SNP Selection and Linkage Disequilibrium (LD) Evaluation

We selected two biologically functional SNPs (T102C: rs6313 and -A1438G: rs6311; Myers et al. 2007; Spurlock et al. 1998). Because we detected r^2 less than 0.800 for all phenotypes (r^2 = healthy controls: 0.719 and MDD: 0.709; Kishi et al. 2009c), we selected two biologically functional SNPs (-A1438G: rs6311 and T102C: rs6313) in this study (Myers et al. 2007; Spurlock et al. 1998). In addition, we also included rs7997012 and rs1928040 in *HTR2A* because McMahon et al. (2006) reported an association between these two SNPs and outcome of citalopram treatment in a very large sample of outpatients with MDD. These four SNPs were used in the following association analysis. Detailed information about SNP selection was described in our previous article.

SNP Genotyping

We used TaqMan assays (Applied Biosystems, Inc., Foster City, CA,) for all SNPs. One allelic probe was labeled with FAM dye and the other with fluorescent VIC dye. The plates were heated for 2 min at 50 and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 58°C for 1 min. Please refer to ABI for the primer sequence. Detailed information, including primer sequences, and reaction conditions, 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 with a likelihood ratio test using the COCAPHASE2.403 program (Dudbridge 2003). In the haplotype analysis, we determined that the cutoff for testing haplotype frequency was 0.05. We used the permutation test option as provided in the haplotype analysis to avoid spurious results and correct for multiple testing. Permutation test correction was performed using 1,000 iterations (random permutations). In addition, Bonferroni's correction was used to control inflation of the type I error rate in the single marker association analysis and in the individual haplotype-wise analysis. For Bonferroni correction, we employed the following numbers of multiple tests: 4 for each sample set in allele- and genotype analysis (4 examined SNPs); and 3 for each sample set in the individual haplotype-wise analysis (3 common haplotypes).

The significance level for all statistical tests was 0.05. Power calculation was performed using the Genetic Power Calculator (Purcell et al. 2003).

Results

Among the clinical characteristics of patients in this pharmacogenetic study, significant differences between either responders or nonresponders and remitters or nonremitters were detected in total SIGH-D score at the baseline ($P_{\text{response}} = 0.0161$ and $P_{\text{remission}} = 0.0136$; Table 1). Genotype frequencies of all SNPs were in HWE (Table 2). We found *HTR2A* to be associated with SSRI therapeutic response and remission in Japanese MDD patients in an all markers haplotype-wise analysis ($P_{\text{response}} = 0.0136$ and $P_{\text{response}} = 0.0400$) (Tables 3 and 4). When we performed a haplotype-wise analysis using the sliding window fashion method, a three-marker haplotype (rs6311-rs6313-rs1928040) showed the strongest association with the SSRI therapeutic response in MDD (P value = 0.000707; Tables 3 and 5). Also, this three-marker haplotype (rs6311-rs6313-rs1928040) showed the strongest association with remission in MDD (P value = 0.0324) (Tables 4 and 6). We also detected a significant association between rs1928040 in *HTR2A* and SSRI response and remission in MDD in an allele-wise analysis ($P_{\text{response}} = 0.0252$ and $P_{\text{remission}} = 0.0418$), but the significance disappeared after Bonferroni correction ($P_{\text{response}} = 0.101$ and $P_{\text{remission}} = 0.167$) (Table 2).

In addition, regarding genotyping quality control measures, we added 32 randomly selected samples that were genotyped again as a measure of genotyping quality control, and the genotype consistency rates for all four SNPs were 100%.

We obtained power of more than 80% for the detection of association when we set the genotype relative risk at 1.65–1.78 in all 265 samples, under a multiplicative model of inheritance (Purcell et al. 2003).

Discussion

We performed an association study for the SSRI therapeutic response in Japanese MDD patients using a larger sample than in two original Japanese studies. In one of those studies, Kato et al. (2006) reported an association between -A1438G (rs6311) and the SSRI therapeutic response in Japanese MDD, whereas Sato et al. (2002) found no such association. In this study, we found an association between *HTR2A* and the SSRI therapeutic response and remission in MDD in the haplotype-wise analysis.

Table 2 Genotype and allele distributions of *HTR2A* in both definition groups

SNPs[a]	Phenotype	MAF[b]	*N*	Genotype distribution[c]			*P* value[e]			Corrected *P* value[f]	
				M/M	M/m	m/m	HWE[d]	Genotype	Allele	Genotype	Allele
rs6311	Responders	0.410	150	47	83	20	0.0784				
(-1438A/G)	Nonresponders	0.428	115	40	53	22	0.743	0.567	0.670		
Intronl	Remitters	0.389	103	36	54	13	0.293				
	Nonremitters	0.432	162	51	82	29	0.690	0.502	0.319		
rs6313	Responders	0.493	150	35	82	33	0.252				
(102T/C)	Nonresponders	0.487	115	31	56	28	0.875	0.624	0.884		
Exonl	Remitters	0.495	103	24	56	23	0.375				
	Nonremitters	0.488	162	42	82	38	0.869	0.827	0.867		
rs 1928040	Responders	0.323	150	64	75	11	0.0806				
T>C	Nonresponders	0.235	115	66	44	5	0.487	0.0540	**0.0252**		0.101
Intron2	Remitters	0.335	103	42	53	8	0.116				
	Nonremitters	0.253	162	88	66	8	0.323	0.0910	**0.0418**		0.167
rs7997012	Responders	0.177	150	99	49	2	0.132				
G>A	Nonresponders	0.186	115	74	39	2	0.215	0.938	0.761		
Intron2	Remitters	0.189	103	65	37	1	0.0840				
	Nonremitters	0.176	162	108	51	3	0.275	0.664	0.696		

[a] Major allele > minor allele, SNP position

[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 by Bonferroni's correction

Table 3 Haplotype-wise analysis between *HTR2A* and SSRIs response in MDD

	Global *P* value[a]		
	2 window	3 window	4 window
rs6311	0.518		
rs6313	**0.0101**	**0.000707**	**0.0136**
rsl928040	0.0535	0.106	
rs7997012			

[a] Bold numbers represent significant *P* value

Table 4 Haplotype-wise analysis between *HTR2A* and SSRIs remission in MDD

	Global *P* value[a]		
	2 window	3 window	4 window
rs6311	0.736		
rs6313	**0.0451**	**0.0324**	**0.0400**
rsl928040	0.0604	**0.0423**	
rs7997012			

[a] Bold numbers represent significant *P* value

Haplotype analysis to investigate SSRI response and resmissin in MDD indicated three common haplotypes (rs6311- rs6313-rs1928040: A-T-T, G-C-T and G-C–C). The G-C-T haplotype was less prevalent in subjects with an SSRI therapeutic response (corrected $P = 0.00723$), while G-C–C was very prevalent in subjects with an SSRI therapeutic response (corrected $P = 0.00864$). Therefore, we considered that *HTR2A* was associated with SSRI therapeutic response in MDD in the Japanese population. On the other hand, The G-C-T haplotype was less prevalent in subjects with remission on SSRIs (uncorrected $P = 0.0200$). This significance disappeared after Bonferroni correction (corrected $P = 0.0600$). As a result, there are possibilities of type I errors in an association between *HTR2A* and SSRI therapeutic remission in MDD of the haplotype-wise analysis statistically.

In this study, we detected a marginal association between rs1928040 and SSRI therapeutic response in Japanese MDD in the allele-wise analysis (uncorrected $P_{response} = 0.0252$ and uncorrected $P_{remission} = 0.0418$). Therefore, we considered that an association between haplotype in *HTR2A* and SSRI response in this study might

Table 5 Haplotype-wise analysis between rs6311-rs6313-rs1928040 in *HTR2A* and SSRIs response in MDD

rs6311-rs6313-rs1928040	Phenotype	Individual haplotype frequency	OR[a]	95% CI[b]	Individual *P* value[c]	Corrected *P* value[d]
A-T-T	Responders	0.551	1.00	1.00–1.00	0.816	
	Nonresponders	0.539				
G-C-T	Responders	0.267	1.84	1.07–3.15	**0.00241**	**0.00723**
	Nonresponders	0.142				
G-C-C	Responders	0.182	0.558	0.337–0.924	**0.00288**	**0.00864**
	Nonresponders	0.319				

[a] *OR* odds ratio

[b] *95% CI* 95% confidence interval

[c] Bold numbers represent significant *P* value

[d] Calculated by Bonferroni's correction (3 tests)

Table 6 Haplotype-wise analysis between rs6311-rs6313-rs1928040 in *HTR2A* and remission in MDD

rs6311-rs6313-rs1928040	Phenotype	Individual haplotype frequency	OR[a]	95% CI[b]	Individual *P* value[c]	Corrected *P* value[d]
A-T-T	Remitters	0.538	1.00	1.00–1.00	0.741	
	Nonremitters	0.556				
G-C-T	Remitters	0.237	1.76	3.16–5.41	**0.0200**	0.0600
	Nonremitters	0.139				
G-C-C	Remitters	0.225	0.759	0.466–1.24	0.0791	
	Nonremitters	0.306				

[a] *OR* odds ratio

[b] *95% CI* 95% confidence interval

[c] Bold numbers represent significant *P* value

[d] Calculated by Bonferroni's correction (3 tests)

be reflected rs1928040. According to the HapMap database, MAFs of rs7997012 and rs1928040 in Caucasians were different to those in Japanese. Haplotype frequencies and LD between rs6313, rs6311, rs1928040 and rs7997012 in Caucasians were significantly different than in Japanese.

Because we detected r^2 less than 0.800 for all phenotypes (r^2 = Control 0.719 and MDD 0.709) (Kishi et al. 2009c), we selected two biologically functional SNPs (T102C: rs6313 and -A1438G: rs6311) in this study (Myers et al. 2007; Spurlock et al. 1998). Although Wilkie and colleagues recently reported an association between rs6314 (C1354T) in *HTR2A* and both response and remission to paroxetine in MDD (Wilkie et al. 2008), this SNP was shown to have "minor allele frequencies: 0%" in the HapMap database (Japanese population).

A few points of caution should be noted in interpreting our results. First, our sample sizes were small, and there is a possibility of statistical errors in our results. Secondly, because we did not perform an association analysis based on LD and a mutation scan of *HTR2A*, a replication study using a larger sample and based on LD may be required for conclusive results. Thirdly, we measured plasma levels of administered sertraline and paroxetine excepting fluvoxamine. However, these effects should be minimal because no correlation between plasma SSRI concentration and clinical response has been reported (Kasper et al. 1993; Saito et al. 2006). Fourthly, because we investigated SSRIs response in MDD patients who were able to take each SSRIs without side effects during the treatment protocol, we did not examine the number of drop out patients due to side effects in this study. Fifthly, we did not investigate several demographic informations (education, income, etc.) of the participated patients in this study. Finally, our subjects did not undergo structured interviews. MDD patients who are not diagnosed by structured interview may develop bipolar disorder in the future (Bowden 2001; Stensland et al. 2008). Also, we did not perform a screening to exclude Axis II disorders. 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 (Kishi et al. 2008, 2009a, b, c, d). In addition, when we found a misdiagnosis, we promptly excluded the misdiagnosed case in consideration of the precision of our sample.

In conclusion, we suggest that *HTR2A* may play an important role in the pathophysiology of the SSRI therapeutic response in Japanese MDD patients. However, it will be important to replicate and confirm these findings in other independent studies using large samples.

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References

Bowden, C. L. (2001). Strategies to reduce misdiagnosis of bipolar depression. *Psychiatric Services, 52*(1), 51–55.

Dudbridge, F. (2003). Pedigree disequilibrium tests for multilocus haplotypes. *Genetic Epidemiology, 25*(2), 115–121.

Kasper, S., Dotsch, M., Kick, H., Vieira, A., & Moller, H. J. (1993). Plasma concentrations of fluvoxamine and maprotiline in major depression: Implications on therapeutic efficacy and side effects. *European Neuropsychopharmacology, 3*(1), 13–21.

Kato, T. (2007). Molecular genetics of bipolar disorder and depression. *Psychiatry and Clinical Neurosciences, 61*(1), 3–19.

Kato, M., Fukuda, T., Wakeno, M., Fukuda, K., Okugawa, G., Ikenaga, Y., et al. (2006). Effects of the serotonin type 2A, 3A and 3B receptor and the serotonin transporter genes on paroxetine and fluvoxamine efficacy and adverse drug reactions in depressed Japanese patients. *Neuropsychobiology, 53*(4), 186–195.

Kato, M., & Serretti, A. (2008). Review and meta-analysis of antidepressant pharmacogenetic findings in major depressive disorder. *Mol Psychiatry* (in press).

Kishi, T., Kitajima, T., Ikeda, M., Yamanouchi, Y., Kinoshita, Y., Kawashima, K., et al. (2009a). Association study of clock gene (CLOCK) and schizophrenia and mood disorders in the Japanese population. *European Archives of Psychiatry and Clinical Neuroscience, 259*(5), 293–297.

Kishi, T., Kitajima, T., Ikeda, M., Yamanouchi, Y., Kinoshita, Y., Kawashima, K., et al. (2009b). CLOCK may predict the response to fluvoxamine treatment in Japanese major depressive disorder patients. *Neuromolecular Medicine, 11*(2), 53–57.

Kishi, T., Kitajima, T., Ikeda, M., Yamanouchi, Y., Kinoshita, Y., Kawashima, K., et al. (2008). Association analysis of nuclear receptor Rev-erb alpha gene (NR1D1) with mood disorders in the Japanese population. *Neuroscience Research, 62*(4), 211–215.

Kishi, T., Kitajima, T., Tsunoka, T., Ikeda, M., Yamanouchi, Y., Kinoshita, Y., et al. (2009c). Genetic association analysis of serotonin 2A receptor gene (HTR2A) with bipolar disorder and major depressive disorder in the Japanese population. *Neuroscience Research, 64*(2), 231–234.

Kishi, T., Kitajima, T., Tsunoka, T., Okumura, T., Ikeda, M., Okochi, T., et al. (2009d). Possible association of prokineticin 2 receptor gene (PROKR2) with mood disorders in the Japanese population. *Neuromolecular Medicine, 11*(2), 114–122.

McMahon, F. J., Buervenich, S., Charney, D., Lipsky, R., Rush, A. J., Wilson, A. F., et al. (2006). Variation in the gene encoding the serotonin 2A receptor is associated with outcome of antidepressant treatment. *American Journal of Human Genetics, 78*(5), 804–814.

Myers, R. L., Airey, D. C., Manier, D. H., Shelton, R. C., & Sanders-Bush, E. (2007). Polymorphisms in the regulatory region of the human serotonin 5-HT2A receptor gene (HTR2A) influence gene expression. *Biological Psychiatry, 61*(2), 167–173.

Perlis, R. H., Fijal, B., Adams, D. H., Sutton, V. K., Trivedi, M. H., & Houston, J. P. (2009). Variation in catechol-O-methyltransferase is associated with duloxetine response in a clinical trial for major depressive disorder. *Biological Psychiatry, 65*(9), 785–791.

Peters, E. J., Slager, S. L., Jenkins, G. D., Reinalda, M. S., Garriock, H. A., Shyn, S. I., et al. (2009). Resequencing of serotonin-related genes and association of tagging SNPs to citalopram response. *Pharmacogenet Genomics, 19*(1), 1–10.

Peveler, R., & Kendrick, T. (2005). Selective serotonin reuptake inhibitors: THREAD trial may show way forward. *British Medical Journal, 330*(7488), 420–421.

Purcell, S., Cherny, S. S., & Sham, P. C. (2003). Genetic power calculator: Design of linkage and association genetic mapping studies of complex traits. *Bioinformatics, 19*(1), 149–150.

Saito, S., Takahashi, N., Ishihara, R., Ikeda, M., Suzuki, T., Kitajima, T., et al. (2006). Association study between vesicle-associated membrane protein 2 gene polymorphisms and fluvoxamine response in Japanese major depressive patients. *Neuropsychobiology, 54*(4), 226–230.

Sato, K., Yoshida, K., Takahashi, H., Ito, K., Kamata, M., Higuchi, H., et al. (2002). Association between -1438G/A promoter polymorphism in the 5-HT(2A) receptor gene and fluvoxamine response in Japanese patients with major depressive disorder. *Neuropsychobiology, 46*(3), 136–140.

Serretti, A., & Artioli, P. (2004a). From molecular biology to pharmacogenetics: A review of the literature on antidepressant treatment and suggestions of possible candidate genes. *Psychopharmacology (Berl), 174*(4), 490–503.

Serretti, A., & Artioli, P. (2004b). The pharmacogenomics of selective serotonin reuptake inhibitors. *The Pharmacogenomics Journal, 4*(4), 233–244.

Serretti, A., Calati, R., Giegling, I., Hartmann, A. M., Moller, H. J., Colombo, C., et al. (2007a). 5-HT2A SNPs and the Temperament and Character Inventory. *Progress in Neuro-Psychopharmacology and Biological Psychiatry, 31*(6), 1275–1281.

Serretti, A., Drago, A., & De Ronchi, D. (2007b). HTR2A gene variants and psychiatric disorders: A review of current literature and selection of SNPs for future studies. *Current Medicinal Chemistry, 14*(19), 2053–2069.

Serretti, A., & Mandelli, L. (2008). The genetics of bipolar disorder: Genome 'hot regions', genes, new potential candidates and future directions. *Molecular Psychiatry, 13*(8), 742–771.

Spurlock, G., Heils, A., Holmans, P., Williams, J., D'Souza, U. M., Cardno, A., et al. (1998). A family based association study of T102C polymorphism in 5HT2A and schizophrenia plus identification of new polymorphisms in the promoter. *Molecular Psychiatry, 3*(1), 42–49.

Stensland, M. D., Schultz, J. F., & Frytak, J. R. (2008). Diagnosis of unipolar depression following initial identification of bipolar disorder: A common and costly misdiagnosis. *Journal of Clinical Psychiatry, 69*(5), 749–758.

Wilkie, M. J., Smith, G., Day, R. K., Matthews, K., Smith, D., Blackwood, D., et al. (2008). Polymorphisms in the SLC6A4 and HTR2A genes influence treatment outcome following antidepressant therapy. *The Pharmacogenomics Journal.*

Williams, J. B. (1988). A structured interview guide for the Hamilton Depression Rating Scale. *Archives of General Psychiatry, 45*(8), 742–747.

Copy Number Variation in Schizophrenia in the Japanese Population

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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-

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Table 1. Global CNV Burden Analysis

CNV Type	Size	CNV Burden				CNVs Intersecting Genes			
		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

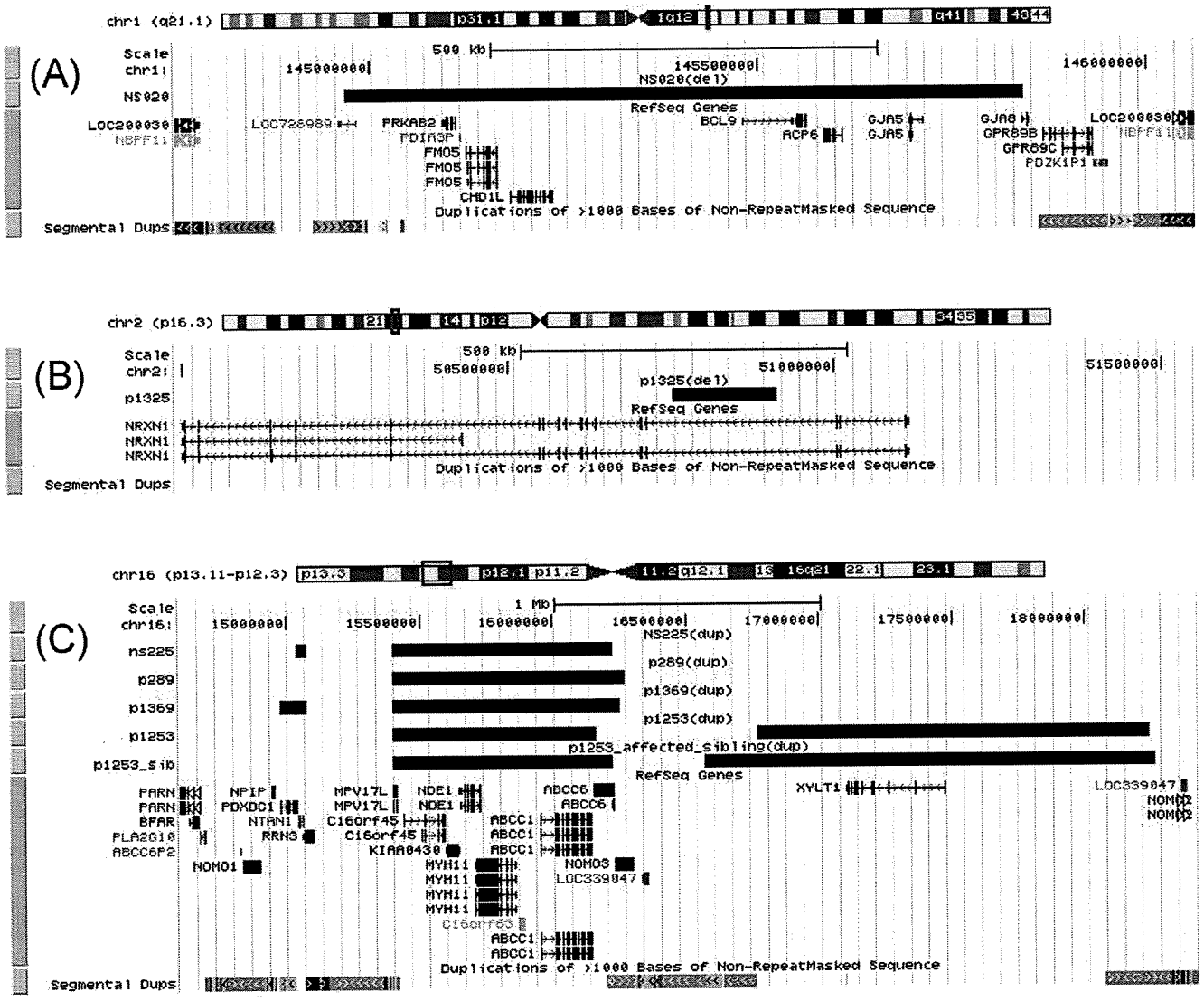

Figure 1. Positions of CNVs according to the validation experiments. CNV validation was undertaken using Illumina HumanHap 660W quad bead arrays (for CNVs at 1q21.1 and *NRXN1*) or 610-quad bead arrays (for CNVs at 16p13.1). Figures are produced on the UCSC Genome Browser according to NCBI Build 36.1, March 2006, hg18 (http://www.genome.ucsc.edu/) and indicate the positions of the CNVs: **(A)** 1q21.1; **(B)** *NRXN1*; and **(C)** 16p13.1: the last trace is that of the affected sibling of "p1253." CNV, copy number variation; NCBI, National Center for Biotechnology Information; UCSC, University of California, Santa Cruz.

implicated CNV risk factors for schizophrenia (4,8). Among large duplications, the most notable is that on 16p13.1, which was found in four cases and one control subject, while one more control subject had the reciprocal deletion (Fisher exact test $p = .19$, one-tailed). These CNVs in cases were confirmed using Illumina arrays (Figure S1 and Tables S1 and S2 in Supplement 2). One of the patients with 16p13.1 duplication had an affected sibling and unaffected mother who had also provided DNA. The duplication was found in the affected sibling but not the unaffected mother (DNA from the father was not available and there is no indication that he suffers with mental illness). The duplication in this family extends further on the centromeric side compared with the region usually included in CNVs of this region (Figure 1).

Of the remaining susceptibility loci reported in the recent studies (4,7,8), we found no deletions at 22q11.2 or 15q13.3. We also find no support for the 15q11.2 locus, where three deletions were found in control subjects and only one in a case (Fisher exact test $p = .37$, two-tailed, a trend in the opposite direction).

We also searched for CNVs that intersected genes and were present only in cases, reasoning as have others (2,3) that such CNVs are good candidates (Tables S3 and S4 in Supplement 2). One of the singleton deletions was in *NRXN1*, a gene implicated in previous studies (2,6,7,9,10) (Figure S1 and Table S2 in Supplement 2). Several more contain intriguing candidate genes (e.g., deletions in *PARK2*, *GRIK2*, *MAGEL2*, and *ATXN2L* and duplications in *CHRNA7* and *NRG4*), which have been implicated in neurodegenerative disorders or have possible functional relevance for neurodevelopment.

Discussion

In this study, we do not find a significant increase in the burden of CNVs in schizophrenia, either overall or for any

specific size range of CNVs, as proposed in previous studies (2–4,7). We did, however, find several trends in the same direction and of a similar magnitude as the largest global CNV survey of schizophrenia (4). Not all research has found such an increased burden, e.g., no evidence was obtained from a study in the Chinese population (5). It is possible that genuine population differences might drive this discrepancy between Caucasian and Asian samples, as might our exclusion of subjects with mental retardation or epilepsy. Sample size could also have played a role. Our sample had a modest power of ~.65 to detect a single CNV in a case for the following very strong candidate loci: 1q21.1, 15q13.3, and 22q11.2 and *NRXN1*, where approximately .2% of affected persons have deletions. In fact, we did find one deletion each in two of these loci (1q21.1 and *NRXN1*).

We found stronger support for association with duplications at 16p13.1, which contain the candidate gene *NDE1*. It is within the interval duplicated in all patients (Figure 1). Deletions and duplications of this region were implicated in autism (13) and schizophrenia (7), while deletions have been implicated in mental retardation (14). The most recent study surveying children with unexplained intellectual disability also reported significant association for both deletions and duplications at this locus ($p = 4.7 \times 10^{-5}$) (15), suggesting that this duplication is also pathogenic for a broad range of neuropsychiatric disorders. Our result for an excess of duplications in schizophrenic probands does not reach statistical significance; however, the frequency of the duplication is fourfold higher in cases than in control subjects (.8% vs. .2%), which is very similar to the rate found in our previous study from the United Kingdom (.6% vs. .2%) (7) and in the ISC study (.4% vs. .2%) (4). We found an identical duplication in an affected sibling. Larger CNVs in this locus, as in one of our probands, were also found in three cases and two control subjects in the ISC (4). The four probands in our study who carry 16p13.1 duplications do not appear to share any specific clinical features (Table S2 in Supplement 2).

We also found one deletion in a case at 1q21.1 and *NRXN1* and none in control subjects, which is close to the reported frequency of .2% in cases. Unlike those deletions of *NRXN1* that were associated with schizophrenia in a previous study (10), the CNV reported here does not intersect exons (10). However, it is large compared with most exon-sparing deletions reported in control subjects (10), and a new reanalysis of all *NRXN1* deletions shows that large (>100 kb) deletions in this gene might be almost as relevant as those affecting exons (16). The relevance to schizophrenia of the other CNVs found only in cases can only be assessed in future meta-analyses of such studies, but we note here that the three deletions we found in *PARK2* are of particular interest, as they have been implicated as a susceptibility factor for autism (17).

In summary, we provide support for the role of CNVs at 16p13.1, 1q21.1, and *NRXN1* in the etiology of schizophrenia. Although we find similar, but not significant, trends for an increased overall burden of CNVs, as well as for the involvement of duplications in the 100 kb to 200 kb range as proposed in the ICS study (4), in this population we could not find an increased burden of very large CNVs (>500 kb) in schizophrenia, which has been the main finding in recent studies (4,7). The discrepancy with previous studies could be due to our exclusion of patients with neurodevelopmental disorders, epilepsy, or known mental retardation, as such features are found in many of the carriers of large CNVs, e.g., 15q13.3 (15). Given the rarity of the CNVs that have been implicated so far in schizophrenia, there is a need for more large studies, studies in non-European populations, and meta-analyses.

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Supplementary material cited in this article is available online.

1. Cook EH Jr, Scherer SW (2008): Copy-number variations associated with neuropsychiatric conditions. *Nature* 455:919–923.
2. Walsh T, McClellan JM, McCarthy SE, Addington AM, Pierce SB, Cooper GM, *et al.* (2008): Rare structural variants disrupt multiple genes in neurodevelopmental pathways in schizophrenia. *Science* 320:539–543.
3. Xu B, Roos JL, Levy S, van Rensburg EJ, Gogos JA, Karayiorgou M (2008): Strong association of de novo copy number mutations with sporadic schizophrenia. *Nat Genet* 40:880–885.
4. The International Schizophrenia Consortium (2008): Rare chromosomal deletions and duplications increase risk of schizophrenia. *Nature* 455: 237–241.
5. Shi YY, He G, Zhang Z, Tang W, Zhang J Jr, Zhao Q, *et al.* (2008): A study of rare structural variants in schizophrenia patients and normal controls from Chinese Han population. *Mol Psychiatry* 13:911–913.
6. Need AC, Ge D, Weale ME, Maia J, Feng S, Heinzen EL, *et al.* (2009): A genome-wide investigation of SNPs and CNVs in schizophrenia. *PLoS Genet* 5:e1000373.
7. Kirov G, Grozeva D, Norton N, Ivanov D, Mantripragada KK, Holmans P, *et al.* (2009): Support for the involvement of large CNVs in the pathogenesis of schizophrenia. *Hum Mol Genet* 18:1497–1503.
8. Stefansson H, Rujescu D, Cichon S, Pietilainen OP, Ingason A, Steinberg S, *et al.* (2008): Large recurrent microdeletions associated with schizophrenia. *Nature* 455:232–236.
9. Kirov G, Gumus D, Chen W, Norton N, Georgieva L, Sari M, *et al.* (2008): Comparative genome hybridization suggests a role for NRXN1 and APBA2 in schizophrenia. *Hum Mol Genet* 17:458–465.
10. Rujescu D, Ingason A, Cichon S, Pietilainen OP, Barnes MR, Toulopoulou T, *et al.* (2009): Disruption of the neurexin 1 gene is associated with schizophrenia. *Hum Mol Genet* 18:988–996.
11. Korn JM, Kuruvilla FG, McCarroll SA, Wysoker A, Nemesh J, Cawley S, *et al.* (2008): Integrated genotype calling and association analysis of SNPs, common copy number polymorphisms and rare CNVs. *Nat Genet* 40: 1253–1260.
12. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, *et al.* (2007): PLINK: A tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 81:559–575.
13. Ullmann R, Turner G, Kirchhoff M, Chen W, Tonge B, Rosenberg C, *et al.* (2007): Array CGH identifies reciprocal 16p13.1 duplications and deletions that predispose to autism and/or mental retardation. *Hum Mutat* 28:674–682.
14. Hannes FD, Sharp AJ, Mefford HC, de Ravel T, Ruivenkamp CA, Breuning MH, *et al.* (2008): Recurrent reciprocal deletions and duplications of 16p13.11: The deletion is a risk factor for MR/MCA while the duplication may be a rare benign variant. *J Med Genet* 46:223–232.
15. Mefford HC, Cooper GM, Zerr T, Smith JD, Baker C, Shafer N, *et al.* (2009): A method for rapid, targeted CNV genotyping identifies rare variants associated with neurocognitive disease. *Genome Res* 19:1579–1585.
16. Kirov G, Rujescu D, Ingason A, Collier DA, O'Donovan MC, Owen MJ (2009): Neurexin 1 (NRXN1) deletions in schizophrenia. *Schizophr Bull* 35:851–854.
17. Glessner JT, Wang K, Cai G, Korvatska O, Kim CE, Wood S, *et al.* (2009): Autism genome-wide copy number variation reveals ubiquitin and neuronal genes. *Nature* 459:569–573.

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ORIGINAL PAPER

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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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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

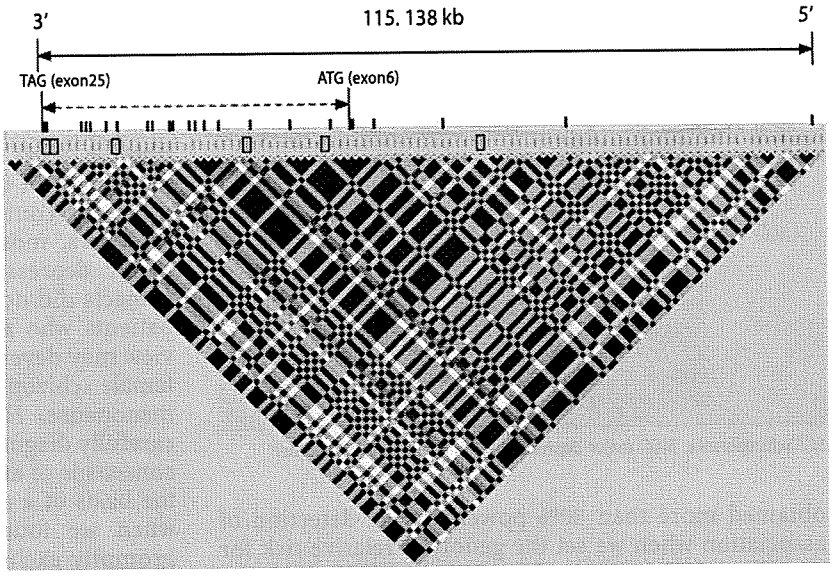

Fig. 1 LD evaluation and tagging SNPs in *CLOCK*. *Black bars* represent exons of *CLOCK*. Tagging SNPs selected from HapMap database are represented by *black boxes*. The *color* scheme is based on r^2 value. LD structure of *CLOCK* is very tight and roughly one block. The *color* scheme is based on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

1. Bailer U, Wiesegger G, Leisch F, Fuchs K, Leitner I, Letmaier M, Konstantinidis A, Stastny J, Sieghart W, Hornik K, Mitterauer B, Kasper S, Aschauer HN (2005) No association of clock gene T3111C polymorphism and affective disorders. Eur Neuropsychopharmacol 15:51–55
2. Barbini B, Benedetti F, Colombo C, Guglielmo E, Campori E, Smeraldi E (1998) Perceived mood and skin body temperature rhythm in depression. Eur Arch Psychiatry Clin Neurosci 248:157–160
3. Barnard AR, Nolan PM (2008) When clocks go bad: neurobehavioural consequences of disrupted circadian timing. PLoS Genet 4:e1000040
4. Barrett JC, Fry B, Maller J, Daly MJ (2005) Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics (Oxford, England) 21:263–265
5. Benedetti F, Dallaspezia S, Fulgosi MC, Lorenzi C, Serretti A, Barbini B, Colombo C, Smeraldi E (2007) Actimetric evidence that CLOCK 3111 T/C SNP influences sleep and activity patterns in patients affected by bipolar depression. Am J Med Genet B Neuropsychiatr Genet 144B:631–635

6. Benedetti F, Serretti A, Colombo C, Barbini B, Lorenzi C, Campori E, Smeraldi E (2003) Influence of CLOCK gene polymorphism on circadian mood fluctuation and illness recurrence in bipolar depression. Am J Med Genet B Neuropsychiatr Genet 123B:23–26
7. Berk M, Dodd S, Kauer-Sant'anna M, Malhi GS, Bourin M, Kapczinski F, Norman T (2007) Dopamine dysregulation syndrome: implications for a dopamine hypothesis of bipolar disorder. Acta Psychiatr Scand 434:41–49
8. Blackwood DH, Visscher PM, Muir WJ (2001) Genetic studies of bipolar affective disorder in large families. Br J Psychiatry 41:s134–s136
9. Bowden CL (2001) Strategies to reduce misdiagnosis of bipolar depression, vol 52. Psychiatric Services, Washington, pp 51–55
10. Buckley TM, Schatzberg AF (2005) On the interactions of the hypothalamic-pituitary-adrenal (HPA) axis and sleep: normal HPA axis activity and circadian rhythm, exemplary sleep disorders. J Clin Endocrinol Metabol 90:3106–3114
11. Camp NJ, Cannon-Albright LA (2005) Dissecting the genetic etiology of major depressive disorder using linkage analysis. Trends Mol Med 11:138–144
12. Camp NJ, Lowry MR, Richards RL, Plenk AM, Carter C, Hensel CH, Abkevich V, Skolnick MH, Shattuck D, Rowe KG, Hughes DC, Cannon-Albright LA (2005) Genome-wide linkage analyses of extended Utah pedigrees identifies loci that influence recurrent, early-onset major depression and anxiety disorders. Am J Med Genet B Neuropsychiatr Genet 135B:85–93
13. Dudbridge F (2003) Pedigree disequilibrium tests for multilocus haplotypes. Genet Epidemiol 25:115–121
14. Gallagher P, Watson S, Smith MS, Young AH, Ferrier IN (2007) Plasma cortisol-dehydroepiandrosterone (DHEA) ratios in schizophrenia and bipolar disorder. Schizophr Res 90:258–265
15. Ginns EI, St Jean P, Philibert RA, Galdzicka M, Damschroder-Williams P, Thiel B, Long RT, Ingraham LJ, Dalwaldi H, Murray MA, Ehlert M, Paul S, Remortel BG, Patel AP, Anderson MC, Shaio C, Lau E, Dymarskaia I, Martin BM, Stubblefield B, Falls KM, Carulli JP, Keith TP, Fann CS, Lacy LG, Allen CR, Hostetter AM, Elston RC, Schork NJ, Egeland JA, Paul SM (1998) A genome-wide search for chromosomal loci linked to mental health wellness in relatives at high risk for bipolar affective disorder among the Old Order Amish. Proc Natl Acad Sci USA 95:15531–15536
16. Hampp G, Ripperger JA, Houben T, Schmutz I, Blex C, Perreau-Lenz S, Brunk I, Spanagel R, Ahnert-Hilger G, Meijer JH, Albrecht U (2008) Regulation of monoamine oxidase A by circadian-clock components implies clock influence on mood. Curr Biol 18:678–683
17. Kaneko N, Muratake T, Kuwabara H, Kurosaki T, Takei M, Ohtsuki T, Arinami T, Tsuji S, Someya T (2007) Autosomal linkage analysis of a Japanese single multiplex schizophrenia pedigree reveals two candidate loci on chromosomes 4q and 3q. Am J Med Genet B Neuropsychiatr Genet 144B:735–742
18. Kelly DL, Conley RR (2004) Sexuality and schizophrenia: a review. Schizophr Bull 30:767–779
19. Lang UE, Puls I, Muller DJ, Strutz-Seebohm N, Gallinat J (2007) Molecular mechanisms of schizophrenia. Cell Physiol Biochem 20:687–702
20. Lehnkering H, Siegmund R (2007) Influence of chronotype, season, and sex of subject on sleep behavior of young adults. Chronobiol Int 24:875–888
21. Maier W, Hofgen B, Zobel A, Rietschel M (2005) Genetic models of schizophrenia and bipolar disorder: overlapping inheritance or discrete genotypes? Eur Arch Psychiatry Clin Neurosci 255:159–166
22. Mansour HA, Wood J, Logue T, Chowdari KV, Dayal M, Kupfer DJ, Monk TH, Devlin B, Nimgaonkar VL (2006) Association study of eight circadian genes with bipolar I disorder, schizoaffective disorder and schizophrenia. Genes Brain Behav 5:150–157
23. McAuley EZ, Blair IP, Liu Z, Fullerton JM, Scimone A, Van Herten M, Evans MR, Kirkby KC, Donald JA, Mitchell PB, Schofield PR (2008) A genome screen of 35 bipolar affective disorder pedigrees provides significant evidence for a susceptibility locus on chromosome 15q25–26. Mol Psychiatry (in press)
24. McClung CA (2007) Circadian genes, rhythms and the biology of mood disorders. Pharmacol Ther 114:222–232
25. McClung CA (2007) Circadian rhythms, the mesolimbic dopaminergic circuit, and drug addiction. Sci World J 7:194–202
26. McClung CA (2007) Clock genes and bipolar disorder: implications for therapy. Pharmacogenomics 8:1097–1100
27. McClung CA (2007) Role for the Clock gene in bipolar disorder. Cold Spring Harb Symp Quant Biol 72:637–644
28. McClung CA, Sidiropoulou K, Vitaterna M, Takahashi JS, White FJ, Cooper DC, Nestler EJ (2005) Regulation of dopaminergic transmission and cocaine reward by the Clock gene. Proc Natl Acad Sci USA 102:9377–9381
29. Muller N, Schwarz MJ (2008) A psychoneuroimmunological perspective to Emil Kraepelins dichotomy: schizophrenia and major depression as inflammatory CNS disorders. Eur Arch Psychiatry Clin Neurosci 258(Suppl 2):97–106
30. Neale BM, Sham PC (2004) The future of association studies: gene-based analysis and replication. Am J Hum Genet 75:353–362
31. Nestler EJ, Carlezon WA Jr (2006) The mesolimbic dopamine reward circuit in depression. Biol Psychiatry 59:1151–1159
32. Nievergelt CM, Kripke DF, Barrett TB, Burg E, Remick RA, Sadovnick AD, McElroy SL, Keck PE Jr, Schork NJ, Kelsoe JR (2006) Suggestive evidence for association of the circadian genes PERIOD3 and ARNTL with bipolar disorder. Am J Med Genet B Neuropsychiatr Genet 141B:234–241
33. Purcell S, Cherny SS, Sham PC (2003) Genetic power calculator: design of linkage and association genetic mapping studies of complex traits. Bioinformatics (Oxford, England) 19:149–150
34. Roybal K, Theobold D, Graham A, DiNieri JA, Russo SJ, Krishnan V, Chakravarty S, Peevey J, Oehrlein N, Birnbaum S, Vitaterna MH, Orsulak P, Takahashi JS, Nestler EJ, Carlezon WA Jr, McClung CA (2007) Mania-like behavior induced by disruption of CLOCK. Proc Natl Acad Sci USA 104:6406–6411
35. Serretti A, Benedetti F, Mandelli L, Lorenzi C, Pirovano A, Colombo C, Smeraldi E (2003) Genetic dissection of psychopathological symptoms: insomnia in mood disorders and CLOCK gene polymorphism. Am J Med Genet B Neuropsychiatr Genet 121B:35–38
36. Shi J, Wittke-Thompson JK, Badner JA, Hattori E, Potash JB, Willour VL, McMahon FJ, Gershon ES, Liu C (2008) Clock genes may influence bipolar disorder susceptibility and dysfunctional circadian rhythm. Am J Med Genet B Neuropsychiatr Genet 147B(7):1047–1055
37. Stensland MD, Schultz JF, Frytak JR (2008) Diagnosis of unipolar depression following initial identification of bipolar disorder: a common and costly misdiagnosis. J Clin Psychiatry 69:749–758
38. Straub RE, MacLean CJ, Ma Y, Webb BT, Myakishev MV, Harris-Kerr C, Wormley B, Sadek H, Kadambi B, O'Neill FA, Walsh D, Kendler KS (2002) Genome-wide scans of three independent sets of 90 Irish multiplex schizophrenia families and follow-up of selected regions in all families provides evidence for multiple susceptibility genes. Mol Psychiatry 7:542–559
39. Takao T, Tachikawa H, Kawanishi Y, Mizukami K, Asada T (2007) CLOCK gene T3111C polymorphism is associated with Japanese schizophrenics: a preliminary study. Eur Neuropsychopharmacol 17:273–276
40. Voderholzer U, Riemann D, Huwig-Poppe C, Kuelz AK, Kordon A, Bruestle K, Berger M, Hohagen F (2007) Sleep in obsessive compulsive disorder: polysomnographic studies under baseline conditions and after experimentally induced serotonin deficiency. Eur Arch Psychiatry Clin Neurosci 257:173–182
41. Weber M, Lauterburg T, Tobler I, Burgunder JM (2004) Circadian patterns of neurotransmitter related gene expression in motor regions of the rat brain. Neurosci Lett 358:17–20

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ORIGINAL PAPER

CLOCK may Predict the Response to Fluvoxamine Treatment in Japanese Major Depressive Disorder Patients

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Abstract Recent studies have shown that selective serotonin reuptake inhibitors (SSRIs) have circadian properties, suggesting that the antidepressive action of SSRIs may also be attributable to circadian mechanisms. Another study reported an association between clock gene (*CLOCK*) and improvements in insomnia symptoms from SSRIs treatment. Therefore, we examined the association between *CLOCK* and the efficacy of fluvoxamine treatment in 121 patients with Japanese major depressive disorder (MDD). The MDD patients in this study had scores of 12 or higher on the 17 items of the Structured Interview Guide for Hamilton Rating Scale for Depression (SIGH-D). We defined a therapeutic response as a decrease of more than a 50% in baseline SIGH-D within 8 weeks, and clinical remission as a SIGH-D score of less than seven at 8 weeks. We selected three tagging SNPs in *CLOCK* for the subsequent statistical association analysis. We detected a significant association between rs3736544, a synonymous polymorphism in exon 20, and the fluvoxamine therapeutic response in MDD in the allele/genotype-wise analyses. In addition, remission with fluvoxamine was also significantly associated with rs3736544. These associations remained significant after Bonferroni correction. Moreover, haplotype analysis findings supported these significant associations, which appeared to be due mainly to rs3736544, in the fluvoxamine therapeutic remission. Our results indicate that *CLOCK* genotype may be a predictor of fluvoxamine treatment response in Japanese MDD. However, our sample size was small, and a replication study using larger samples may be required for conclusive results.

Keywords Major depressive disorder · *CLOCK* · Tagging SNPs · Fluvoxamine · SSRIs

Introduction

Major depressive disorder (MDD) patients commonly present not only abnormalities in sleep–wake rhythms but also disruptions in biological circadian rhythms. Therefore, disruptions in circadian rhythms have been suggested to be involved in the pathogenesis of MDD (Barnard and Nolan 2008; Kishi et al. 2008a, 2008b). All psychotropic drugs act on the systems of neurotransmitters such as dopamine and serotonin in the brain (Barnard and Nolan 2008), and recently these neurotransmitter systems have been reported to have reciprocal interactions with circadian rhythms (Monteleone and Maj 2008).

Selective serotonin reuptake inhibitors (SSRIs) such as fluvoxamine, which are major therapeutic agents for MDD, inhibit serotonin transport in the presynaptic neuron, and increase the extracellular serotonin level. This mechanism is believed to relieve depressive symptoms (Peveler and

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Kendrick 2005). On the other hand, many animal and in vitro studies have shown that serotonin directly affects circadian rhythms (Monteleone and Maj 2008), and SSRIs have also been reported to have circadian properties. SSRIs have a phase shifting effect in neural firing in the rat suprachiasmatic nucleus (Sprouse et al. 2006), and change the expression of clock genes in the striatum and hippocampus of mice (Uz et al. 2005), suggesting that the antidepressive action of SSRIs may also be attributable to circadian mechanisms. Therefore, we considered that clock genes might be therapeutic targets for SSRIs.

The clock gene (*CLOCK*, OMIM *601851, 25 exons in this genomic region spanning 115.138 kb), located on 4q12, is one of the major components of the cellular clock gene mechanism. It is known to be associated with human circadian preference (morningness/eveningness) (Katzenberg et al. 1998; Mishima et al. 2005). Several clinical subgroup analyses have shown a significant association between an SNP (rs1801260: T3111C) in *CLOCK* and sleep disregulation in mood disorders including MDD and bipolar disorder (BP) (Serretti et al. 2003) and a higher recurrence rate in BP (Benedetti et al. 2003). In addition, Serretti and colleagues reported an association between T3111C and improved insomnia from fluvoxamine or paroxetine treatment (Serretti et al. 2005). However, three genetic studies, including our previous study, reported no association between *CLOCK* and MDD (Bailer et al. 2005; Desan et al. 2000; Kishi et al. 2008a). Thus, there is disagreement in the results of these studies as to treatment response and the pathophysiology of MDD (Gratacos et al. 2008).

In this study, we examined the association between *CLOCK* and the efficacy of fluvoxamine treatment in Japanese MDD patients. To do this, we applied the recently recommended strategy of "gene-based" association analysis (Neale and Sham 2004).

Materials and Methods

Subjects

The subjects were 121 MDD patients (60 males and 61 females: mean age ± standard deviation (SD) 44.5 ± 16.5 years). 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 consensus of at least two experienced psychiatrists on the basis of a review of medical records. Fluvoxamine was taken two or three times a day for 8 weeks. The initial total dose was 50–100 mg per day, and the dosage was then increased gradually to a maximum of 150 mg, depending on the patients' condition. Patients with insomnia and severe anxiety were prescribed benzodiazepine drugs, but no other psychotropic drugs were permitted during the study. The study was described to subjects and written informed consent was obtained from each. This study was approved by the Ethics Committee at Fujita Health University.

Data Collection

The MDD patients in this study had scores of 12 or higher on the 17 items of the Structured Interview Guide for Hamilton Rating Scale for Depression (SIGH-D). Patients with this moderate range of severity tend to respond to antidepressants (Saito et al. 2006). We defined a therapeutic response as a decrease of more than 50% in baseline SIGH-D within 8 weeks, and a clinical remission as a SIGH-D score of less than 7 at 8 weeks. Detailed information on data collection was described in a previous paper (Saito et al. 2006). The clinical characteristics of the patients in this study, classified according to these definitions, can be seen in Table 1.

SNPs Selection and Linkage Disequilibrium (LD) Evaluation

We selected three "tagging SNPs" (rs3736544: synonymous polymorphism in exon 20, rs1801260: 3′ untranslated region (UTR) in exon 23, rs3749474: 3′ UTR in exon 23) in *CLOCK*. Detailed information can be seen in our previous paper (Kishi et al. 2008a).

SNPs Genotyping

We used TaqMan assays (Applied Biosystems, Inc., Foster City, CA,) for all SNPs. Detailed information can be seen in our previous paper (Kishi et al. 2008a).

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 associations with the chi-square test (SAS/Genetics, release 8.2, SAS Japan Inc, Tokyo, Japan), and haplotype-wise association analysis was done with a likelihood ratio test using the COCAPHASE 2.403 program (Dudbridge 2003). Bonferroni's correction was used to control inflation of the type I error rate. Power calculation was performed using the Genetic Power Calculator (Purcell et al. 2003).

The significance level for all statistical tests was 0.05.

Table 1 Clinical characteristics of the patients in both definition groups

	N			Age (mean ± SD)	Baseline SIGH-D (avg ± SD)	Fluvoxamine dose at 8 weeks (mg/day) (avg ± SD)	Number of previous episode (avg ± SD)
	Total	Male	Female				
Overall	121	60	61	44.5 ± 16.5	20.2 ± 5.88	122 ± 40.9	1.39 ± 0.658
Clinical response group[a]							
Responders	60	31	29	44.4 ± 16.3	21.5 ± 6.19	118 ± 41.1	1.36 ± 0.574
Nonresponders	61	29	32	44.3 ± 17.3	18.8 ± 5.28	125 ± 40.7	1.43 ± 0.774
P-value	0.645			0.819	0.0145	0.391	0.480
Clinical remission group[b]							
Remitters	45	22	23	43.7 ± 15.9	19.6 ± 5.06	113 ± 43.9	1.37 ± 0.598
Nonremitters	76	38	38	45.1 ± 17.1	20.5 ± 6.34	127 ± 38.2	1.41 ± 0.715
P-value	0.722			0.750	0.750	0.101	0.856

[a] Clinical response was defined as a 50% or greater decrease in the baseline SIGH-D score

[b] Clinical remission was defined as a final SIGH-D score of less than seven

Results

The LD structures of *CLOCK* from the HapMap database were described in our previous paper (Kishi et al. 2008a). Among the clinical characteristics of the patients in this study, only one significant difference with total SIGH-D score was detected at the baseline in relation to fluvoxamine therapeutic response (*P*-value = 0.0145) (Table 1). Genotype frequencies of all SNPs were in HWE. We detected a significant association between rs3736544 and the fluvoxamine therapeutic response in MDD in the allele/genotype-wise analysis (Table 2). In addition, remission with fluvoxamine was significantly associated with rs3736544 (Table 2). Moreover, the significance of these associations remained after Bonferroni correction (Table 2). We also found an association between rs3749474 and the fluvoxamine therapeutic response in MDD in the genotype-wise analysis (*P*-value: 0.0251) (Table 2). However, this might have resulted from type I error due to multiple testing (*P*-value: 0.0752 after Bonferroni's correction) (Table 2). The haplotype-wise analysis provided evidence for a significant association that appears to be due mainly to rs3736544 in fluvoxamine therapeutic remission (Table 3).

Table 2 Association analysis of tagging SNPs in *CLOCK*

SNP[a]	Phenotype	MAF	*N*	Genotype distribution[b]			*P*-value[d]			Corrected *P*-value[d,e]	
				M/M	M/m	m/m	HWE[c]	Genotype	Allele	Genotype	Allele
rs3736544 G > T	Responders	0.267	60	30	28	2	0.135				
	Nonresponders	0.115	61	48	12	1	0.804	**0.00434**	**0.00261**	**0.00130**	**0.00738**
	Remission	0.289	45	21	22	2	0.203				
	Nonremission	0.132	76	57	18	1	0.751	**0.00651**	**0.00257**	**0.0195**	**0.00771**
rs1801260 T > C	Responders	0.133	60	46	12	2	0.297				
	Nonresponders	0.189	61	39	21	1	0.328	0.187	0.243		
	Remission	0.156	45	33	10	2	0.301				
	Nonremission	0.164	76	52	23	1	0.378	0.390	0.855		
rs3749474 T > C	Responders	0.417	60	19	32	9	0.452				
	Nonresponders	0.336	61	27	27	7	0.949	0.358	0.196		
	Remission	0.467	45	12	24	9	0.632				
	Nonremission	0.322	76	34	35	7	0.637	0.0734	**0.0251**		0.0752

[a] major allele > minor allele

[b] M: major allele, m: minor allele

[c] HWE: Hardy–Weinberg equilibrium

[d] Bold numbers represent significant *P*-value

[e] Caluculated by Bonferroni's correction

Table 3 Haplotype-wise analysis of tagging SNPs in *CLOCK*

Common haplotypes rs3736544-rs1801260-rs3749474	Phenotype	Individual haplotype frequency	Individual *P*-value[a]	Phenotype	Global *P*-value[a]
GTT	Responders	0.600	0.173		
	Nonresponders	0.686		Responders	0.436
	Remission	0.548	**0.0191**	Nonresponders	
	Nonremission	0.703		Remission	**0.015**
GCC	Responders	0.146	0.401	Nonremission	
	Nonresponders	0.188			
	Remission	0.167	1.00		
	Nonremission	0.167			
TTC	Responders	0.255	**0.0137**		
	Nonresponders	0.125			
	Remission	0.286	**0.00417**		
	Nonremission	0.130			

[a] Bold numbers represent significant *P*-value

Discussion

In this study, we detected a significant association between rs3736544 in *CLOCK*, which is a synonymous polymorphism in exon 20, and the fluvoxamine therapeutic response and remission in the allele/genotype-wise analysis. This significance remained after Bonferroni correction. Haplotype analysis indicated three common haplotypes (rs3736544-rs1801260-rs3749474: GTT, GCC and TTC). Among them, the TTC haplotype was less prevalent in subjects with a fluvoxamine therapeutic response ($P = 0.0137$) and was associated with remission on fluvoxamine ($P = 0.00417$). The GTT haplotype was also significantly associated with remission on fluvoxamine ($P = 0.0191$). In a recent study, we selected six tagging SNPs among 106 SNPs covering all of *CLOCK*, including 5′-flanking regions about 2 kb upstream (5′) from the initial exon and about 5 kb downstream (3′) from the last exon (HapMap database contig number chr4: 55990340.. 56108588), with the criteria of an r^2 threshold greater than 0.8 in "pair-wise tagging only" mode using the Tagger program. LD structures of *CLOCK* from the HapMap database were described in our previous paper (Kishi et al. 2008a). However, the LD structure of *CLOCK* in our sample was very tight except for rs1801260 and rs3749474 (Kishi et al. 2008a). Also, the LD structures of MDD samples treated with fluvoxamine and control samples were almost the same (Kishi et al. 2008a). As these results show, rs3736544 covers a wide and important region including the exons and the promoter region in *CLOCK*. Therefore, it is possible that rs3736544 influences biological function in the brain. In previous genetic analyses of *CLOCK*, only T3111C (rs1801260) was selected. T3111C (rs1801260) has been detected at position 3111 in the *CLOCK* mRNA 3′ untranslated region, and was reported to be associated with a substantial delay in preferred timing for activity and sleep in a human study (Katzenberg et al. 1998). As for function, T3111C (rs1801260) has been speculated to affect mRNA (Katzenberg et al. 1998); however, one study with luciferase reported no significant effect on mRNA translatability from T3111C (Robilliard et al. 2002). We found an association of rs3736544 but not T3111C (rs1801260) with treatment outcome in this study. These findings suggest that functional analyses for other regions of the *CLOCK* should be performed in future studies.

A subgroup analysis has shown a significant association between an SNP (rs1801260: T3111C) in *CLOCK* and sleep disregulation in mood disorders (Serretti et al. 2003). Because benzodiazepine drugs are surely effective for insomnia and severe anxiety in MDD patients, which might mask the sleep disruption in MDD due to circadian abnormality, the analysis which takes the usage of benzodiazepines into account may also need to be carried out in the future. Because we had only a few MDD fluvoxamine treatment samples without benzodiazepine drugs, and we wanted to avoid statistical error, we did not perform such an analysis among these samples. Another subgroup analysis has shown a higher recurrence rate in BP in relation to T3111C (Benedetti et al. 2003), but we lacked data on recurrence in our sample, so we could not perform such analysis.

Our recent study found no association between *CLOCK* and MDD in the Japanese population (Kishi et al. 2008a). Thus, there is disagreement in the results among studies as to the treatment response and the pathophysiology of MDD (Gratacos et al. 2008).

A few points of caution should be noted in interpreting our results. First, it will be necessary to investigate the possibility that rs3736544 reflects biological function,

which we did not do in the present study. 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., minor allele frequencies less than 0.01) from the viewpoint of power. Third, our sample sizes were small. A replication study using larger samples may be required for conclusive results.

In conclusion, our results indicate that *CLOCK* may be associated with fluvoxamine treatment outcome in Japanese MDD.

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References

Bailer, U., Wiesegger, G., Leisch, F., Fuchs, K., Leitner, I., Letmaier, M., et al. (2005). No association of clock gene T3111C polymorphism and affective disorders. *European Neuropsychopharmacology, 15*(1), 51–55. doi:10.1016/j.euroneuro.2004.05.004.

Barnard, A. R., & Nolan, P. M. (2008). When clocks go bad: Neurobehavioural consequences of disrupted circadian timing. *PLoS Genetics, 4*(5), e1000040. doi:10.1371/journal.pgen.1000040.

Benedetti, F., Serretti, A., Colombo, C., Barbini, B., Lorenzi, C., Campori, E., et al. (2003). Influence of CLOCK gene polymorphism on circadian mood fluctuation and illness recurrence in bipolar depression. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics, 123B*(1), 23–26. doi:10.1002/ajmg.b.20038.

Desan, P. H., Oren, D. A., Malison, R., Price, L. H., Rosenbaum, J., Smoller, J., et al. (2000). Genetic polymorphism at the CLOCK gene locus and major depression. *American Journal of Medical Genetics, 96*(3), 418–421. doi:10.1002/1096-8628(20000612)96:3<418::AID-AJMG34>3.0.CO;2-S.

Dudbridge, F. (2003). Pedigree disequilibrium tests for multilocus haplotypes. *Genetic Epidemiology, 25*(2), 115–121. doi:10.1002/gepi.10252.

Gratacos, M., Soria, V., Urretavizcaya, M., Gonzalez, J. R., Crespo, J. M., Bayes, M., et al. (2008). A brain-derived neurotrophic factor (BDNF) haplotype is associated with antidepressant treatment outcome in mood disorders. *The Pharmacogenomics Journal, 8*(2), 101–112. doi:10.1038/sj.tpj.6500460.

Katzenberg, D., Young, T., Finn, L., Lin, L., King, D. P., Takahashi, J. S., et al. (1998). A CLOCK polymorphism associated with human diurnal preference. *Sleep, 21*(6), 569–576.

Kishi, T., Kitajima, T., Ikeda, M., Yamanouchi, Y., Kinoshita, Y., Kawashima, K., et al. (2008a). Association study of clock gene (CLOCK) and schizophrenia and mood disorders in the Japanese population. *European archives of psychiatry and clinical neuroscience* (in press).

Kishi, T., Kitajima, T., Ikeda, M., Yamanouchi, Y., Kinoshita, Y., Kawashima, K., et al. (2008b). Association analysis of nuclear receptor Rev-erb alpha gene (NR1D1) with mood disorders in the Japanese population. *Neuroscience Research, 62*(4), 211–215. doi:10.1016/j.neures.2008.08.008.

Mishima, K., Tozawa, T., Satoh, K., Saitoh, H., & Mishima, Y. (2005). The 3111T/C polymorphism of hClock is associated with evening preference and delayed sleep timing in a Japanese population sample. *American Journal of Medical Genetics. Part B, Neuropsychiatric genetics, 133B*(1), 101–104. doi:10.1002/ajmg.b.30110.

Monteleone, P., & Maj, M. (2008). The circadian basis of mood disorders: Recent developments and treatment implications. *European Neuropsychopharmacology, 18*(10), 701–711. doi:10.1016/j.euroneuro.2008.06.007.

Neale, B. M., & Sham, P. C. (2004). The future of association studies: Gene-based analysis and replication. *American Journal of Human Genetics, 75*(3), 353–362.

Peveler, R., & Kendrick, T. (2005). Selective serotonin reuptake inhibitors: THREAD trial may show way forward. *BMJ, 330*(7488), 420–421. doi:10.1136/bmj.330.7488.420-b.

Purcell, S., Cherny, S. S., & Sham, P. C. (2003). Genetic Power Calculator: Design of linkage and association genetic mapping studies of complex traits. *Bioinformatics, 19*(1), 149–150. doi:10.1093/bioinformatics/19.1.149.

Robilliard, D. L., Archer, S. N., Arendt, J., Lockley, S. W., Hack, L. M., English, J., et al. (2002). The 3111 Clock gene polymorphism is not associated with sleep and circadian rhythmicity in phenotypically characterized human subjects. *Journal of Sleep Research, 11*(4), 305–312. doi:10.1046/j.1365-2869.2002.00320.x.

Saito, S., Takahashi, N., Ishihara, R., Ikeda, M., Suzuki, T., Kitajima, T., et al. (2006). Association study between vesicle-associated membrane protein 2 gene polymorphisms and fluvoxamine response in Japanese major depressive patients. *Neuropsychobiology, 54*(4), 226–230. doi:10.1159/000100777.

Serretti, A., Benedetti, F., Mandelli, L., Lorenzi, C., Pirovano, A., Colombo, C., et al. (2003). Genetic dissection of psychopathological symptoms: Insomnia in mood disorders and CLOCK gene polymorphism. *American Journal of Medical Genetics. Part B, Neuropsychiatric genetics, 121B*(1), 35–38. doi:10.1002/ajmg.b.20053.

Serretti, A., Cusin, C., Benedetti, F., Mandelli, L., Pirovano, A., Zanardi, R., et al. (2005). Insomnia improvement during antidepressant treatment and CLOCK gene polymorphism. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics, 137B*(1), 36–39. doi:10.1002/ajmg.b.30130.

Sprouse, J., Braselton, J., & Reynolds, L. (2006). Fluoxetine modulates the circadian biological clock via phase advances of suprachiasmatic nucleus neuronal firing. *Biological Psychiatry, 60*(8), 896–899. doi:10.1016/j.biopsych.2006.03.003.

Uz, T., Ahmed, R., Akhisaroglu, M., Kurtuncu, M., Imbesi, M., Dirim Arslan, A., et al. (2005). Effect of fluoxetine and cocaine on the expression of clock genes in the mouse hippocampus and striatum. *Neuroscience, 134*(4), 1309–1316. doi:10.1016/j.neuroscience.2005.05.003.

Identification of Novel Candidate Genes for Treatment Response to Risperidone and Susceptibility for Schizophrenia: Integrated Analysis Among Pharmacogenomics, Mouse Expression, and Genetic Case-Control Association Approaches

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Background: Pharmacogenomic approaches based on genomewide sets of single nucleotide polymorphisms (SNPs) are now feasible and offer the potential to uncover variants that influence drug response.

Methods: To detect potential predictor gene variants for risperidone response in schizophrenic subjects, we performed a convergent analysis based on 1) a genomewide (100K SNP) SNP pharmacogenetic study of risperidone response and 2) a global transcriptome study of genes with mRNA levels influenced by risperidone exposure in mouse prefrontal cortex.

Results: Fourteen genes were highlighted as of potential relevance to risperidone activity in both studies: *ATP2B2, HS3ST2, UNC5C, BAG3, PDE7B, PAICS, PTGFRN, NR3C2, ZBTB20, ST6GAL2, PIP5K1B, EPHA6, KCNH5,* and *AJAP1*. The SNPs related to these genes that were associated in the pharmacogenetic study were further assessed for evidence for association with schizophrenia in up to three case-control series comprising 1564 cases and 3862 controls in total (Japanese [JPN] 1st and 2nd samples and UK sample). Of 14 SNPs tested, one (rs9389370) in *PDE7B* showed significant evidence for association with schizophrenia in a discovery sample (p_{allele} = .026 in JPN_1st, two-tailed). This finding replicated in a joint analysis of two independent case-control samples (p_{JPN_2nd+UK} = .008, one-tailed, uncorrected) and in all combined data sets (p_{all} = .0014, two-tailed, uncorrected and p_{all} = .018, two-tailed, Bonferroni correction).

Conclusions: We identified novel candidate genes for treatment response to risperidone and provide evidence that one of these additionally may confer susceptibility to schizophrenia. Specifically, *PDE7B* is an attractive candidate gene, although evidence from integrated methodology, including pharmacogenomics, pharmacotranscriptomic, and case-control association approaches.

Key Words: Expression: PDE7B, pharmacogenomics, risperidone, schizophrenia

Schizophrenia is a severe psychiatric disorder with a lifetime risk of approximately 1%. With its early onset, typically in late teens to early 20s, frequent relapse and chronic course, schizophrenia imposes a considerable burden on sufferers, their families, and society. Worldwide, it is a major source of morbidity, but it is often overlooked that it is also associated with a considerable truncation in life span, the mortality rate in individuals with schizophrenia being more than twice that of the age- and sex-matched population (1). A large number of antipsychotics have been developed as treatment agents. However, individual response to these drugs is highly variable, and identifying the optimal treatment for any patient is often a trial and error process that can span many years and even then, response is often poor. There is a pressing need both to identify new treatments and to attempt to improve the information based on which response to treatment can be predicted.

Genetic factors are generally assumed to contribute to variable treatment response (2), and on this basis, a number of pharmacogenetic studies have been performed. Here, the aim was to detect DNA sequence predictors for treatment response. Most studies have focused on genes encoding neurotransmitter receptors, such as dopamine or serotonin receptors, the logic being that antipsychotics usually have high affinities with members of these classes of receptor. Although a number of variants have been correlated with treatment response in several stud-

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ies—for example, dopamine D2 and D3 receptor variants (2)—there are no definitive predictors of response.

Pharmacogenetics has been driven by a candidate gene approach. This approach has the disadvantage that targets for study are limited by our current understanding of the mechanisms of drugs, and therefore, this method cannot identify unsuspected predictor genes. Approaches that are independent of prior functional hypotheses of gene action based on genome-wide surveys of SNPs are, however, now feasible. The genome-wide approach has its disadvantages, but one of the most important is that, with effectively random sets of SNPs, the low prior probability that any is truly associated with disease requires a stringent type I error rate to control the enormous potential for reporting false positives. One way to address this issue is to use very large (and therefore highly powered) studies in which such stringent statistical support might realistically be achieved. Another approach that is more economical in genotyping costs is to undertake multistage analysis in which candidate variants from a screening sample are validated by replication in other data sets (3). However, because the samples for pharmacogenomics require a large amount of clinical data and are preferably prospective, large samples are difficult to collect.

Another approach is to try to enhance the prior probability for a given gene by integrating pharmacogenomic data with other sources of data—for example, from studies of gene expression (4). Under the hypothesis that genes related to drug response may be regulated by exposure to that drug, genes in which expression is altered in animals exposed to that drug have a higher probability of being genuinely associated in a pharmacogenetic study than any random gene. If this is correct, genes in which expression is altered by drug exposure require less stringent statistical support.

We aimed to detect predictor genes for risperidone response in schizophrenic patients using this convergent approach (4). Specifically, we compared data from a pharmacogenetic study based on first-episode, previously drug-naive subjects with schizophrenia who were treated with risperidone with data from a pharmacotranscriptomic study based on mice exposed to the same drug. Moreover, candidate variants from genes implicated by convergent data were also tested for evidence for association to schizophrenia per se because variants that are related to drug response may also be related to disease risk (5). Evidence that this occurs can be considered an additional independent line of circumstantial support that the convergence between the pharmacogenetic and transcriptomics does not merely reflect chance.

Methods and Materials

Subjects and Collection of Clinical Data

We performed an open-labeled pharmacogenetic study involving 108 first-episode, previously antipsychotic-naive schizophrenic patients. All received risperidone monotherapy after enrollment. Details are described elsewhere (6,7). Briefly, patients were entered into the study if they 1) met DSM-IV-TR criteria for schizophrenia (and then remained in follow-up to at least 6 months), 2) were physically healthy and had all laboratory parameters within normal limits, and 3) had neither a current nor a past DSM-IV-TR diagnosis of mood disorders or substance abuse. Consensus diagnoses were made by at least two experienced psychiatrists on the basis of unstructured interviews with patients and families and review of medical records. Duration of untreated psychosis (DUP) was defined as the period from the onset of psychotic symptoms to that of first antipsychotic exposure. Sixty subjects were recruited from outpatient clinics, and 48 subjects were treated as inpatients.

Subjects received risperidone monotherapy (starting dosage: .5–4 mg/day, mean starting dosage: 2.5 mg), and dosage was adjusted in accordance with symptomatic response by trained psychiatrists (1–8 mg/day, mean dosage: 3.4 mg at 8 weeks) for 8 weeks. Patients with insomnia were prescribed brotizolam, .25 mg or .5 mg, at bedtime. No other psychotropic drugs were permitted.

Clinical symptoms were evaluated at the first visit and after 8 weeks of treatment by the use of the Positive and Negative Syndrome Scale (PANSS). Evaluations were carried out by qualified psychiatrists and psychologists (the interrater reliability was measured by intraclass correlation coefficient was .90, unpublished data).

The clinical characteristics of subjects that we used as potential covariates were selected from another report (8): sex (57 male, 51 female), age (mean 30.2 ± 9.5 years), DUP (1.5–32 months, mean 7.6 ± 7.1 months), and baseline PANSS total score (mean 83.0 ± 22.9).

Samples used in the schizophrenia case-control association analysis consisted of three sets: (1) JPN_1st: this was used for identifying genes of potential interest and comprised 540 patients with schizophrenia (275 male and 265 female; aged 43.3 ± 15.0 years) and 425 healthy controls (236 male and 189 female; aged 36.3 ± 13.9 years) from the Japanese population; 2) JPN_2nd sample (used to follow up genes of interest) comprised 545 patients with schizophrenia (282 male and 263 female; aged 50.7 ± 14.9 years) and 500 controls (279 male and 221 female; aged 40.8 ± 15.4 years) from the Japanese population; 3) Additional follow-up data for SNPs of interest were extracted from a UK genomewide association study (GWAS) of schizophrenia comprising 479 patients with schizophrenia and 2937 controls from the UK population (9).

Controls in the Japanese population were screened for past history of mental disorders. All individuals were unrelated. After explanation of the study, written informed consent was obtained from each subject. This study was approved by the Ethics Committee at Fujita Health University, University of Occupational and Environmental Health, Nagoya University Graduate School of Medicine, Osaka University Graduate School of Medicine and by multiple ethics committees across the UK where sample recruitment was performed.

Microarray Experiments

See also Methods in Supplement 1.

SNP Chip. Genomewide genotyping was carried out using Illumina Sentrix human 1 Genotyping BeadChip (109,363 SNPs randomly distributed throughout the genome) according to the manufacturer's instructions (Illumina, San Diego, California). Details are given in the Supplement 1.

Mouse Expression Chip. We compared mRNA levels of the prefrontal cortex (PFC) between control ($n = 3$) and risperidone-exposed mice (2.4 mg/kg given orally, once a day for 21 days, $n = 3$). Affymetrix Mouse Gene 1.0 St. Array, which profiles the expression of 28,853 genes (Affymetrix, Santa Clara, California), was used to measure the amount of mRNA.

The procedures involving animals and their care were conducted in conformity with the international guidelines, Principles of Laboratory Animal Care (National Institutes of Health Publication 85-23, revised 1985).

Experimental Procedures and Statistical Analysis

Study 1: Pharmacogenomics. Quality control (QC) regarding population stratification (Figure S1 in the Supplement 1),