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ORIGINAL PAPER

Taro Kishi · Tsuyoshi Kitajima · Masashi Ikeda · Yoshio Yamanouchi · Yoko Kinoshita
Kunihiro Kawashima · Tomo Okochi · Takenori Okumura · Tomoko Tsunoka · Toshiya Inada
Norio Ozaki · Nakao Iwata

Association study of clock gene (*CLOCK*) and schizophrenia and mood disorders in the Japanese population

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Abstract Recently the clock genes have been reported to play some roles in neural transmitter systems, including the dopamine system, as well as to regulate circadian rhythms. Abnormalities in both of these mechanisms are thought to be involved in the pathophysiology of major mental illness such as schizophrenia and mood disorders including bipolar disorder (BP) and major depressive disorder (MDD). Recent genetic studies have reported that *CLOCK*, one of the clock genes, is associated with these psychiatric disorders. Therefore, we investigated the association between the six tagging SNPs in *CLOCK* and the risk of these psychiatric disorders in Japanese patients diagnosed with schizophrenia (733 patients), BP (149) and MDD (324), plus 795 Japanese controls. Only one association, with schizophrenia in females, was detected in the haplotype analysis ($P = 0.0362$). However, this significance did not remain after Bonferroni correction ($P = 0.0724$). No significant association was found with BP and MDD. In conclusion, we suggest that *CLOCK* may not play a major role in the pathophysiology of Japanese schizophrenia, BP and MDD patients. However, it will be important to replicate and confirm these findings in other independent studies using large samples.

Key words schizophrenia · bipolar disorder · major depressive disorder · *CLOCK* · tagging SNP

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T. Kishi and T. Kitajima have contributed equally to this work.

T. Kishi (✉) · T. Kitajima · M. Ikeda · Y. Yamanouchi
Y. Kinoshita · K. Kawashima · T. Okochi · T. Okumura
T. Tsunoka · N. Iwata
Department of Psychiatry
Fujita Health University School of Medicine
Toyoake, Aichi 470-1192, Japan
Tel.: +81-562/93-9250
Fax: +81-562/93-1831
E-Mail: tarok@fujita-hu.ac.jp

N. Ozaki
Department of Psychiatry
Nagoya University Graduate School of Medicine
Nagoya 466-8850, Japan

T. Inada
Neuropsychiatric Research Institute
Seiwa Hospital
Tokyo 162-0851, Japan

M. Ikeda
Department of Psychological Medicine
School of Medicine
Cardiff University
Heath Park
Cardiff CF14 4XN, UK

Introduction

Sleep disturbances are commonly observed in psychiatric disorders, and sleep manipulations can influence clinical status. Abnormalities in circadian rhythms have been reported to be involved in the pathophysiology of major mental illness such as schizophrenia and mood disorders [2, 24, 26, 27]. Also, because all psychotropic drugs have actions on the systems of neurotransmitters such as dopamine and serotonin in the brain, altered neural transmission is hypothesized to be a susceptibility factor for major mental illness [21, 29]. Recently these neurotransmitter systems have been reported to have reciprocal interactions with circadian rhythms [3, 40].

Clock genes were also discussed to regulate not only circadian rhythms but dopamine neural transmission [25]. Recently, *Per2*, one of the circadian clock genes, was shown to alter dopamine levels in the caudate putamen and the nucleus accumbens, mediated by reduced expression and activity of monoamine oxidase A, and its mutant mice showed

behaviors that resembled human mood disorders [16]. Abnormalities in dopamine neural transmission are known to be involved in the pathophysiology of schizophrenia [19], bipolar disorder (BP) [7] and major depressive disorder (MDD) [31]. A recent study has reported that plasma cortisol levels are elevated in schizophrenia and BP patients compared with controls [14]. Adrenal steroid hormones levels change based on circadian rhythms, it has been suggested and this mechanism may be involved in the development of insomnia and psychiatric disorders [10]. In addition, some genetic studies showed significant associations between schizophrenia/schizo-affective disorder and timeless homolog gene (*TIMELESS*) or period homolog 3 gene (*PER3*), between BP and Bmal1 gene (*ARNTL*) *or TIMELESS* or *PER3* [22, 32]. These facts suggest a crucial relationship between circadian rhythms and psychiatric disorders, and so genes associated with the molecular clock mechanism are good candidates for the etiology of psychiatric disorders. We thought these psychiatric disorders may have some shared mechanisms as to circadian rhythms and considered that it was reasonable to assess all these disorders.

Recent genetic studies showed significant associations of a SNP (T3111C: rs1801260) in *CLOCK*, one of the clock genes, with Japanese schizophrenia [39] and clinical features of BP such as a high recurrence rate [5, 6, 35]. In an animal study using *CLOCK* mutant mice that showed mania-like behavior, this behavior was reversed by lithium treatment [34]. In addition, *CLOCK* mutant mice showed altered regulation of dopamine release in the ventral tegmental area mediated tyrosine hydroxylase regulated by circadian rhythms [28, 41]. Therefore, *CLOCK* would seem to be a good candidate gene for the pathophysiology of psychiatric disorders.

The *CLOCK* gene (OMIM *601851, 25 exons in this genomic region spanning 115.138 kb) is located on 4q12. This genomic region was shown to be closely related to susceptibility for schizophrenia [17, 38], BP [8, 15, 23] and MDD [11, 12]. Therefore, in this study, we aim to examine the genetic association between *CLOCK* and schizophrenia, BP and MDD in the Japanese population. To do this, we applied the recently recommended strategy of 'gene-based' association analysis [30]. We conducted a case–control association analysis using relatively large samples by selecting 'tagging SNPs' from the HapMap database.

Materials and methods

Subjects

The subjects in the association analysis were 733 schizophrenia patients [393 males and 340 females; mean age ± standard deviation (SD) 36.3 ± 18.4 years], 149 with BP (79 males and 70 females: 95 patients with bipolar I disorder and 54 patients with bipolar II disorder; 47.8 ± 14.6 years), 324 with MDD (159 males and 165 females; 47.5 ± 16.1 years) and 795 healthy controls (347 males and 448 females; 37.6 ± 14.3 years). Patients were grouped according to the following DSM-IV subtypes of schizophrenia: Paranoid Type (n = 216), Disorganized Type (n = 221), Catatonic Type (n = 29), Residual Type (n = 142), Undifferentiated Type (n = 125). The patients were diagnosed according to DSM-IV criteria with the consensus of at least two experienced psychiatrists on the basis of unstructured interviews and a review of medical records. All healthy control subjects were also psychiatrically screened based on unstructured interviews. None had severe medical complications such as cirrhosis, renal failure, heart failure or other Axis-I disorders according to DSM-IV. No structured methods were used to assess psychiatric symptoms in the controls, who included hospital staff, their families and medical students. None of the subjects were known to be related to each other, and all were ethnically Japanese. Written informed consent was obtained from each subject. This study was approved by the ethics committees at Fujita Health University and Nagoya University Graduate School of Medicine.

SNP selection and linkage disequilibrium evaluation

We first consulted the HapMap database (release#23.a.phase2, Mar 2008, http://www.hapmap.org, population: Japanese Tokyo: minor allele frequencies (MAFs) of more than 0.1) and included 106 SNPs covering *CLOCK* (5′-flanking regions including about 2 kb from the initial exon and about 5 kb downstream (3′) from the last exon: HapMap database contig number chr4: 55990340.. 56108588). Then six 'tagging (tag) SNPs' including rs1801260: T3111C (called SNP5 in this study) associated with Japanese schizophrenia [39] were selected with the criteria of an r^2 threshold greater than 0.8 in 'pairwise tagging only' mode using the 'Tagger' program (Paul de Bakker, http://www/broad.mit.edu/mpg/tagger) of the HAPLOVIEW software [4], in the following association analysis.

SNP genotyping

We used TaqMan assays (Applied Biosystems, Foster City, CA, USA) for all SNPs. Detailed information is available on request.

Statistical analysis

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by chi-square test (SAS/Genetics, release 8.2, SAS Japan Inc., Tokyo, Japan). Marker-trait association analysis was used to evaluate allele- and genotype-wise association with the chi-square test (SAS/Genetics, release 8.2, SAS Japan Inc., Tokyo, Japan), and haplotype-wise association analysis was evaluated by a likelihood ratio test using the COCAPHASE2.403 program [13]. Bonferroni's correction was used to control inflation of the type I error rate. Power calculation was performed using genetic power calculator [33]. The significance level for statistical tests was 0.05.

Results

Genotype frequencies were in HWE for this SNP. Linkage disequilibrium structures from the HapMap database can be seen in Fig. 1. The LD structures of schizophrenia, BP, MDD and control samples were almost the same (Fig. 1). In addition, LD from SNP1 to SNP4 was very tight in our control samples (r^2 more than 0.9), although we selected tag SNPs from HapMap database with the criteria of r^2 more than

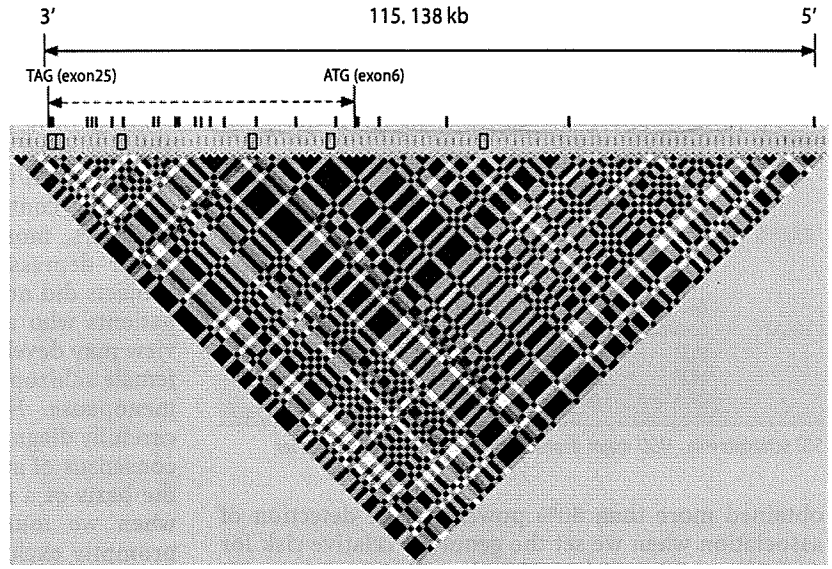

Fig. 1 LD evaluation and tagging SNPs in *CLOCK*. *Black bars* represent exons of *CLOCK*. Tagging SNPs selected from HapMap database are represented by *black boxes*. The *color* scheme is based on r^2 value. LD structure of *CLOCK* is very tight and roughly one block. The *color* scheme is based on r^2 value. Other information can be seen at the HAPLOVIEW website

Table 1 Association analysis of tagging SNPs in CLOCK

SNP ID[a]		Phenotype	MAF	*n*	Genotype distribution			*P* value		
					M/M	M/m	m/m	HWE	Genotype	Allele
SNP1	rs11939815	Control	0.216	795	490	266	39	0.708		
	G > T	SCZ	0.207	733	460	243	30	0.767	0.513	0.724
		MDD	0.221	324	192	121	11	0.123	0.822	0.302
		BP	0.228	149	88	54	7	0.724	0.694	0.406
SNP2	rs11931061	Control	0.221	795	485	268	42	0.532		
	A > G	SCZ	0.212	733	454	247	32	0.827	0.536	0.699
		MDD	0.224	324	190	123	11	0.0948	0.902	0.208
		BP	0.228	149	88	54	7	0.724	0.795	0.821
SNP3	rs11133385	Control	0.220	795	485	270	40	0.760		
	A > G	SCZ	0.222	733	452	236	45	0.0615	0.881	0.541
		MDD	0.221	324	193	119	12	0.222	0.977	0.482
		BP	0.235	149	88	52	9	0.723	0.574	0.837
SNP4	rs3736544	Control	0.220	795	487	266	42	0.472		
	G > A	SCZ	0.205	733	460	246	27	0.402	0.296	0.317
		MDD	0.222	324	192	120	12	0.199	0.914	0.334
		BP	0.232	149	88	53	8	0.996	0.663	0.875
SNP5	rs1801260	Control	0.161	795	563	208	24	0.373		
	T > C	SCZ	0.148	733	532	185	16	0.986	0.321	0.519
		MDD	0.157	324	231	84	9	0.684	0.833	0.972
		BP	0.158	149	106	39	4	0.856	0.887	0.976
SNP6	rs3749474	Control	0.380	795	311	364	120	0.427		
	T > C	SCZ	0.359	733	301	338	94	0.953	0.228	0.412
		MDD	0.356	324	119	160	45	0.450	0.793	0.547
		BP	0.376	149	58	70	21	0.987	0.895	0.940

[a]Major allele > minor allele

SCZ schizophrenia, *MDD* major depressive disorder, *BP* bipolar disorder, *MAF* minor allele frequency, *M* major allele, *m* minor allele, *HWE* Hardy–Weinberg equilibrium

0.8. We did not find an association between these tag SNPs and Japanese schizophrenia, BP or MDD in any of the analyses (Tables 1, 2). It is known that there are sex differences in not only the pathophysiology of schizophrenia [18] but also in circadian rhythms [20], and we detected slight gender differences in LD structures constructed of tag SNPs of both schizophrenia samples in this study (Supplementary Figure 1). To further investigation of these associations, we performed an explorative single marker and haplotype-wise analysis of subjects divided by sex. Only one association was detected, with schizophrenia females, in the haplotype-wise analysis ($P = 0.0362$) (Supplementary Table 4). However, this significance did not remain after Bonferroni correction ($P = 0.0724$) (Supplementary Table 4). Also, no association was detected in either sex in MDD or BP (Supplementary Tables 1, 2, 3, 4). In the power analysis, we

Table 2 Haplotype-wise analysis of tagging SNPs in *CLOCK*

Haplotype	Phenotype	Individual haplotype frequency	Individual *P* value	Phenotype	Global *P* value
GAAGTT	Control	0.625			
	SCZ	0.651	0.190	SCZ	0.340
	MDD	0.617	0.672	MDD	0.883
	BP	0.629	0.948	BP	0.957
GAAGCC	Control	0.158			
	SCZ	0.139	0.202		
	MDD	0.165	0.648		
	BP	0.150	0.782		
TGGATC	Control	0.217			
	SCZ	0.210	0.671		
	MDD	0.218	0.926		
	BP	0.220	0.867		

SCZ schizophrenia, *MDD* major depressive disorder, *BP* bipolar disorder

obtained more than 80% power for the detection of association when we set the genotype relative risk for *CLOCK* at 1.25–1.52 in schizophrenia, 1.76–1.85 in BP and 1.58–1.95 in MDD, under a multiplicative model of disease risk.

Discussion

In this study, only one association with schizophrenia in females was detected in the haplotype analysis ($P = 0.0362$), but this significance did not remain after Bonferroni correction ($P = 0.0724$). Also, we could not replicate the association between SNP5 (rs1801260: T3111C) and schizophrenia found in an earlier study [39], using larger Japanese schizophrenia and control samples. At this SNP, Takao et al. [39] showed higher MAFs of schizophrenia (MAFs: 0.224) compared with those of controls (MAFs: 0.141), although our study did not detect a significant difference with MAFs in schizophrenia or any specific gender subgroup compared with control. Also, there has been opened MAFs: 0.198 in Japanese HapMap database. In addition, LD from SNP1 to SNP4 was very tight in our control samples (r^2 more than 0.9), despite our selection of tag SNPs from HapMap database with the criteria of r^2 more than 0.8 (Minimum r^2 from SNP1 to SNP4 was 0.754 according to the database). So the differences of MAFs and r^2 in the Takao's study and the HapMap database with this study might be influenced by the sample size of each studies [39].

Similar to our study, several other investigations have found no association between *CLOCK* and BP or MDD using case–control samples and family based samples [1, 22, 32, 35, 36].

A few points of caution should be noted in interpreting our results. First, the lack of association may be due to biased samples, such as small sample sizes, especially BP and MDD samples or unmatched age- or gender-samples. Because our BP and MDD samples are small, there are possibilities of typeIIerrors in the results of association analysis for mood disorders statistically. Also, although we included subgroup analyses divided by gender, careful interpretation is needed with respect to the association of schizophrenia itself. On average, the controls are much younger than the patients. This means that a number of young controls may go on to develop one these disorders, most likely MDD, since the incidence of major depression is as high as 5% or more. Our subjects did not undergo structured interviews. MDD patients who are not diagnosed by structured interview may develop BP in the future [9, 37]. In addition, female schizophrenia has possibility to develop in the menopause. However, in this study patients were carefully diagnosed according to DSM-IV criteria with consensus of at least two experienced psychiatrists on the basis of a review of medical records. In addition, when we found a misdiagnosis of a patient, we promptly excluded the misdiagnosed case in consideration of the precision of our sample. Second, we did not include a mutation scan to detect rare variants with functional effects. However, it is difficult to evaluate the association of such extremely rare variants (e.g. MAFs < 0.01) from the viewpoint of power.

In conclusion, we suggest that *CLOCK* may not play a major role in the pathophysiology of schizophrenia, BP and MDD in the Japanese population. However, it will be important to replicate and confirm these findings in other independent studies using larger samples.

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No Association Between Polymorphisms of Neuronal Oxide Synthase 1 Gene (*NOS1*) and Schizophrenia in a Japanese Population

Takenori Okumura · Tomo Okochi · Taro Kishi · Masashi Ikeda · Tsuyoshi Kitajima · Yoshio Yamanouchi · Yoko Kinoshita · Kunihiro Kawashima · Tomoko Tsunoka · Hiroshi Ujike · Toshiya Inada · Norio Ozaki · Nakao Iwata

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Abstract The neuronal nitric oxide synthase gene (*NOS1*) is located on 12q24, in a susceptibility region for schizophrenia, and produces nitric oxide (NO) in the brain. NO plays a role in neurotransmitter release and is the second messenger of the *N*-methyl-D-aspartate (NMDA) receptor. Furthermore, it is connected to the dopaminergic and serotonergic neural transmission systems. Therefore, abnormalities in the NO pathway are thought to be involved in the pathophysiology of schizophrenia. Several genetic studies showed an association of *NOS1* with schizophrenia. However, results of replication studies have been inconsistent. Therefore, we conducted a replication study of *NOS1* with schizophrenia in a Japanese sample. We selected seven SNPs (rs41279104, rs3782221, rs3782219, rs561712, rs3782206, rs2682826, and rs6490121) in *NOS1* that were positively associated with schizophrenia in previous studies. Two SNPs showed an association with Japanese schizophrenic patients (542 cases and 519 controls, rs3782219: *P* allele = 0.0291 and rs3782206: *P* allele = 0.0124, *P* genotype = 0.0490), and almost these significances remained with an increased sample size (1154 cases and 1260 controls, rs3782219: *P* allele = 0.0197 and rs3782206: *P* allele = 0.0480). However, these associations also might have resulted from type I error on account of multiple testing (rs3782219: *P* allele = 0.133 and rs3782206: *P* allele = 0.168). In conclusion, we could not replicate the association between seven SNPs in *NOS1* and schizophrenia found in several earlier studies, using larger Japanese schizophrenia and control samples.

Keywords Schizophrenia · Neuronal nitric oxide syntase 1 gene (*NOS1*) · Case-control association study

T. Okumura · T. Okochi · T. Kishi (✉) · M. Ikeda · T. Kitajima · Y. Yamanouchi · Y. Kinoshita · K. Kawashima · T. Tsunoka · N. Iwata
Department of Psychiatry, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan
e-mail: tarok@fujita-hu.ac.jp

N. Ozaki
Department of Psychiatry, Nagoya University Graduate School of Medicine, Nagoya, Aichi 466-8850, Japan

M. Ikeda
Department of Psychological Medicine, School of Medicine, Cardiff University, Heath Park, Cardiff CF14 4XN, UK

H. Ujike
Department of Neuropsychiatry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Science, Okayama, Japan

T. Inada
Neuropsychiatric Research Institute, Seiwa Hospital, Shinjuku-ku, Tokyo 162-0851, Japan

Introduction

Schizophrenia is a common psychiatric disease, seen in approximately 1% of the world population. It is characterized by delusions, hallucinations, and cognitive dysfunction. Genetic factors play an important role in susceptibility to schizophrenia (Cardno and Gottesman 2000), and several genetic studies have identified susceptibility genes (Ross et al. 2006).

The nitric oxide synthase 1 gene (*NOS1*) is located on 12q24, and consists of 12 alternative untranslated first exons, termed exon 1a_1l, and 28 exons in a genomic region spanning 149.404 Kb. *NOS1* is considered to be a likely candidate gene for schizophrenia owing to its

chromosomal location, 12q24, which has been reported to be a susceptibility locus from several linkage studies (Bailer et al. 2000, 2002; DeLisi et al. 2002), and to produce nitric oxide (NO). NO is synthesized from L-arginine by a family of isoformic enzymes known as nitric oxide synthases (NOSs). Three isoforms of NOS have been identified: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) (McLeod et al. 2001). NO is involved in a variety of mechanisms, such as neurotransmitter release, *N*-methyl-D-aspartate (NMDA) receptor activation (Joca et al. 2007; Snyder and Ferris 2000), and oxidative stress in the brain (Yao and Reddy 2005). Abnormalities in these mechanisms are thought to be involved in the pathophysiology of psychotic disorders (Bennett 2008). Moreover, evidence from pharmacological studies in animal and postmortem studies supports an association between NO and psychotic disorders (Wass et al. 2009; Yao et al. 2004).

A number of genetic association studies showed that single nucleotide polymorphisms (SNP) in *NOS1* were associated with schizophrenia. Shinkai et al. (2002) examined the association between a synonymous SNP (rs2682826) in exon 29 and schizophrenia in a Japanese population, and showed that it was significant. Fallin et al. (2005) identified a haplotype (rs3782221–rs3782219–rs561712–rs3782206) and reported it to be associated with schizophrenia and schizoaffective disorder. *NOS1* has a complex promoter–exon1 region. Expression of the different mRNA from distinct promoters in *NOS1* is controlled by the 5′ flanking region (Bros et al. 2006). Reif et al. (2006) reported a polymorphism (rs41279104) in the promoter region of exon 1c associated with schizophrenia and prefrontal brain function. Recently, a whole genome association study reported an association between rs6490121 in intron 2 of *NOS1* and schizophrenia (Moskvina et al. 2009).

In this study, we conducted a replication study of association between significant seven SNPs in *NOS1* and schizophrenia in a Japanese samples.

Materials and Methods

Subjects

A total of 542 patients with schizophrenia (276 males and 266 females; mean age ± standard deviation; 43.8 ± 14.8 years) and 519 healthy controls (264 males and 255 females; 36.5 ± 14.1 years) were recruited. For rs3782219 and rs3782206, which showed a significant association in the allele and/or genotype-wise analysis, additional samples were included for this association analysis, bringing the totals to 1154 schizophrenics (additional 612 cases) and 1260 controls (additional 741 controls). All subjects were unrelated to each other and ethnically Japanese. The patients were diagnosed according to DSM-IV criteria with the consensus of at least two experienced psychiatrists on the basis of unstructured interviews and a review of medical records, and who were outpatients or inpatients of psychiatric hospitals. The patients were grouped according to the following DSM-IV subtypes of schizophrenia: Paranoid Type ($n = 429$), Disorganized Type ($n = 441$), Catatonic Type ($n = 39$), Residual Type ($n = 138$), and Undifferentiated Type ($n = 107$). All healthy control subjects were also psychiatrically screened based on unstructured interviews. None had severe medical complications such as cirrhosis, renal failure, heart failure, or other Axis-I disorders according to DSM-IV. No structured methods were used to assess psychiatric symptoms in the controls (hospital staffs and medical students). None of the subjects were known to be related to each other, and all were ethnically Japanese. Written informed consent was obtained from each subject. This study was approved by the ethics committees at Fujita Health University, Okayama University, and Nagoya University Graduate School of Medicine.

SNP Selection and Genotyping

We selected seven SNPs (rs41279104, rs3782221, rs3782219, rs561712, rs3782206, rs2682826, and rs6490121) in *NOS1* shown by previous studies to have a positive association with schizophrenia (Fallin et al. 2005; Moskvina et al. 2009; Reif et al. 2006; Shinkai et al. 2002). We used TaqMan assays (Applied Biosystems, Foster City, CA, USA) for all SNPs. Detailed information is available on request.

Statistical Analysis

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by the chi-square test (SAS/Genetics, release 8.2, SAS Japan Inc., Tokyo, Japan). Marker–trait association was also evaluated by the chi-square test in allele- and genotype-wise analyses. Haplotype frequencies were estimated in a two- to four-marker sliding window fashion and log likelihood ratio tests were performed for global *P* values with COCAPHASE program version 3.0.6 (Dudbridge 2003). In these haplotype-wise analyses, rare haplotypes (less than 0.05) in either of cases and controls were excluded from the association analysis. Power calculation was performed using a statistical program prepared by Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purecell//gpc/). To correct for problems of multiple comparisons, we applied the Benjamini–Hochberg (BH) method, which is a procedure to control for false discovery

rate (FDR) (Dudbridge 2003). The level of significance for all statistical tests was 0.05.

Results

Genotype frequencies of subjects and controls did not deviate significantly from HWE. In the first-set of analysis, two SNPs (rs3782219 and rs3782206) showed a significant association with schizophrenia in allele and/or genotype-wise analysis (rs3782219: *P* allele = 0.0291 and rs3782206: *P* allele = 0.0124, *P* genotype = 0.0490). Five other SNPs did not show evidence of association with schizophrenia (Table 1). There was no evidence of association with schizophrenia in haplotype-wise analysis (Table 2).

To validate the significant association of rs3782219 and rs3782206 found in the first-set samples, and even some significances remained with an increased sample size (1154 cases and 1260 controls, rs3782219: *P* allele = 0.0197 and rs3782206: *P* allele = 0.0480); however, these associations also might have resulted from type I error on account of multiple testing (rs3782219: *P* allele = 0.133 and rs3782206: *P* allele = 0.168) (Table 1).

We obtained power of more than 80% for the detection of association when we set the genotype relative risk at 1.27–1.36 under a multiplicative model of inheritance in the first-set samples.

Discussion

We found marginal associations between two SNPs (rs3782219 and rs3782206) and schizophrenia in allele and/or genotype-wise analysis, and almost these significances remained with an increased sample size. However, we suggested that it might have resulted from type I error due to multiple testing. Fallin et al. (2005) reported that significant associations of haplotypes were identified with four SNPs in intron 2 (rs3782221, rs3782219, rs561712, and rs3782206). However, no association was found in our study. Shinkai et al. (2002) showed a strongly positive association between rs2682826 and schizophrenia in a Japanese population. However, although we examined more large Japanese samples than original study (Shinkai et al. 2002), we found no significant association with schizophrenia. The result of this study was in concordance with replication studies in other ethnic population samples (Liou et al. 2003; Tang et al. 2008). Recently, a whole genome association study reported a possible association between rs6490121 in *NOS1* and schizophrenia (O'Donovan et al. 2008). To avoid multiple testing problems, it is important to conduct replication study. Our samples were provided for replication study and showed a significant association and odds ratio that were opposite to UK samples (O'Donovan et al. 2008). However, although we performed a replication study using larger different samples

Table 1 Association study between *NOS1* and schizophrenia

SNP ID	Position	Phenotype[a]	MAF[b]	*N*	Genotype distribution[c]			*P* value[e]			Corrected *P* value[f]
					M/M	M/m	m/m	HWE[d]	Genotype	Allele	Allele
rs41279104	114886493	SCZ	0.197	542	355	162	25	0.243	0.436	0.230	
	Promoter region of exon 1c	CON	0.175	519	354	148	17	0.751			
rs3782221	114805000	SCZ	0.448	542	173	252	117	0.161	0.488	0.275	
	Intron 1	CON	0.424	519	175	247	97	0.550			
rs3782219	114797355	SCZ	0.411	1154	409	540	205	0.248	0.0655	**0.0197**	0.133
	Intron 1	CON	0.444	1260	394	611	255	0.518			
rs561712	114761232	SCZ	0.176	542	374	145	23	0.0677	0.128	0.856	
	Intron 2	CON	0.179	519	346	160	13	0.274			
rs3782206	114754240	SCZ	0.279	1154	610	443	101	0.111	0.133	**0.0480**	0.168
	Intron 3	CON	0.254	1260	706	467	87	0.415			
rs6490121	114717324	SCZ	0.390	542	203	255	84	0.790	0.244	0.0952	
	Intron 10	CON	0.425	519	175	246	98	0.484			
rs2682826	114662005	SCZ	0.353	542	223	255	64	0.491	0.469	0.228	
	Exon 29	CON	0.328	519	230	237	52	0.424			

[a] *SCZ* schizophrenia, *CON* control

[b] *MAF* minor allele frequency

[c] *M* major allele, *m* minor allele

[d] Hardy–Weinberg equilibrium

[e] Bold numbers represent significant *P* value

[f] Calculated using Benjamini–Hochberg (BH) method

Table 2 Haplotype-wise analysis between *NOS1* and schizophrenia

SNP ID	Global *P* value		
	2 Window	3 Window	4 Window
rs41279104			
	0.228		
rs3782221		0.187	
	0.051		0.106
rs3782219		0.223	
	0.180		0.203[a]
rs561712		0.0770	
	0.0620		0.112
rs3782206		0.0780	
	0.0600		0.223
rs2682826		0.211	
	0.131		
rs6490121			

[a] Fallin et al. reported

than original study, we could not replicate. In other recent study, Tang et al. (2008) reported a significant association with schizophrenia of rs3782206 in a Chinese population. This discordance of results may reflect problems in the replication study, such as population difference, in each sample. Therefore, it is necessary to evaluate a polymorphism for this association with schizophrenia in various ethnic populations.

A few points of caution should be noted in interpreting our results. First, we did not apply a LD-based approach and a mutation scan to detect rare variants with functional effects. Moreover, we did not examine a VNTR in exon 1f within the promoter region in *NOS1*. Reif et al. (2006) reported a significant association of the haplotype constructed by rs41279106 and this VNTR with schizophrenia. These problems are future topics for study. Second, our sample was not matched in terms of age. Moreover, our samples were not assessed by a standard structured interview, and thus there is a chance of false negatives due to misdiagnosis or sampling bias (Kishi et al. 2009).

In conclusion, we suggest that these seven SNPs in *NOS1* may not play a role in the susceptibility to schizophrenia in the Japanese population. However, other functional polymorphisms in *NOS1* may show important roles in the pathophysiology of schizophrenia, and further investigations will be necessary.

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No association between the *Bcl2-interacting killer* (*BIK*) gene and schizophrenia

Kazutaka Ohi[a,d], Ryota Hashimoto[a,b,c,d,*], Yuka Yasuda[a,b,d], Hidenaga Yamamori[a,e], Hiroaki Hori[c], Osamu Saitoh[f], Masahiko Tatsumi[g], Masatoshi Takeda[a,b], Nakao Iwata[h], Norio Ozaki[i], Kunitoshi Kamijima[j], Hiroshi Kunugi[c]

[a] *Department of Psychiatry, Osaka University Graduate School of Medicine, Suita, Osaka, Japan*
[b] *The Osaka-Hamamatsu Joint Research Center for Child Mental Development, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan*
[c] *Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan*
[d] *Japan Science and Technology Agency, CREST, Kawaguchi, Saitama, Japan*
[e] *Department of Molecular Neuropsychiatry, Osaka University Graduate School of Medicine, Suita, Osaka, Japan*
[f] *Department of Psychiatry, National Center Hospital, National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan*
[g] *Yokohama Shinryo Clinic, Yokohama, Kanagawa, Japan*
[h] *Department of Psychiatry, Fujita Health University School of Medicine, Toyoake, Aichi, Japan*
[i] *Department of Psychiatry, Nagoya University Graduate School of Medicine, Nagoya, Aichi, Japan*
[j] *Department of Psychiatry, Showa University School of Medicine, Shinagawaku, Tokyo, Japan*

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ABSTRACT

The *Bcl2-interacting killer* (*BIK*) gene interacts with cellular and viral survival-promoting proteins, such as Bcl-2, to enhance apoptosis. The BIK protein promotes cell death in a manner analogous to Bcl-2-related death-promoting proteins, Bax and Bak. There have been lower Bcl-2 levels and increased Bax/Bcl-2 ratio in the temporal cortex of patients with schizophrenia compared with those in controls. Because the death-promoting activity of BIK was suppressed in the presence of the cellular and viral survival-promoting proteins, the BIK protein is suggested as a likely target for antiapoptotic proteins. The purpose of this study is to investigate the association between genetic variants in the *BIK* gene and schizophrenia in a large Japanese population (1181 patients with schizophrenia and 1243 healthy controls). We found nominal evidence for association of alleles, rs926328 ($\chi^2 = 4.44$, $p = 0.035$, odds ratio = 1.13) and rs2235316 ($\chi^2 = 4.41$, $p = 0.036$, odds ratio = 1.13), with schizophrenia. However, these associations were no longer positive after correction for multiple testing (rs926328: corrected $p = 0.105$, rs2235316: corrected $p = 0.108$). We conclude that *BIK* might not play a major role in the susceptibility of schizophrenia in Japanese population.

Schizophrenia (MIM 181500) is a common complex psychiatric disease and is generally considered as a neurodevelopmental disorder. The lifetime morbidity rate is 0.5–1.0% across distinct populations. Family, twin, and adoption studies of schizophrenia have indicated that there are strong genetic factors with an estimated heritability of approximately 80% [5,20]. Regions on a number of chromosomes (e.g., 1, 6, 8, 10, 13, and 22) have been implicated as sites of potential vulnerability genes [17]. For example, 22q11–13 has been shown as a suggestive region in a number of linkage studies.

Apoptosis, a form of programmed cell death, is regulated by a complex cascade of pro- and anti-apoptotic members of the Bcl-2 family proteins. The ratio of pro-apoptotic (e.g. Bax, Bad) to anti-apoptotic (e.g. Bcl-2, Bcl-X_L) protein levels is a key determinant in regulating cytochrome *c* release and subsequent caspase activation, leading to rapid neuronal death [16,21]. Postmortem brain studies showed that markers of apoptosis, levels of apoptotic regulatory proteins and DNA fragmentation patterns, were altered in schizophrenia [1,2,12,13]. The Bcl-2 levels are 30% lower in temporal cortex in schizophrenia compared with controls [12]. There is a 50% increase in the Bax/Bcl-2 ratio in the temporal cortex of patients with schizophrenia compared to matched controls [13]. The cortical neuropathology in schizophrenia such as synaptic deficits and reduced neuropil without overall neuronal loss, and limited and often layer-specific reductions of neurons appears to be characterized by non-lethal and localized apoptotic activity at the level of synapses and terminal neuritis in schizophrenia [1,2,8,13]. Neuroimaging studies suggest that a progressive loss of cortical gray matter occurs in the early stage of the clinical course of schizophrenia [3,7,19]. Although the mechanisms underlying these data are

* Corresponding author at: The Osaka-Hamamatsu Joint Research Center for Child Mental Development, Osaka University Graduate School of Medicine, D3, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 3074; fax: +81 6 6879 3059.
E-mail address: hashimor@psy.med.osaka-u.ac.jp (R. Hashimoto).

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Table 1
Genotype and allele distributions for SNPs in the *BIK* gene between patients with schizophrenia and controls.

Marker				SCZ			CON			Genotypic p-value ($df = 2$)	SCZ	CON	Allelic p-value ($df = 1$)	OR
dbSNP IDs	Position[a]	M/m	Gene	M/M	M/m	m/m	M/M	M/m	m/m		MAF			
rs926328	22896778	T/A	5′	0.29	0.50	0.21	0.26	0.51	0.24	0.10	0.46	0.49	0.035	0.89
rs4988372	22897148	C/T	5′	0.89	0.10	0.005	0.91	0.09	0.003	0.50	0.06	0.05	0.25	1.16
rs4988374	22897266	C/A	5′	0.89	0.10	0.005	0.91	0.09	0.003	0.45	0.06	0.05	0.21	1.18
rs2235316	22914504	A/C	Intron 3	0.26	0.50	0.24	0.29	0.49	0.22	0.10	0.49	0.46	0.036	1.13

SCZ, patients with schizophrenia; CON, healthy controls; m, minor allele; M, major allele; MAF, minor allele frequency; OR, odds ratio.
[a] db SNP build 129.

unknown, evidence for progressive clinical deterioration and subtle neurostructural changes following the onset of psychosis has led to the hypothesis that the increased apoptotic vulnerability may contribute to the pathophysiology of schizophrenia.

BIK (*Bcl2-interacting killer*) gene (MIM 603392) mapped on chromosome 22q13, which has a modest linkage to schizophrenia [6]. The gene contains 5 exons and spans approximately 19 kb [22]. *BCL-2* gene has been shown to enhance the survival of a variety of cell types exposed to diverse cell death-inducing stimuli. Bcl-2-related proteins either promote cell survival or accelerate cell death. The protein encoded by the *BIK* gene is known to interact with the cellular survival-promoting proteins Bcl-2 and Bcl-X_L, as well as with the viral survival-promoting proteins Epstein–Barr virus (EBV) BHRF1 and adenovirus E1B 19-kD to enhance apoptosis [4,9]. The BIK promotes cell death similar to the Bcl-2-related death-promoting proteins, Bax and Bak. Because the death-promoting activity of BIK was suppressed by coexpression of Bcl-2, Bcl-X_L, EBV BHRF1, and E1B 19-kD, the BIK protein might be associated with anti-apoptotic proteins. In this study, we investigated whether the *BIK* gene is associated with schizophrenia in a large Japanese population.

The subjects consisted of 1181 patients with schizophrenia [51.1% males (604/577), mean age ± SD; 46.1 ± 14.9 years] and 1243 healthy controls [46.5% male (578/665), mean age ± SD; 38.6 ± 15.7 years]. The sex ratio and the mean age differed significantly between groups (sex ratio; $\chi^2 = 5.2$, $p = 0.022$, mean age; $z = -13.0$, $p < 0.001$). All the subjects were biologically unrelated Japanese. Patients were recruited at the National Center Hospital of Neurology and Psychiatry, Showa University School of Medicine, Fujita Health University School of Medicine and Osaka University Graduate School of Medicine. Cases were recruited from both outpatients and inpatients at the hospitals. Each schizophrenic research subject had been diagnosed by at least two trained psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria based on unstructured clinical interviews. Controls, including the hospital and institutional staffs, were recruited through local advertisements. Psychiatrically healthy controls were evaluated using unstructured interviews to exclude individuals who had current or past contact with psychiatric services. We did not assess the controls for the family history of psychiatric disorders, such as schizophrenia, bipolar disorder, or major depressive disorder. All experiments on human subjects were conducted in accordance with the Declaration of Helsinki and all procedures were carried out with the adequate understanding and written consent of the subjects.

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to standard procedures. Four single nucleotide polymorphisms (SNPs) in the *BIK* gene were selected from the public database (HAPMAP: http://www.hapmap.org/index.html.ja) in order to cover the *BIK* gene (4.75 kb per SNP). The four SNPs in the *BIK* gene (rs926328, rs4988372, rs4988374 and rs2235316) were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay (Applied Biosystems, Foster City, California, USA), as described previously [10,11,15]. Primers and probes for detection of the SNPs are available upon request.

Statistical analyses were performed using SNPAlyze V5.1.1 Pro software (DYNACOM, Yokohama, Japan) and SPSS 16.0J software (SPSS Japan Inc., Tokyo, Japan). Differences in clinical characteristics between patients and controls or between genotype groups were analyzed using χ^2 tests for categorical variables and the Mann–Whitney *U*-test for continuous variables. A deviation from Hardy–Weinberg equilibrium (HWE) was tested separately in cases and controls by using the χ^2 tests for goodness of fit. The allelic and genotypic distributions of the *BIK* polymorphisms between patients and controls were analyzed using χ^2 tests. The number of effective independent SNPs assayed was estimated by the spec-

Table 2
Marker-to-marker linkage disequilibrium for all the combinations of the four SNPs in the *BIK* gene.

	rs926328	rs4988372	rs4988374	rs2235316
rs926328	–	1.00	1.00	0.36
rs4988372	1.00	–	1.00	0.88
rs4988374	1.00	1.00	–	0.86
rs2235316	0.39	0.91	0.91	–

For each pair of markers, the standardized D' in controls is shown below the diagonal, and the standardized D' in patients with schizophrenia is shown above the diagonal.

tral decomposition method of Nyholt using SNPSpD software [14]. The pairwise linkage disequilibrium (LD) analyses, expressed by D', were applied to detect the intermarker relationship in each group using the SNPAlyze V5.1.1 Pro software. We performed power calculations using the Power Calculator for Two Stage Association Studies (http://www.sph.umich.edu/csg/abecasis/CaTS/ [18]). Power (>0.80) was calculated under prevalence of 0.01, several allele frequencies in patients (rs926328; 0.46, rs4988372; 0.06, rs4988374; 0.06, or rs2235316; 0.46) and an alpha level of 0.05 using a multiplicative model, assuming varying degrees of the odds ratio. All p-values reported are two tailed. Statistical significance was defined as $p < 0.05$.

The genotype and allele frequencies of four SNPs located in the *BIK* gene and the flanking regions are summarized in Table 1. Genotyping completeness ranged from 96.3% (rs2235316) to 99.3% (rs4988372). The genotype distributions of all examined SNPs in the *BIK* gene were in HWE for both controls and patients with schizophrenia ($p > 0.3$). We found no genotypic association between the four SNPs in the *BIK* gene and schizophrenia (Table 1). On the other hand, there were significant differences in allele frequencies of rs926328 and rs2235316 (Table 1). The major T allele of the rs926328 and the minor C allele of the rs2235316 in the *BIK* gene were in excess in patients with schizophrenia compared with controls (rs926328; $\chi^2 = 4.44$, $p = 0.035$, odds ratio = 1.13, 95% confidential interval 1.01–1.27, rs2235316; $\chi^2 = 4.41$, $p = 0.036$, odds ratio = 1.13, 95% confidential interval 1.01–1.27; Table 1). However, these associations did not survive after SNPSpD correction for multiple testing in this gene (the effective number of independent marker loci: 3.0; rs926328; corrected $p = 0.105$, rs2235316; corrected $p = 0.108$). There was no allelic association with schizophrenia for the other two SNPs, rs4988372 and rs4988374. The LD relationships between markers were shown in Table 2. The LD pattern observed in our patients was nearly identical to those in our controls. The strong LD patterns among four SNPs were observed in both groups ($D' > 0.8$), except for the weak LD between rs926328 and rs2235316 in both groups ($D' < 0.5$).

Nominal associations between the genetic variants, rs926328 and rs2235316, and schizophrenia in this study were no longer positive after correction for multiple testing. Power analysis showed that our subjects had sufficient power (>0.80) to detect an effect of the odds ratio (1.18 or more) for rs926328 or rs2235316. For other two SNPs, rs4988372 and rs4988374, our sample size had power (>0.80) to detect an effect of the odds ratio (1.38 or more). As the odds ratios of the rs926328 and rs2235316 were 1.13, our sample size was not enough to detect such a small contribution to risk for schizophrenia. This could lead to the type II error. There were other limitations in our study. Control samples were not matched by age and sex with the patient population or were not represent general population as they included substantial portion of hospital and institutional staffs. Although the psychiatric phenotypes of the controls were screened by the unstructured interview, we did not assess the family history of controls subjects. Thus, control subjects might carry the risk allele from affected parents. Careful interpretation of our results due to population stratification would be needed in our case control designed study, despite the precaution of ethnic matching of this study. Therefore, it is necessary to carry out further investigations to confirm our findings in other samples with age and sex matched case-control subjects and with much larger sample size.

In conclusion, we have examined a possible association between the *BIK* gene and schizophrenia for the first time and have not found the association with schizophrenia in the Japanese population. Two SNPs gave nominal evidence for association and this association did not survive after correction for multiple testing. *BIK* is not likely to be a major susceptibility gene for schizophrenia in this Japanese population. The diagnosis of schizophrenia itself is too non-specific and that the *BIK* might be more related to differences in patients with and without specific deficits in basic neurophysiological process such as sensory gating, executive function, etc. Further studies are needed to investigate the contribution of pro- or anti-apoptotic genes other than *BIK* in schizophrenia.

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Prepulse inhibition of the startle response with chronic schizophrenia: A replication study

Masatsugu Moriwaki [a,b,1], Taro Kishi [a,c,1,*], Hidetoshi Takahashi [c,d], Ryota Hashimoto [c,d,e], Kunihiro Kawashima [a,c,f], Tomo Okochi [a,c], Tsuyoshi Kitajima [a,c], Osamu Furukawa [b], Kiyoshi Fujita [b], Masatoshi Takeda [d,e], Nakao Iwata [a,c]

[a] Department of Psychiatry, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan
[b] Okehazama Hospital, Toyoake, Aichi 470-1168, Japan
[c] Japan Science and Technology Agency, CREST, Kawaguchi, Saitama, Japan
[d] Department of Psychiatry, Osaka University Graduate School of Medicine, Suita, Osaka, Japan
[e] The Osaka-Hamamatsu Joint Research Center for Child Mental Development, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan
[f] Jindai Hospital, Toyota, Aichi 470-0361, Japan

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ABSTRACT

Prepulse inhibition (PPI) deficit, the acoustic startle reflex (ASR) and habituation (HAB) impairment are considered to be endophenotypes for schizophrenia. The recent two studies have reported that a PPI deficit was detected in Japanese schizophrenic patients. We replicated that study using larger samples (115 schizophrenic patients and 111 normal controls) than the original study and a method same as original study. A startle response monitoring system was used to deliver acoustic startle stimuli, and to record and score the electromyographic activity of the orbicularis oculi muscle. We evaluated the startle measures of mean magnitude of ASR, HAB, and PPI at prepulse sound pressure intensities of 82 dB (PPI82), 86 dB (PPI86), and 90 dB (PPI90). ASR was significantly different between schizophrenic patients and controls. HAB and all PPI session data from schizophrenic patients were significantly lower than in controls. In addition, we detected significant differences for ASR, HAB and each PPI (82, 86 and 90 dB) between schizophrenic patients and controls with the use of multiple regression analysis. The gender and smoking state were not correlated with ASR, HAB or any PPI in multiple regression analysis. In conclusion, we were able to replicate the finding of HAB impairment and PPI deficit in chronic Japanese schizophrenic patients.

1. Introduction

Disruption of sensorimotor gating is considered to lead to abnormalities in cognitive dysfunction, such as attention-related information processing (Braff et al., 1992; McGhie and Chapman, 1961). Several investigations suggested that disruption of sensorimotor gating was measured mainly by prepulse inhibition (PPI) and the acoustic startle reflex (ASR) (Braff et al., 1992; Kunugi et al., 2007; Takahashi et al., 2008). PPI deficit, ASR and habituation (HAB) impairment are considered endophenotypes for schizophrenia (Braff et al., 1992; Kunugi et al., 2007; Takahashi et al., 2008; Walters and Owen, 2007).

Recently, Kunugi et al. (2007) have reported that a PPI deficit was detected in Japanese schizophrenic patients. More recently, Takahashi et al. (2008) evaluated the startle measures of mean magnitude of ASR, HAB, and PPI at prepulse sound pressure intensities of 82, 86, and 90 dB, respectively. Although they found that ASR was not significantly different between the patients and controls, they reported that patients showed significantly reduced HAB and PPI for all prepulse intensities compared to controls (Takahashi et al., 2008). In this study, we performed a replication using larger samples (115 schizophrenic patients and 111 normal controls) than the original study and a method same as original study (Takahashi et al., 2008).

2. Materials and methods

2.1. Subjects

One hundred and fifteen schizophrenic patients (71 males and 40 females: mean age ± standard deviation (SD) 52.0 ± 1.46 years)

* Corresponding author at: Department of Psychiatry, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan. Tel.: +81 562 93 9250; fax: +81 562 93 1831.
E-mail address: tarok@fujita-hu.ac.jp (T. Kishi).
[1] These authors contributed equally to this work.

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and 111 normal controls (72 males and 38 females: mean age ± standard deviation (SD) 32.7 ± 0.674 years) were recruited. All subjects were unrelated to each other, ethnically Japanese, and lived in the central area of Japan. The patients were diagnosed according to DSM-IV criteria with the consensus of at least two experienced psychiatrists on the basis of a structured interview using the Structured Clinical Interview for DSM-IV disorders (SCID-1) and a review of medical records. All the patients met the following inclusion criteria: (1) age 25–70 years, (2) no systemic or neurologic disease, (3) no electroconvulsive therapy, (4) no history of head trauma, (5) no lifetime history of substance dependence or history of substance abuse within 3 months and (6) maintenance of main antipsychotic drug therapy for 2 months. All healthy control subjects were also psychiatrically screened based on structured interviews. None had severe medical complications or other Axis-I disorders according to DSM-IV using SCID-1. No structured methods were used to assess psychiatric symptoms in the controls. The study was described to subjects and written informed consent was obtained from each. This study was approved by the ethics committees at Fujita Health University School of Medicine and Okehazama Hospital.

2.2. Startle response measurement

2.2.1. Apparatus and stimuli

We measured startle measure using a commercial computerized human startle response monitoring system (Startle Eyeblink Reflex Analysis System Map1155SYS, Nihonsanteku Co., Osaka, Japan). Startle eyeblink electromyographic responses were recorded from the left orbicularis oculi muscle with 2 Ag/AgCl disposable electrodes (sensor area 15 mm^2) filled with wet gel. The first electrode (Blue Sensor N-00-S, Ambu, Ballerup, Denmark) was positioned approximately 1 cm directly below the pupil of the left eye and low enough to not touch the lower eyelid, while the second electrode (Blue Sensor M-00-S, Ambu, Ballerup, Denmark) was placed laterally and slightly superior to the first one, with the centers of the electrodes separated by approximately 2 cm. The impedance between the two electrodes was measured and deemed acceptable if below 5 kΩ. The impedance was measured with an electrode impedance meter (MaP811, Nihonsanteku Co., Osaka, Japan) at a measurement frequency = 30 Hz. The ground electrode (Blue Sensor M-00-S) was placed on the left angle of the mandible. We used a method same as Takahashi et al.'s (2008) study. Detailed information can be seen in Takahashi et al.'s (2008) paper.

2.2.2. The stimulus sequence, procedure and response scoring and data reduction

Measurements were made with startle paradigm constructed of 3 blocks with continuous 70 dB SPL background white noise. Pulse stimuli consisted of broadband white noises with an instantaneous rise/fall time lasting for 40 ms and presented at 115 dB SPL. Prepulse stimuli were also broadband white noises with an instantaneous rise/fall time lasting for 20 ms and presented at three different intensities (82, 86, and 90 dB SPL). The lead interval (LI) (from prepulse onset to pulse onset) in our study was set at 120 ms. In block 1, the startle response for pulse alone trials (PA trial) was recorded 6 times. Block 2 consisted of PA trials or trials of pulse with prepulse at three intensities (PP trials), performed eight times for each condition. Block 3 was the same as block 1 to measure the habituation phenomenon. All trials were presented in a fixed pseudorandom order, separated by inter-trial intervals of 15–25 s (20 s on average). The startle paradigm consisted of a total of 44 trials. Together with 5 min acclimation to the background noise, the session lasted approximately 20 min. We used a method same as Takahashi et al.'s (2008) study. Detailed information can be seen in Takahashi et al.'s (2008) paper.

2.3. Statistical analysis

Individual t test and chi-square tests (Fisher's exact test when appropriate) were used to compare means and categorical proportions, respectively. All PPI measures were examined with analysis of variance (ANOVA) with repeated measures of prepulse intensities. In addition, because several investigators have reported significant differences in startle response with gender difference, between smokers and nonsmokers, and with the effect of benzodiazepine in not only schizophrenic patients but also normal controls (Kumari et al., 2004, 1996, 1997; Swerdlow et al., 1999), we performed exploratory analysis of the possible correlations between all startle measures (PPI, ASR and HAB, respectively) and diagnosis (schizophrenia or controls), gender, and smoking history (smoker or nonsmoker) by multiple regression analysis. In these analyses, all startle measures were set as the dependent variable, and diagnosis (schizophrenia or controls), gender and current smoking status (smoker or nonsmoker) were set as the independent variables. We performed another multiple regression analysis on the effect of several clinical variables on all startle measures with schizophrenic patients. In these analyses, all startle measures were set as the dependent variable, and age, duration of illness, gender, smoking status, and medication dosage (antipsychotics and anxiolytics/hypnotics) were set as the independent variables. The significance level for all statistical tests was 0.05.

All statistical analyses were performed using JMP (JMP 5.0. 1J, SAS Japan Inc., Tokyo, Japan).

3. Results

3.1. Patient and control demographics and disposition

Among the subjects (schizophrenia or controls) in this study, only one difference with recruited age was detected (P value ≤ 0.0001) (Table 1). Also, there were 15 nonresponders in the patient group and 4 in the control group. Therefore, we performed the analysis of startle measure with 100 patients and 107 controls. Clinical demographics and disposition of analysed schizophrenic patients are shown in Table 1.

3.2. Schizophrenic patients vs. controls

We detected significant differences for all startle measures between schizophrenic patients and controls (Fig. 1). In addition, we detected significant differences for ASR, HAB and each PPI (82, 86 and 90 dB) between schizophrenic patients and controls with the use of multiple regression analysis (Table 2). The gender and smoking state were not correlated with ASR, HAB or any PPI in multiple regression analysis (Table 2).

3.3. Startle response with schizophrenia reflected clinical variables

There was no significant correlation of ASR, HAB or any PPI for either side with age, duration of illness, gender, smoking status, and medication dosage (antipsychotics and anxiolytics/hypnotics) (data not shown).

4. Discussion

We investigated startle response with schizophrenic patients and controls using larger samples than the original study. We were able to replicate HAB impairment and PPI deficit in chronic Japanese schizophrenic patients using a multiple regression model, controlling for other parameters known to influence the startle measure. To our knowledge, this type of analysis has not been previously used for startle response study. Controlling for

Table 1
Patients and controls demographics and disposition.

	Participated control subjects	Participated schizophrenia subjects	*P* value[a]
N	111	115	
Current smoker/nonsmoker	42 (37.4%)	32 (27.8%)	0.0791
Sex (males/females)	72/38	71/40	0.513
Age, years (mean ± SD)	32.7 ± 0.674	52.0 ± 1.46	**<0.0001**
	Analysed control subjects	**Analysed schizophrenia subjects**	***P* value[a]**
N	107	100	
Current smoker/nonsmoker, *n* (%)	40/67 (62.6)	26/74 (26.0)	0.0781
Sex (males/females)	71/36	62/38	0.514
Age, years (mean ± SD)	32.7 ± 7.16	51.6 ± 15.7	**<0.0001**
Duration of illness (day, mean ± SD)		9750 ± 524	
Clinical diagnosis, *n* (%)			
Paranoid type		30 (30)	
Disorganeized type		9 (9)	
Residual type		61 (61)	
PANSS total score		77.1 ± 23.5	
Medication dosage			
Antipsychotics (mg/day)[b]		693 ± 48.3	
Antiparkinsonian drugs, *n* (%)		0 (0)	
Mood stabilizers, *n* (%)		21 (21)	
Anxiolytics/hypnotics, *n* (%) (mg/day)[c]		69 (69) (8.46 ± 9.51)	

[a] Bold represents significant *P* value.
[b] Chlorpromazine-equivalent.
[c] Diazepam-equivalent.

important covariates, we found that startle response changes did not differ with smoking status or gender. We showed that the only factor influencing startle measure was diagnosis (schizophrenia or controls). None of the startle measures in schizophrenic patients was correlated with age, duration of illness, gender, smoking status, or medication dosage (antipsychotics and anxiolytics/hypnotics). Therefore, we considered that startle response might have diagnosis specificity associated with sensorimotor gating deficit.

Kunugi et al. (2007) reported reduced ASR and PPI despite of no reduced HAB in schizophrenia. On the other hand, Takahashi et al. (2008) reported reduced PPI and HAB despite of no reduction of

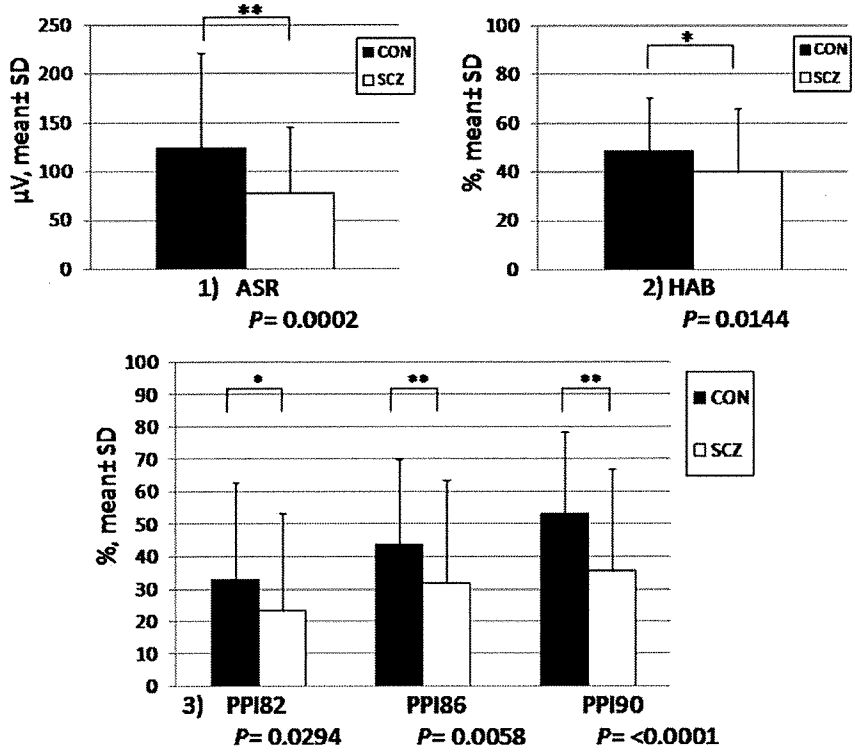

Fig. 1. ANOVA of startle measures with schizophrenia and controls. $^{*}P < 0.05$, $^{**}P < 0.01$. (1) Acoustic startle reflex (ASR) (μV, mean ± SD); schizophrenia: 78.0 ± 67.6; controls: 124 ± 96.5. (2) Habituation (HAB) (%, mean ± SD); schizophrenia: 39.8 ± 26.0; controls: 48.6 ± 21.7. (3) Prepulse inhibition (PPI); PPI 82 (%, mean ± SD); schizophrenia: 23.3 ± 30.2; controls: 33.0 ± 29.9. PPI 86 (%, mean ± SD); schizophrenia: 32.0 ± 31.7; controls: 43.8 ± 26.2. PPI 90 (%, mean ± SD); schizophrenia: 35.5 ± 31.5; controls: 53.1 ± 25.3.

Table 2
Multiple regression analysis of startle measure with clinical variables. All startle measures were set as the dependent variable, and diagnosis (schizophrenia or controls), gender and current smoking status (smoker or nonsmoker) were set as the independent variables.

Startle measure[a]	Clinical variables[b]	*P* value[c]	SRC[d]
ASR	Diagnosis	**<0.0001**	0.402
	Current smoker/nonsmoker	0.189	0.0480
	Sex	0.591	0.110
Habituation	Diagnosis	**0.0194**	0.177
	Current smoker/nonsmoker	0.264	−0.0879
	Sex	0.294	0.0820
PPI 82db	Diagnosis	**0.0249**	0.163
	Current smoker/nonsmoker	0.107	0.124
	Sex	0.0756	−0.136
PPI 86db	Diagnosis	**0.00350**	0.212
	Current smoker/nonsmoker	0.174	0.181
	Sex	0.0818	−0.132
PPI 90db	Diagnosis	**<0.0001**	0.310
	Current smoker/nonsmoker	0.0652	0.139
	Sex	0.428	−0.0592

[a] ASR: acoustic startle reflex; PPI: prepulse inhibition.
[b] Diagnosis: schizophrenia or controls.
[c] Bold represents significant *P* value.
[d] SRC: standardized regression coefficient.

ASR in schizophrenia. In this study, we detected significant differences for all of ASR, HAB and each PPI (82, 86 and 90 dB) between schizophrenic patients and controls with the use of multiple regression analysis. We considered that the difference of some results in these studies might be the following reasons: (1) Taking different antipsychotic in patient group (Kumari and Sharma, 2002; Kumari et al., 2002; Wynn et al., 2007). Several studies showed the difference of the kind of the antipsychotics influenced startle response (Kumari and Sharma, 2002; Kumari et al., 2002; Wynn et al., 2007). (2) A difference of the numbers of the samples in each studies. Schizophrenic patients and healthy controls of Kunugi's study and Takahashi's study were reported to be 20 patients and 16 controls, and 51 patients and 55 controls, respectively (Kunugi et al., 2007; Takahashi et al., 2008). We replicated this study using larger samples (115 schizophrenic patients and 111 normal controls) than the two original studies (Kunugi et al., 2007; Takahashi et al., 2008).

A few points of caution should be noted in interpreting our results. First, the significant association may be due to biased samples, such as unmatched age samples. We were apprehensive that the age might influence ASR, HAB and all PPI. Therefore, we performed another exploratory analysis of the possible correlations between all startle measures (PPI, ASR and HAB, respectively) and diagnosis (schizophrenia or controls), age, gender, and smoking history (smoker or nonsmoker) by multiple regression analysis for confirmation. However, the gender, age and smoking state were not correlated with ASR, HAB or any PPI in multiple regression analysis (data not shown). Second, 21 patients needed to take mood stabilizers such as valproic acid and carbamazepine. Since a recent animal study using DBA/2NCrl mice reported that mood stabilizers increased PPI (Flood et al., 2009), our results for these 21 schizophrenic patients may have been influenced by the mood stabilizers.

In conclusion, we were able to replicate the HAB impairment and PPI deficit in chronic Japanese schizophrenic patients. However, because our samples are small, it will be necessary to conduct a longer term replication study using a larger patient group in the future.

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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.neures.2009.07.009.

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Copy Number Variation in Schizophrenia in the Japanese Population

Masashi Ikeda, Branko Aleksic, George Kirov, Yoko Kinoshita, Yoshio Yamanouchi, Tsuyoshi Kitajima, Kunihiro Kawashima, Tomo Okochi, Taro Kishi, Irina Zaharieva, Michael J. Owen, Michael C. O'Donovan, Norio Ozaki, and Nakao Iwata

Background: Copy number variants (CNVs) have been shown to increase the risk to develop schizophrenia. The best supported findings are at 1q21.1, 15q11.2, 15q13.3, and 22q11.2 and deletions at the gene neurexin 1 (*NRXN1*).

Methods: In this study, we used Affymetrix 5.0 arrays to investigate the role of rare CNVs in 575 patients with schizophrenia and 564 control subjects from Japan.

Results: There was a nonsignificant trend for excess of rare CNVs in schizophrenia ($p = .087$); however, we did not confirm the previously implicated association for very large CNVs (>500 kilobase [kb]) in this population. We provide support for three previous findings in schizophrenia, as we identified one deletion in a case at 1q21.1, one deletion within *NRXN1*, and four duplications in cases and one in a control subject at 16p13.1, a locus first implicated in autism and later in schizophrenia.

Conclusions: In this population, we support some of the previous findings in schizophrenia but could not find an increased burden of very large (>500 kb) CNVs, which was proposed recently. However, we provide support for the role of CNVs at 16p13.1, 1q21.1, and *NRXN1*.

Key Words: Deletion, duplication, *NRXN1*, 16p13.1, 1q21.1, schizophrenia

Copy number variations (CNVs) are deletions and duplications of DNA ranging from a kilobase (kb) to several megabases (Mb). Recently, rare CNVs were shown to play a role in the etiology of a number of neuropsychiatric disorders, particularly schizophrenia, autism, and mental retardation (1).

Several studies have reported a greater prevalence of rare CNVs in people with schizophrenia (2-4). However, some have found no such excess (5,6) and even among the positive studies, there is marked variation in the magnitude of the observed effect. For example, in the International Schizophrenia Consortium (ISC) study (4), cases had only a 1.15-fold excess of rare CNVs, rising to 1.67-fold for deletions greater than 500 kb. An increase only among very large CNVs (>1 Mb) in cases was found by Kirov *et al.* (7). Another study showed an odds ratio of 3.37 for CNVs, rising to 4.82 for early-onset schizophrenia (2). This may, in part, reflect differences in the sensitivity of CNV assays, definitions of low-frequency CNVs, or variation in the phenotypic composition of the samples, as cases with early onset or lower IQ were particularly enriched for CNVs in one study (2).

In addition to increased CNV burden, a number of specific CNVs have been associated with schizophrenia (4,7,8). There is strong replicated evidence for deletions at 1q21.1, 15q11.2, 15q13.3, and 22q11.2 and emerging evidence for duplications at 16p13.1 (4,7). Deletions of the neurexin 1 gene (*NRXN1*) have also been reported in multiple studies on schizophrenia (2,6,7,9,10). Given the discrepancy in estimates of the effect size of CNV burden as a risk factor for schizophrenia and in particular the absence of association in the only Asian sample reported to date (5), we aimed to test for an excess burden of CNVs in a population from Japan. We also sought supportive evidence for a contribution for the specific loci listed above.

Methods and Materials

We analyzed 1139 age- and gender-matched unrelated subjects of Japanese ethnicity (575 schizophrenic patients and 564 control subjects). Control subjects were members of the general public who had no personal history of mental disorders. This was ascertained during face-to-face interviews where subjects were asked if they had suffered an episode of depression, mania, or psychotic experiences or if they had received treatment for any psychiatric disorder. Patients were entered into the study if they 1) met DSM-IV criteria for schizophrenia; 2) were physically healthy and had normal routine laboratory tests; and 3) had no mood disorders, substance abuse, neurodevelopmental disorders, epilepsy, or known mental retardation. Consensus diagnoses were made by at least two experienced psychiatrists according to DSM-IV criteria on the basis of unstructured interviews with patients and families and review of medical records. After description of the study, written informed consent was obtained from each subject. This study was approved by the ethics committees of each participating university.

We used Affymetrix 5.0 Arrays (Affymetrix, Santa Clara, California), following the manufacturer's protocols (http://www.affymetrix.com). This array includes 470K single nucleotide polymorphism (SNP) probes and 420K nonpolymorphic probes. The CNVs discussed below in more detail (at *NRXN1*, 1q21.1, and 16p13.1) were validated using the Illumina HumanHap 660W- or 610-quad bead arrays (Illumina, San Diego, California), following the manufacturer's protocols (http://www.illumina.com).

Copy number variations were called using the Birdsuite program (http://www.broadinstitute.org/science/programs/medical-and-

From the Medical Research Council (MRC) Centre for Neuropsychiatric Genetics and Genomics (MI, GK, IZ, MJO, MCO), Department of Psychological Medicine and Neurology, School of Medicine, Cardiff University, Cardiff, United Kingdom; Department of Psychiatry (MI, YK, YY, TKit, KK, TO, TKis, NI), Fujita Health University School of Medicine, Toyoake, Aichi; Department of Psychiatry (BA, NO), Nagoya University Graduate School of Medicine, Nagoya; and Core Research for Environmental Science and Technology (CREST) (BA, YK, TKit, KK, TO, TKis, NO, NI), Japan Science and Technology Agency, Kawaguchi, Saitama, Japan.

Address correspondence to George Kirov, M.D., MRC Centre for Neuropsychiatric Genetics and Genomics, Department of Psychological Medicine and Neurology, School of Medicine, Cardiff University, Cardiff CF14 4XN, UK; E-mail: kirov@cardiff.ac.uk.

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Table 1. Global CNV Burden Analysis

		CNV Burden				CNVs Intersecting Genes			
CNV Type	Size	SCZ	CON	CNV Rate SCZ/CON	*p* Value	SCZ	CON	CNV Rate SCZ/CON	*p* Value
Deletions and Duplications	All	567	485	1.1/.95	.087	382	320	.74/.62	.084
	100–200 kb	285	229	.55/.45	.046	182	145	.35/.28	.074
	200–500 kb	221	192	.43/.37	.20	150	134	.29/.26	.30
	500 kb–1 Mb	48	52	.09/.10	.72	38	32	.07/.06	.31
	>1 Mb	13	12	.025/.023	.52	12	9	.023/.018	.35
Deletions Only	All	174	157	.34/.31	.30	91	87	.18/.17	.46
	100–200 kb	98	84	.19/.16	.26	52	47	.10/.09	.38
	200–500 kb	65	60	.13/.12	.42	29	35	.06/.07	.79
	500 kb–1 Mb	8	8	.015/.016	.62	8	3	.015/.006	.12
	>1 Mb	3	5	.006/.010	.86	2	2	.004/.004	.69
Duplications Only	All	393	328	.76/.64	.10	291	233	.56/.45	.075
	100–200 kb	187	145	.36/.28	.070	130	98	.25/.19	.071
	200–500 kb	156	132	.30/.26	.21	121	99	.23/.19	.18
	500 kb–1 Mb	40	44	.077/.086	.73	30	29	.058/.057	.53
	>1 Mb	10	7	.019/.014	.33	10	7	.019/.014	.33

p values are one-tailed and based on 10,000 permutations.
CNV, copy number variation; CON, control; kb, kilobase; Mb, megabase; SCZ, schizophrenia.

population-genetics/birdsuite/birdsuite-0) (11). The software first assigns copy number across regions of known copy number polymorphisms, then calls SNP genotypes (for samples and SNPs believed to have two copies of the locus), then searches for novel CNVs via a hidden Markov model, and generates an integrated sequence and copy number genotype at every locus. It takes into account genotypes within CNVs, e.g., A-null, AAB, and BBB, in addition to AA, AB, and BB calls (11).

We observed a batch effect, similar to what we reported in our previous study (7): arrays from different batches gave poor results if analyzed together. Therefore, we identified the batches and analyzed together samples within the same batch, as recommended in the Birdsuite manual (11). After initial filtering for quality control, using the standard criteria implemented in the Genotyping Console software (www.affymetrix.com), including quality control call rate (>86%), SNP call rate (>95%), and population stratification based upon principal components analysis, 1107 samples (560 cases and 547 control subjects) were retained for further analysis. They had 16,466 CNVs (eight subjects showed no CNVs). We then excluded low-confidence CNVs (logarithm of odds <10), CNVs <100 kb, and those with the lowest 1% density for probe coverage (52 segments). We removed 50 samples that had high sample-specific measures of noise (variance >2), as those had a mean of 175 CNV segments, indicating they were false-positives. We also removed 17 samples that had more than 20 apparent CNVs (the mean number of CNVs for these samples was 156), as such samples are also likely to be false-positives (4,7). The filtering left 1032 samples: 519 cases aged 43.4 ± 14.7 years (258 male and 261 female cases) and 513 control subjects aged 43.8 ± 14.5 years (252 male and 261 female control subject). They had a total of 5180 CNVs (~5 per person). Finally, following previous studies (4,7), we filtered common CNVs (found in >1% of the total sample), leaving 1052 rare and larger than 100 kb CNVs for the analysis (~1 per person). This filtering was also performed for CNVs found at >5% in the total sample, resulting in 2081 CNVs. All CNVs that passed filtering and were present in <1% of the samples are available as an University of California, Santa Cruz (UCSC)-friendly file in Supplement 1.

Copy number variations were considered to colocalize if they overlapped by at least 50% of their length, as implemented in PLINK ver1.0.4 (http://pngu.mgh.harvard.edu/~purcell/plink/) (12) as used for the analysis of CNV loci in previous datasets (4,7).

Results

The numbers of rare CNVs stratified by size in cases and control subjects are listed in Table 1. Overall, we found an excess of CNVs in subjects with schizophrenia (case-control ratio = 1.16). Although not significant ($p = .087$, one-tailed permutation test), this is similar to that reported by the largest CNV study (4) where the case-control ratio was 1.15. The effect in that study (4) was coming mostly from deletions >500 kb and duplications in the 100 kb to 200 kb range. No subcategory of CNV defined by size or nature (deletion or duplication) was significantly associated with disease in the current study. Copy number variations in the 100 kb to 200 kb range were more common in cases than in control subjects, ratio = 1.23, $p = .046$; however, this does not survive correction for the multiple testing of four size ranges and two types of CNVs. Duplications (but not deletions) within the same size range were the most significantly associated general category in the ISC study ($p = 1 \times 10^{-4}$) with virtually an identical effect (case-control ratio = 1.26). However, no specific duplications of this size overlapped between the two studies (4). We did not replicate the finding of an excess of large deletions (>500 kb) that was reported in the ISC study (4) or of deletions and duplications >1 Mb reported in the study by Kirov *et al.* (7).

Analysis of the burden of CNVs intersecting genes revealed no significant excess of genes disrupted in subjects with schizophrenia, either overall or for any size range, with similar trends to the results from the general burden analysis (Table 1).

We repeated the same analysis for CNVs <5% in the sample. This resulted in 388 and 368 deletions and 698 and 627 duplications in cases and control subjects, respectively. The trends between cases and control subjects were virtually identical to those in Table 1 (data not presented).

Although we found no enrichment of large CNVs in schizophrenia, we present the details of large CNVs (>1 Mb) in Table S1 in Supplement 2 because these have been most consistently implicated by others (4,7). Of those, one case but no control subjects had a deletion on 1q21.1, one of the most convincingly