	Genotype[c]			*P* value	Allele[d]		*P* value	Haplotype *P* value
				M/M	M/m	m/m		M	m		
Cx 36	rs752876	I_{cx36}	Case	121	186	74	0.807	56.2	43.8	0.877	0.807
	G > A		Control	127	177	77		56.6	43.4		
	rs3743123		Case	138	176	67	0.803	59.3	40.7	0.958	
	C > T		Control	133	185	63		59.2	40.8		
Panx 2	rs17284210	/	Case	134	51	7	0.680	83.1	16.9	0.372	/
	T > G		Control	141	46	5		85.4	14.6		
	rs3817816	/	Case	90	79	23	0.987	67.4	32.6	0.878	/
	G > A		Control	89	79	24		66.9	33.1		
	rs4838859	I_{panx2}	Case	107	74	11	0.949	75.0	25.0	0.802	0.386
	A > G		Control	110	71	11		75.8	24.2		
	rs7292533		Case	84	94	14	0.344	68.2	31.8	0.539	
	T > C		Control	84	86	22		66.1	33.9		

[a] Upper value is dbSNP designation while lower value represents major > minor allele

[b] Dotted line represents LD block

[c] *M* Major allele, *m* minor allele in absolute numbers

[d] *M* Major allele, *m* minor allele in percents

haplotype association test if the risk haplotype was linked to variants (Chakravarti 1999). Therefore, regarding the Japanese population, the data presented in this article do not provide sufficient evidence for the involvement of the two gap junction coding genes in conferring that susceptibility.

Definition of phenotypes is vital for genetic association study; therefore, endophenotypes (being more specific than phenotypes) are thought to be important for this field (Gottesman and Gould 2003; Braff et al. 2007). We did not take advantage of endophenotypes in order to test for genetic association, and that might be a limitation of our study. However, here we have to mention that several studies showed gamma band oscillations, recorded in schizophrenic patients, that indeed have reduced power and synchrony in comparison to nonaffected subjects (Spencer et al. 2003, 2004). Therefore, rather that segregation of the sample in respect to the characteristic of gamma band oscillations, we implemented a crude phenotype approach (schizophrenia phenotype in toto) in the study design. Moreover, regarding Cx36, our data are in concordance with previous research regarding the aforementioned gene and schizophrenia (Meyer et al. 2002).

In conclusion, neither Panx2 nor Cx36 has influence on the pathogenesis of schizophrenia in the Japanese population. However, since this is the first study regarding Panx2 and schizophrenia, it might be interesting to explore the possible association in different population settings.

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