receptor *trkB* was knocked out show behavioral changes (hyper-locomotion, stereotyped behaviors, and cognitive impairments) related to the symptomatology of schizophrenia (Zorner et al., 2003). In addition, *BDNF* maps to chromosome 11p13, a region with potentially significant linkages to schizophrenia (Suarez et al., 2006).

A number of genetic association studies have shown that SNPs in *BDNF* are associated with schizophrenia (Nanko et al., 2003; Szekeres et al., 2003), and a meta-analysis study also showed a weak association between C270T and schizophrenia (Zintzaras, 2007), but not between Val66Met and schizophrenia (Kanazawa et al., 2007; Naoe et al., 2007; Xu et al., 2007; Zintzaras, 2007).

A recent study focused on the complex microsatellite polymorphism *BDNF*-LCPR located ~1.0 kbp upstream of the translation initiation site of *BDNF* (Okada et al., 2006). This polymorphism contained 23 novel allelic variants, including four major alleles (A1–A4). A luciferase assay showed a significantly lower expression level of the A1 allele than the other three alleles of *BDNF*-LCPR. Furthermore, the A1 allele frequency was significantly higher in bipolar disorder patients than in controls. Therefore, *BDNF*-LCPR can be seen as an important schizophrenia-susceptibility factor, but determination of genotype distributions of this polymorphism is difficult due to technical limitations.

Two main goals of the present study were to examine 1) whether tagging SNPs from the HapMap database can represent *BDNF*-LPCR through linkage disequilibrium (LD) evaluation, and 2) whether these tagging SNPs are associated with schizophrenia in a Japanese population. We also performed meta-analysis regarding two polymorphisms (Val66Met and C270T), which have been intensively examined in relation to *BDNF*.

2. Materials and methods

2.1. Subjects

The subjects in the LD evaluation of *BDNF*-LCPR were 66 healthy controls (35 males and 31 females; age 50.33 ± 11.03 (mean ± SD) years) who had participated in a previous study (Okada et al., 2006). The sample used in the association analysis comprised 1,117 schizophrenia patients (628 males and 489 females; 47.4 ± 15.3 years) and 1,102 healthy controls (504 males and 598 females; 37.1 ± 14.4 years). All participants were unrelated Japanese people living in central areas of Japan. The patients were diagnosed according to DSM-IV criteria with consensus from at least two experienced psychiatrists on the basis of an unstructured interview and review of medical records. Patients with any other axis-I disorder were excluded.

All healthy controls were also psychiatrically screened based on unstructured interviews to exclude subjects with brain/psychotic disorders, or those with first-degree relatives with a psychotic disorder. A trained psychiatrist interviewed each participant with respect to current or past mental states (psychotic, mood, anxiety, obsessive–compulsive symptoms) and family history (Ikeda et al., 2005). Healthy controls were mainly recruited from the staff of participating hospitals.

Written informed consent was obtained from each subject. This study was approved by the Ethics Committee of each institution involved.

2.2. Tagging SNP selection and LD evaluation

First we consulted the HapMap database (release#16.c.1, Jun 2005 www.hapmap.org, population: Japanese Tokyo: minor allele frequencies (MAFs) of more than 0.05) and selected 38 SNPs covering the *BDNF* gene (5′-flanking regions ranging from 9,467 bp away from the initial exon to 4,454 bp downstream (3′) from the last exon: HapMap database contig number chr11: 27709339..27628562). Then, seven 'tagging SNPs' (rs1491851, rs11030121, rs7934165, rs12291063, rs11030101, rs6265 [Val66-Met], rs1519480: Table 1) were selected with the criterion of an r^2 threshold greater than 0.8 in 'pair-wise tagging only' mode using the 'Tagger' program (de Bakker et al., 2005) (http://www/broad.mit.edu/mpg/tagger), a tool within the HAPLOVIEW software (Barrett et al., 2005). In addition to these tagging SNPs, we included the C270T polymorphism, which has been intensively examined in other papers. A total of eight SNPs were selected for the following LD evaluation and case-control association analysis.

2.3. Genotyping

Information on genotypic distributions of *BDNF*-LCPR, which had been determined previously (Okada et al., 2006), was used for LD evaluation (66 healthy controls). Genotyping

Table 1
Association analysis of tagging SNPs in *BDNF*.

Gene	Marker IDs		Distance to next SNP (bp)	N[a]		MAF[b]		M/M[c]		M/m[d]		m/m[e]		*p*-values	
				SCZ	CON	SCZ	CON	SCZ	CON	SCZ	CON	SCZ	CON	Genotype	Allele
BDNF (minus strand)	M1	rs1491851	0	1114	1100	0.234	0.247	647	622	413	413	54	65	0.491	0.312
	M2	rs11030121	16556	1111	1097	0.047	0.045	1011	999	96	97	4	1	0.409	0.79
	M3	rs7934165	4224	1109	1099	0.46	0.468	332	320	534	529	243	250	0.861	0.581
	M4	C270T	10185	1115	1102	0.032	0.03	1046	1036	67	66	2	0	0.372	0.716
	M5	rs12291063	27697	1117	1095	0.206	0.202	698	695	378	358	41	42	0.842	0.736
	M6	rs11030101	13357	1110	1099	0.344	0.34	484	485	489	480	137	134	0.969	0.813
	M7	rs6265	828	1111	1100	0.413	0.428	394	365	516	529	201	206	0.528	0.326
	M8	rs1519480	4204	1112	1100	0.246	0.235	631	640	414	403	67	57	0.621	0.375

[a] N = number, SCZ = schizophrenia, CON = control.
[b] MAF = minor allele frequency.
[c] M/M = major allele/major allele.
[d] M/m = major allele/minor allele.
[e] m/m = minor allele/minor allele.

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of remaining SNPs was performed by allelic discrimination assay (Applied Biosystems, CA).

2.4. Statistical analysis

2.4.1. LD evaluation and gene-based case-control association study

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by χ^2 goodness-of-fit test.

For LD evaluation, multiallelic $D'_{\wedge}$ was calculated by the COCAPHASE 2.403 program (Dudbridge, 2003). Haplotype frequencies of tagging SNPs (M6, M7 and M8) and *BDNF*-LCPR were then estimated using PHASE software (http://www.stat.washington.edu/stephens/software.html) (Stephens and Donnelly, 2003). Because this region shows high LD, a block-like structure was conserved, and the sum of haplotype frequencies was calculated to examine whether these haplotypes could capture the specific allele of *BDNF*-LCPR in accordance with the haplotype-tagging method (Kamatani et al., 2004).

Marker-trait association was evaluated with the use of a likelihood ratio test (allele-wise and haplotype-wise analyses) and χ^2 test (genotype-wise analysis). For exhaustive screening, we examined eight-marker haplotype analysis in sliding-window fashion using the COCAPHASE 2.403 program (Dudbridge, 2003). A power calculation was performed using a statistical algorithm implemented in the Genetic Power Calculator program (http://pngu.mgh.harvard.edu/~purcell/gpc/).

We also carried out imputation of SNPs that are not directly genotyped but are present in the HapMap database (JPT + CHB founders, release 23, filtered by MAF greater than 0.01 and genotyping rate greater than 0.95) by PLINK software (version 1.04) (Purcell et al., 2007). A total of 108 SNPs were included for this analysis, from rs12574598 (chr11..27531152) to rs11030149 (chr11..27798413; the location is based on Human, Mar, 2006, hg18 assembly). We used the "-proxy -drop" option and picked up only imputed SNPs with an INFO value greater than 0.8 in accordance with the authors' recommendation.

The level of significance for all statistical tests was set at 0.05.

2.4.2. Meta-analysis

To identify studies eligible for the meta-analysis, we searched PubMed citations through December 2008 using the terms "*BDNF*," "brain-derived neurotrophic factor," and "schizophrenia" as key words.

Regarding selection of studies, we included the case-control genetic association studies of the Val66Met and/or C270T polymorphism. Studies with data for only schizophrenic patients or only healthy controls were excluded, as were family-based studies.

We also carried out quality assessment using the Newcastle–Ottawa Scale (NOS) for case-control studies. The Newcastle–Ottawa Scale uses a "star" rating system to judge the quality based on three aspects of the study: selection of study groups, comparability of study groups and ascertainment of either the exposure or outcome of interest. The maximum number of stars a study may receive in each of these categories is 4, 2 and 3, respectively, for a total of 9 possible stars. The validity of these tools has been previously established (http://www.ohri.ca/programs/clinical_epidemiology/oxford.htm).

We then assessed the HWE in the patients and control subjects of each study using the χ^2 goodness-of-fit test, and we explored whether heterogeneity was present using Q statistics. Finally, we performed a fixed-effects as well as random-effects model meta-analysis within groups of homogeneous odds ratios (ORs). The fixed effects model assumes that all existing studies are included in the meta-analysis, and therefore weights each study only by the inverse of the variance of that study. A random-effect model, in contrast, assumes that between-study variation is due to chance and/or random variation and an individual study effect. Random-effect models are more conservative than fixed effects models and generate a wider confidence interval (CI). The significance of the pooled OR was determined using a Z-test. Fourth, publication bias was assessed using a linear regression analysis to measure funnel plot asymmetry. A probability level of $p < 0.05$ was used as a threshold for statistical significance. Data were analyzed using the "Comprehensive Meta Analysis" (Version 2.2.046) statistical software package.

3. Results

3.1. LD evaluation of BDNF in the Japanese population

Sixteen variants of *BDNF*-LCPR were found in our sample ($N_{allele} = 132$): three samples for the A1 allele [$(CG)_{del}(CA)_{12}(GA)_3$], 21 samples for the A2 allele [$(CG)_4(CA)_{12}(GA)_3$], 39 samples for the A3 allele [$(CG)_5(CA)_{12}(GA)_2$], 42 samples for the A4 allele [$(CG)_5(CA)_{13}(GA)_3$], and 27 samples for the A5 allele [combination of remaining rare alleles]. The results for LD between *BDNF*-LCPR and our eight tagging SNPs are

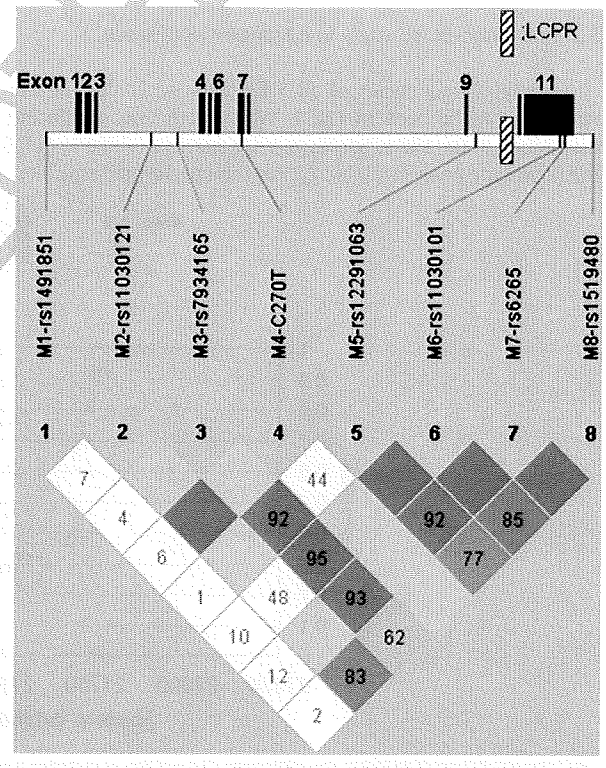

Fig. 1. LD structure in the *BDNF* gene.

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Table 2
Sliding-window analysis of tagging SNPs in *BDNF*.

Gene	Marker IDs	*p*-values						
		2-window	3-window	4-window	5-window	6-window	7-window	8-window
BDNF	M1							
		0.4111						
	M2		0.7189					
		0.8163		0.7133				
	M3		0.9294		0.9801			
		0.6334		0.9513		0.9883		
	M4		0.8741		0.9962		0.9851	
		0.7537		0.9594		0.9697		0.975
	M5		0.9088		0.9827		0.8971	
		0.9304		0.8325		0.9167		
	M6		0.8204		0.7188			
		0.5905		0.6972				
	M7		0.4048					
		0.4796						
	M8							

shown in Supplementary Table 1. The LD analysis of *BDNF*-LCPR and M6–M8 polymorphisms showed D' values ranging from 0.955 to 0.975, indicating tight linkage between M6, M7 and M8 and *BDNF*-LCPR (Supplementary Table 1).

On the other hand, single marker tests may be inefficient when each single marker carries a small to moderate amount of association information about the trait. In this situation, the association might not be detected when markers are analyzed individually, whereas combining genotypes from neighboring markers may provide a more powerful test. Therefore, in order to perform exploratory multimarker association testing, we estimated the haplotype frequencies and summed the haplotype frequencies to examine whether the haplotypes could capture the specific alleles of *BDNF*-LCPR in accordance with the haplotype-tagging method (Kamatani et al., 2004) (Supplementary Table 2).

3.2. Population-based study in a Japanese population

Genotype frequencies of all SNPs were in HWE. The LD structure can be seen in Fig. 1. We detected no significant association in the allele/genotype-wise analyses (Table 1) or in the haplotype analysis (Table 2). We then performed stratified analysis by gender, since a recent paper reported the effect of gender on *BDNF* in major depressive disorder (Verhagen et al., 2008). Again, we could not find any association in this subgroup analysis (Supplementary Tables 3 and 4).

The A1 allele of *BDNF*-LCPR is a low-frequency allele (around 2.3%). Therefore, considering both the low frequency of A1 and the number of alleles in the LCPR polymorphism, we included imputation analysis around *BDNF*, because additional SNPs might provide better coverage of this very polymorphic repeat. 35 SNPs passed our quality control, but no evidence for association could be detected between imputed SNPs and schizophrenia in the Japanese population (Fig. 2).

We obtained more than 80% power for the detection of an association when we set the genotype relative risk at 1.19–1.57 in schizophrenia under a multiplicative model of inheritance.

3.3. Meta-analysis

3.3.1. Val66Met polymorphism (rs6265, high-resolution plot is shown in Supplementary Figure 1)

An updated meta-analysis (total of 22 population-based association studies), in which three studies (Donohoe et al., 2007; Han et al., 2008; Takahashi et al., 2008) and our current

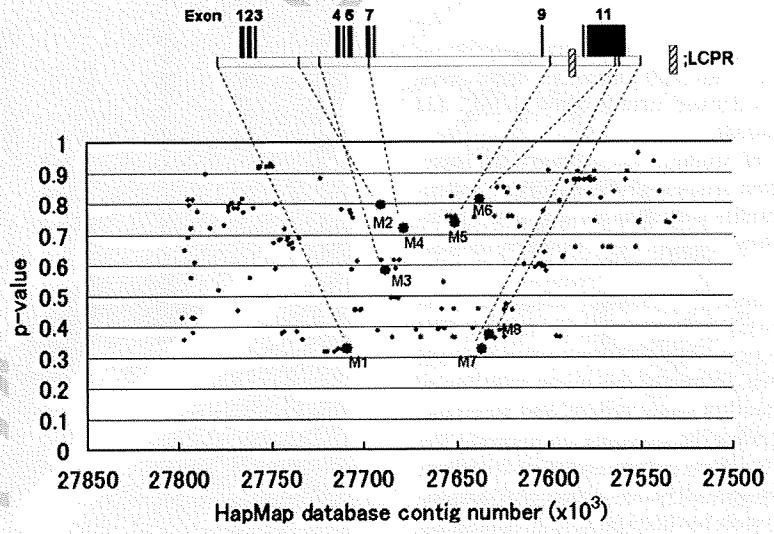

Fig. 2. Results of imputing SNP in the *BDNF* gene. The weights of evidence were calculated using imputed genotypes (small circles) and observed genotypes (big circles). The SNP position from the HapMap database is plotted on the *X* axis.

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results were added to the last meta-analysis (Kanazawa et al., 2007), can be seen in Table 4 (Egan et al., 2003; Hong et al., 2003; Nanko et al., 2003; Skibinska et al., 2004; Anttila et al., 2005; de Krom et al., 2005; Neves-Pereira et al., 2005; Schumacher et al., 2005; Tan et al., 2005; Chen et al., 2006; Jonsson et al., 2006; Numata et al., 2006; Tochigi et al., 2006; Watanabe et al., 2006; Zhang et al., 2006; Donohoe et al., 2007; Naoe et al., 2007; Qian et al., 2007; Xu et al., 2007; Han et al., 2008; Takahashi et al., 2008). All studies were independent and in HWE. We did not observe significant heterogeneity among ORs ($Q=18.99$, $df=21$, $p=0.586$). The pooled OR derived from all studies comprising 6568 patients and 8824 control subjects was not significant in each model (fixed model: pooled OR = 0.976, 95% CI = 0.928–1.026, $p=0.345$, random model: OR = 0.976, 95% CI = 0.928–1.026, $p=0.345$; Table 3). Next, in order to limit the ethnic heterogeneity, we analyzed the Caucasian and Asian samples separately. These subgroup analyses also showed no significant

Table 3
Meta-analysis of case-control studies between the rs6265 and schizophrenia.

First author	Year	Ethnicity	No.		No. of G(Val) major allele		No. of A(Met) minor allele		Diagnostic system	OR	95% CI	p-value	NOS
			SCZ	CON	SCZ	CON	SCZ	CON					
Egan	2003	American	203	133	332	218	74	48	DSM-IV	1.012	0.68–1.51	0.952	7 (selection 3/4 comparability 2/2 exposure 2/3)
Skinbinska	2004	Polish	336	375	565	613	107	137	DSM-IV	0.847	0.64–1.12	0.242	7 (selection 3/4 comparability 2/2 exposure 2/3)
Antilla	2005	Finnish	94	98	156	166	32	30	DSM-IV	1.135	0.66–1.96	0.648	7 (selection 3/4 comparability 2/2 exposure 2/3)
Neves-Pereira	2005	Scotish	321	350	541	547	101	153	DSM-IV	0.667	0.51–0.88	0.004	8 (selection 3/4 comparability 2/2 exposure 3/3)
Shumacher	2005	German	533	1097	842	1777	224	417	DSM-IV	1.134	0.95–1.36	0.176	8 (selection 3/4 comparability 2/2 exposure 3/3)
de Krom	2005	Dutch	273	580	437	928	109	232	DSM-IV	0.998	0.77–1.29	0.986	7 (selection 3/4 comparability 2/2 exposure 2/3)
Jonsson	2006	Swdish	187	275	312	452	60	98	DSM-III-R	0.887	0.62–1.26	0.504	7 (selection 3/4 comparability 2/2 exposure 2/3)
Zhang	2006	American	84	250	135	406	33	94	DSM-IV	1.056	0.68–1.64	0.810	8 (selection 3/4 comparability 2/2 exposure 3/3)
Donohoe	2007	Irish	359	745	598	1241	120	249	DSM-IV	1.000	0.79–1.27	0.999	7 (selection 3/4 comparability 2/2 exposure 2/3)
Hong	2003	Han Chinese	93	198	85	189	101	207	DSM-IV	1.085	0.76–1.54	0.648	7 (selection 3/4 comparability 2/2 exposure 2/3)
Nanko	2003	Japanese	178	332	209	382	147	282	DSM-IV	0.953	0.73–1.24	0.716	7 (selection 3/4 comparability 2/2 exposure 2/3)
Tan	2005	Han Chinese	108	145	117	165	99	125	DSM-IV	1.117	0.78–1.59	0.541	8 (selection 3/4 comparability 2/2 exposure 3/3)
Chen	2006	Han Chinese	560	576	573	607	547	545	DSM-IV	1.063	0.90–1.25	0.465	7 (selection 3/4 comparability 2/2 exposure 2/3)
Numata	2006	Japanese	159	318	198	364	120	272	DSM-IV	0.811	0.62–1.07	0.137	7 (selection 3/4 comparability 2/2 exposure 2/3)
Tochigi	2006	Japanese	401	569	487	675	315	463	DSM-IV	0.943	0.78–1.13	0.533	7 (selection 3/4 comparability 2/2 exposure 2/3)
Watanabe	2006	Japanese	349	423	407	491	291	355	DSM-IV	0.989	0.81–1.21	0.914	7 (selection 3/4 comparability 2/2 exposure 2/3)
Naoe	2007	Japanese	211	205	249	258	173	152	DSM-IV	1.179	0.89–1.56	0.247	7 (selection 3/4 comparability 2/2 exposure 2/3)
Qian	2007	Han Chinese	604	650	616	657	592	643	DSM-IV	0.982	0.84–1.15	0.820	7 (selection 3/4 comparability 2/2 exposure 2/3)
Xu	2007	Han Chinese	275	297	292	297	258	297	DSM-IV	0.884	0.70–1.11	0.296	7 (selection 3/4 comparability 2/2 exposure 2/3)
Han	2008	Korean	96	79	97	84	95	74	DSM-IV	1.112	0.11–0.22	0.622	7 (selection 3/4 comparability 2/2 exposure 2/3)
Takahashi	2008	Japanese	33	29	39	37	27	21	ICD-10	1.220	0.20–0.37	0.592	7 (selection 3/4 comparability 2/2 exposure 2/3)
Current study	2007	Japanese	1111	1100	1304	1259	918	941	DSM-IV	0.942	0.86–1.06	0.326	7 (selection 3/4 comparability 2/2 exposure 2/3)
Pooled (22)[a] fixed		All	6568	8824	8591	11813	4543	5835		0.973	0.93–1.02	0.293	
Pooled (22)[a] random		All								0.973	0.93–1.02	0.293	
Pooled (9)[b] fixed		Caucasian	2390	3903	3918	6348	860	1458		0.967	0.88–1.06	0.480	
Pooled (9)[b] random		Caucasian								0.956	0.85–1.08	0.444	
Pooled (13)[c] fixed		Asian	4178	4921	4673	5465	3683	4377		0.980	0.92–1.04	0.501	
Pooled (13)[c] random		Asian								0.980	0.92–1.04	0.501	

SCZ, schizophrenia patients; CON, control.
OR, odd ratio; CI, confidence interval; NOS, The Newcastle–Ottawa Scale.
[a] $Q=18.99$, $df=21$, $p=0.586$ for heterogeneity.
[b] $Q=11.54$, $df=8$, $p=0.173$ for heterogeneity.
[c] $Q=7.39$, $df=12$, $p=0.831$ for heterogeneity.

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association in either ethnic sample (Table 3). No publication bias was found ($t=0.369$, $p=0.716$).

3.3.2. C270T polymorphism (high-resolution plot is shown in Supplementary Figure 2)

For C270T polymorphism, nine population-based association studies and our current study met our criteria for the updated meta-analysis (Watanabe et al., 2007). (Table 4) (Nanko et al., 2003; Szekeres et al., 2003; Anttila et al., 2005; Galderisi et al., 2005; Szczepankiewicz et al., 2005; Jonsson et al., 2006; Watanabe et al., 2006; Zhang et al., 2006; Xu et al., 2007). Total sample sizes for patients and control subjects were 2887 and 3336, respectively. All studies were independent and in HWE. We did not observe significant heterogeneity among ORs ($Q=13.57$, $df=9$, $p=0.139$). The ORs and 95% CIs for the 10 population-based studies are shown in Table 3. When the analysis was run within a fixed model, the T allele was significantly associated with schizophrenia (pooled OR = 1.219, 95% CI = 1.013–1.468, $Z=2.100$, $p=0.036$). However, in a random effects model, there was no evidence of a significant association (pooled OR = 1.268, 95% CI = 0.997–1.614, $Z=1.934$, $p=0.053$). When the study that showed the most significant results (Szekeres et al., 2003) was removed, the association in the fixed model did not remain significant (pooled OR = 1.165, 95% CI = 0.966–1.407, $Z=1.596$, $p=0.110$).

The subgroup analyses based on the ethnic differences were then analyzed separately. The pooled OR derived from the six Caucasian studies comprising 970 patients and 1182 control subjects was not significant (fixed model: pooled OR = 1.210, 95% CI = 0.922–1.589, $p=0.169$, random model: OR = 1.273, 95% CI = 0.884–1.834, $p=0.195$), nor was the pooled OR derived from the four Asian studies comprising 1917 patients and 2154 control subjects (fixed model: pooled OR = 1.227, 95% CI = 0.953–1.580, $p=0.112$, random model: pooled OR = 1.293, 95% CI = 0.894–1.870, $p=0.173$). No publication bias was found ($t=1.89$, $p=0.101$).

4. Discussion

In this study, we carried out a detailed LD evaluation of the region harboring *BDNF*-LCPR, a gene-based association study in the Japanese population, and we updated the meta-analysis of two functional SNPs, Val66Met and C270T. From these results, we conclude that the commonly identified variants in *BDNF* are not associated with schizophrenia.

Regarding single marker based LD tagging, our data showed that *BDNF*-LCPR could be represented well. On the other hand, as for multimarker tagging, our data showed that *BDNF*-LCPR could be represented moderately well by the haplotypes constructed with our tagging SNPs (73.5% coverage) since the most important functional allele in *BDNF*-LCPR, the A1 allele [$(CG)_{del}(CA)_{12}(GA)_3$], could not be captured adequately by our tagging SNPs. These included C270T, which showed a trend for significance in the meta-analysis ($p_{fix\ model}=0.036$, $r^2=0.000945$). In other words, we could not evaluate the tagging SNPs haplotype coverage (M6–M7–M8)

Table 4
Meta-analysis of case-control studies between the C270T polymorphism and schizophrenia.

First author	Year	Ethnicity	No.		No. of major (C) allele		No. of minor (T) allele		Diagnostic system	OR	95% CI	p-value	NOS
			SCZ	CON	SCZ	CON	SCZ	CON					
Szekeres	2003	Finnish	101	68	174	132	28	4	DSM-IV	5.310	1.82–15.51	0.002	7 (selection 3/4 comparability 2/2 exposure 2/3)
Antilla	2005	Finnish	94	98	171	182	17	14	DSM-IV	1.292	0.62–2.70	0.495	8 (selection 3/4 comparability 2/2 exposure 3/3)
Galderisi	2005	Itarian	107	111	201	211	13	11	DSM-IV	1.241	0.54–2.83	0.609	8 (selection 3/4 comparability 2/2 exposure 3/3)
Szczepankiewicz	2005	Polish	397	380	755	725	39	35	DSM-IV	1.070	0.67–1.71	0.777	8 (selection 3/4 comparability 2/2 exposure 3/3)
Jonsson	2006	Swedish	187	275	354	521	20	29	DSM-IV	1.015	0.57–1.82	0.96	7 (selection 3/4 comparability 2/2 exposure 2/3)
Zhang	2006	American	84	250	158	470	10	30	DSM-IV	0.992	0.47–2.07	0.982	8 (selection 3/4 comparability 2/2 exposure 3/3)
Nanko	2003	Japanese	178	332	339	649	17	15	DSM-IV	2.170	1.07–2.13	0.032	8 (selection 3/4 comparability 2/2 exposure 3/3)
Watanabe	2006	Japanese	349	423	672	827	26	19	DSM-IV	1.684	0.92–3.07	0.089	7 (selection 3/4 comparability 2/2 exposure 2/3)
Xu	2007	Han Chinese	275	297	534	574	16	20	DSM-IV	0.860	0.44–1.68	0.658	7 (selection 3/4 comparability 2/2 exposure 2/3)
Current study	2007	Japanese	1115	1102	2159	2138	71	66	DSM-IV	1.065	0.76–1.50	0.716	7 (selection 3/4 comparability 2/2 exposure 2/3)
Pooled (10)[a] fixed		All	2887	3336						1.219	1.01–1.47	0.036	
Pooled (10)[a] random		All								1.268	0.997–1.614	0.053	
Pooled (6)[b] fixed		Caucasian	970	1182						1.210	0.92–1.59	0.169	
Pooled (6)[b] random		Caucasian								1.273	0.88–1.83	0.195	
Pooled (4)[c] fixed		Asian	1917	2154						1.227	0.95–1.58	0.112	
Pooled (4)[c] random		Asian								1.293	0.89–1.87	0.173	

SCZ, schizophrenia patients; CON, control.
OR, odd ratio; CI, confidence interval; NOS, The Newcastle–Ottawa Scale.
[a] $Q=13.57$, $df=9$, $p=0.139$ for heterogeneity.
[b] $Q=8.24$, $df=5$, $p=0.143$ for heterogeneity.
[c] $Q=5.32$, $df=3$, $p=0.150$ for heterogeneity.

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for the A1 allele appropriately, due to the small size of the LD evaluation sample and the low population frequency of A1 (only 3 subjects had the A1 allele). Therefore, we included the imputation analysis of SNPs around *BDNF* to examine whether unobserved SNPs would be associated with schizophrenia. This is because we expected to increase the possibility of capturing the A1 allele in *BDNF*-LCPR by multi-marker information in the HapMap database. Nevertheless, we could find no evidence for association from imputed SNPs with schizophrenia. Thus, taking these findings together, we speculate that: 1) Variants in *BDNF* including *BDNF*-LCPR may not be associated with schizophrenia, and 2) we merely overlooked the association of the A1 allele in *BDNF*-LCPR in our analysis. The aforementioned scenario also implies that the LD structure between the Al allele and common SNPs examined in the LD evaluation (or the imputation) may be unique. Therefore, further LD evaluation and a mutation scan will be needed to obtain conclusive results and in order to capture the A1 allele, considering the technical difficulty regarding direct genotyping of this complex polymorphic region.

Our gene-based association study and meta-analysis of Val66Met did not show an association with schizophrenia. Our sample size in these analyses was large enough to rule out type II error. Meanwhile, meta-analysis of C270T showed a trend for significance in the fixed model. In general, the meta-analytic strategy has the advantage of increasing the statistical power; however the interpretation of positive results from meta-analysis in genetic association studies is difficult. Speaking conservatively, the pooling of results can be truly reasonable only when the causal variants have been identified (i.e. biological evidence for such variants has been established clearly) (Sand, 2007). Of course we cannot safely say in every case, therefore we have to try to reduce the possible confounding effects, such as LD differences among populations; it is well known that LD structures are often unstable and cannot be generalized across populations of different origin (Ingles et al., 1997). In relation to this, the author proposed that the MAFs among populations must be examined for meta-analysis (Sand, 2007). In our case, a two-fold difference in C270T allele frequency was found between Caucasian samples (5.2%) and Asian samples (2.7%), suggesting that there is genetic heterogeneity among populations. Thus, we assume from the viewpoint of this heterogeneity or just from multiple testing that this trend for association may be derived from false-positives. To correct the p-value using the Benjamini–Hochberg (BH) method, which is a type of false discovery rate (FDR) controlling procedure, the Q-value in the C270T meta-analysis ($Q = 0.0136$) is higher than the p-value in the fixed model ($p = 0.036$). This gap suggests no significance in the C270T meta-analysis. Therefore, our case-control analysis based on LD and meta-analyses does not provide evidence for an association of the *BDNF* gene with schizophrenia.

Several caveats should be noted. Firstly, we did not include a systematic mutation scan in the 5′ flanking region or exon regions. Secondly, our phenotypic diagnosis is not based on structured interviews and control samples are significantly younger than case samples.

To conclude, our results indicate that common SNPs in the *BDNF* gene do not play a major role in patients with schizophrenia. However, if there is a possibility for an association of *BDNF* with schizophrenia, the A1 allele in *BDNF*-LCPR is the most attractive candidate variant. Further LD evaluation or an association study in which *BDNF*-LCPR is genotyped directly will be required for a conclusive result.

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Contributors

KK and TK performed laboratory assays and the data-analysis. KK, TK and MI drafted the manuscript. MI, TK, YY, YK, NT, SS, KO, YY, RH, BA, MT and TI advised on data-analysis. NO and NI participated in the design of the study, interpretation of the data, and drafting of the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.schres.2009.03.040.

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Association analysis of Group II metabotropic glutamate receptor genes (*GRM2* and *GRM3*) with mood disorders and fluvoxamine response in a Japanese population

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ABSTRACT

Background: Several lines of evidence implicate abnormalities in glutamate neural transmission in the pathophysiology of mood disorders, including major depressive disorder (MDD) and bipolar disorder (BP). Preclinical antidepressant effects were also reported for group II metabotropic glutamate receptor (Group II mGluRs) antagonists show dose-dependent antidepressant-like effects in murine models of depression. Also, it has been suggested that abnormalities in the hypothalamic-pituitary-adrenal axis and serotonergic neural transmission are important mechanisms in the pathophysiology of mood disorders. Group II mGluRs play an important role in regulating the function of these mechanisms. From these results, it has been suggested that abnormalities in Group II mGluRs might be involved in the pathophysiology of mood disorders, including MDD) and BP, and may influence the clinical response to treatment with SSRIs in MDD. Therefore, we studied the association between Group II mGluR genes (*GRM2* and *GRM3*) and mood disorders and the efficacy of fluvoxamine treatment in Japanese MDD patients.
Materials and methods: Using three tagging SNPs in *GRM2* and an SNP (rs6465084) reported functional variant in *GRM3*, we conducted a genetic association analysis of case-control samples (325 MDD patients, 155 BP patients and 802 controls) in the Japanese population. In addition, we performed an association analysis of *GRM2* and *GRM3* and the efficacy of fluvoxamine treatment in 117 Japanese patients with 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 clinical response as a decrease of more than 50% in baseline SIGH-D within 8 weeks, and clinical remission as an SIGH-D score of less than 7 at 8 weeks.
Results: We found an association between rs6465084 in *GRM3* and MDD in the allele-wise analysis after Bonferroni's correction (P-value = 0.0371). However, we did not find any association between *GRM3* and BP or the fluvoxamine therapeutic response in MDD in the allele/genotype-wise analysis. We also did not detect any association between *GRM2* and MDD, BP or the fluvoxamine therapeutic response in MDD in the allele/genotype-wise or haplotype-wise analysis.
Discussion: We detected an association between only one marker (rs6465084) in GRM3 and Japanese MDD patients. However, because we did not perform an association analysis based on LD and a mutation scan of GRM3, a replication study using a larger sample and based on LD may be required for conclusive results.

1. Introduction

Several lines of evidence implicate abnormalities in glutamate neural transmission in the pathophysiology of mood disorders, including major depressive disorder (MDD) (Hashimoto et al., 2007; Paul and Skolnick, 2003) and bipolar disorder (BP) (Hashimoto et al., 2007; Witkin et al., 2007), and in the mechanisms of therapeutic actions of antidepressants (Palucha and Pilc, 2002). Glutamate exerts its actions through activation of receptors such as *N*-methyl-d-aspartate (NMDA) receptor, metabotropic

Abbreviations: Group II mGluRs, Group II metabotropic glutamate receptors; MDD, major depressive disorder; BP, bipolar disorder; SIGH-D, Structured Interview Guide for Hamilton Rating Scale for Depression; LD, linkage disequilibrium; NMDA, *N*-methyl-D-aspartate; mGluRs, metabotropic glutamate receptors; AMPA, a-amino-3-hydroxy-5-methyl-4-isoxazole propionate; MAFs, minor allele frequencies; HWE, Hardy-Weinberg equilibrium; MDR, Multifactor Dimensionality Reduction; TSNAX-DISC1 gene, Translin-associated factor X-Disrupted-in-Schizophrenia-1 gene; GWAS, whole genome association study.

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glutamate receptors (mGluRs) and a-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA). NMDA receptor antagonists such as dizocilpine and memantine show antidepressant-like actions in mice exhibiting depressive behavior, such as in the forced swim test (Poleszak et al., 2007). Preclinical antidepressant effects were also reported for group II metabotropic glutamate receptor (mGluR2 and mGluR3) antagonists, MGS0039 and LY341495 show dose-dependent antidepressant-like effects in murine models of depression, such as the forced swim and tail suspension tests (Bespalov et al., 2008).

The mGluRs are classified into three groups (group I mGluRs: mGluR1 and mGluR5, group II mGluRs: mGluR2 and mGluR3, and group III mGluRs: mGluR4 and mGluR6-8). Postmortem study has shown decreased mGluR3 in the perirhinal cortex in MDD patients compared with control subjects (Beneyto et al., 2007). Group II mGluRs are highly expressed in brain structures apparently related to emotional states, including the forebrain and limbic areas (Tamaru et al., 2001; Wright et al., 2001). It has been suggested that abnormalities in the hypothalamic-pituitary-adrenal axis (HPA axis) (Buckley and Schatzberg, 2005) and serotonergic neural transmission (Levinson, 2006; Serretti and Mandelli, 2008) are important mechanisms in the pathophysiology of mood disorders. Group II mGluRs have been shown to regulate the function of HPA axis activity (Holsboer and Barden, 1996; Scaccianoce et al., 2003). In animal studies, MGS0039 elevated serotonin levels in the rat medial prefrontal cortex in an *in vivo* microdialysis study (Karasawa et al., 2005) and MGS0039 and LY341495 increased the activity of serotonin neurons in the rat dorsal raphe nucleus (Kawashima et al., 2005). In addition, Matrisciano and colleagues reported that reported that both the expression and function of group II mGluRs are amplified in rat hippocampus when rat was administered imipramine chronically (Matrisciano et al., 2002). From these results, group II mGluRs appear to be good candidates both for involvement in the pathophysiology of, and as therapeutic targets in, MDD.

The mGluR2 gene (*GRM2*: OMIM *604099, 7 exons in a genomic region spanning 10.466 Kb) is located on 3p21. The mGluR3 gene (*GRM3* OMIM *601115, 6 exons in a genomic region spanning 221.763 Kb) is 7q21. This genomic region has been shown to be closely related to susceptibility for BP (Detera-Wadleigh et al., 1997). Therefore, we studied the association between *GRM2* or *GRM3* and mood disorders and the efficacy of fluvoxamine treatment in Japanese MDD patients.

2. Materials and methods

2.1. Subjects

The subjects in the association analysis were 325 MDD patients (159 males and 166 females; mean age ± standard deviation 47.3 ± 14.9years), 155 BP patients (80 males and 75 females; 96 patients with bipolar I disorder and 59 patients with bipolar II disorder; 47.9 ± 14.2 years) and 802 healthy controls (351 males and 451 females; 37.2 ± 15.9 years). Of the 325 MDD patients, 117 (58 males and 59 females; 44.8 ± 16.7 years) were treated with fluvoxamine and diagnosed according to DSM-IV criteria with the consensus of at least two experienced psychiatrists on the basis of a review of medical records and assessments with the Structured Interview Guide for Hamilton Rating Scale for Depression (SIGH-D). The remaining MDD 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 subjects were unrelated to each other, ethnically Japanese, and lived in the central area of Japan. All healthy controls were also psychiatrically screened based on unstructured interviews. None had severe medical complications such as liver cirrhosis, renal failure, heart failure, or other Axis-I disorders according to DSM-IV.

2.2. Data collection

The 117 MDD patients in this study had scores of 12 or higher on the 17 items of the SIGH-D (Peveler and Kendrick, 2005). We defined a clinical response as a decrease of more than 50% in baseline SIGH-D within 8 weeks, and 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 patients in this study, classified according to these definitions, can be seen in Table 1.

2.3. SNP selection

We first consulted the HapMap database (release#23.a.phase2, Mar 2008, www.hapmap.org, population: Japanese Tokyo: minor allele frequencies (MAFs) of more than 0.05) and included 4 SNPs covering *GRM2* (5′-flanking regions including about 6.3 Kb from the initial exon and about 1 kb downstream (3′) from the last exon: HapMap database contig number chr17: 51711684.. 51730152). Then three 'tagging SNPs' were selected with the criteria of r^2 threshold greater than 0.8 in 'pair-wise tagging only' mode using the 'Tagger' program (Paul de Bakker, http://www/broad.mit.edu/mpg/tagger), an implement of the HAPLOVIEW software (Barrett et al., 2005), for the following association analysis. In addition, we selected rs6465084 in *GRM3*, which is reported to be associated with prefrontal brain functioning, for use in the later association analysis.

2.4. SNP genotyping

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

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	117	58	59	44.8 ± 16.7	20.1 ± 5.84	122 ± 41.0	1.38 ± 0.656
Clinical response group[a]							
Responders	60	29	31	45.1 ± 16.5	21.4 ± 6.19	119 ± 41.0	1.36 ± 0.574
Nonresponders	57	29	28	44.4 ± 17.2	18.7 ± 5.14	125 ± 41.2	1.41 ± 0.780
P-value	0.783			0.849	0.0102	0.468	0.750
Clinical remission group[b]							
Remitters	46	22	24	44.3 ± 16.2	19.5 ± 5.05	114 ± 43.7	1.37 ± 0.598
Nonremitters	71	37	34	45.2 ± 17.3	20.5 ± 6.30	127 ± 38.6	1.39 ± 0.718
P-value	0.650			0.809	0.350	0.118	0.879

[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 7.

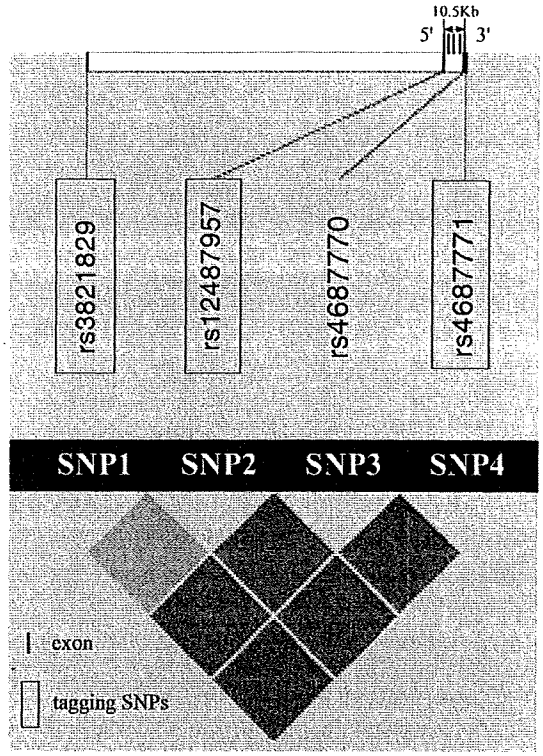

Fig. 1. LD evaluation and tagging SNPs in *GMR2*. Black bars represent exons of *GMR2*. Tagging SNPs selected from HapMap database are represented by black boxes. The color scheme is based on *D'* values. Other information can be seen at the HAPLOVIEW website.

2.5. 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 a statistical program prepared by Purcell et al. (2003).

3. Results

The linkage disequilibrium (LD) structure of *GRM2* can be seen in Fig. 1. Genotype frequencies were in HWE for all SNPs. In addition, regarding genotyping quality control measures, we added twenty-five 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 found an association between rs6465084 in *GRM3* and MDD in the allele-wise analysis after Bonferroni's correction (*P*-value = 0.0371). However, we did not find any association between *GRM3* and BP in the allele/genotype-wise (Table 2). We also did not detect any association between *GRM2* and MDD or BP in the allele/genotype-wise or haplotype-wise analysis (MDD: *P*-value = 0.2537 and BP: *P*-value = 0.1349)(Table 2).

With regard to the clinical characteristics of patients, only one difference was detected between responders and nonresponders in baseline SIGH-D scores (*P*-value = 0.0102) (Table 1). One patient each was prescribed alprazolam, loflazepate and etizolam. Two patients each were prescribed lorazepam, brotizolm, flunitrazepam and zopiclone. We did not find any association between *GRM2* or *GRM3* and the fluvoxamine therapeutic response in MDD patients in allele/genotype-wise (Table 3) or haplotype-wise analysis (response: *P*-value = 0.2459 and remission: *P*-value = 0.3181).

Moreover, to evaluate the interactions with each SNP in these genes, we analyzed the gene-gene interactions with the use of the Multifactor Dimensionality Reduction (MDR) method (Hahn et al., 2003). In this analysis, however, no interactions were obtained with MDD and BP (data not shown).

In the power analysis, we obtained more than 80% power for the detection of association when we set the genotype relative risk at 1.31–1.63 and 1.55–2.18in MDD and BP, respectively, for *GRM2* and at 1.51 and 2.02 in MDD and BP, respectively, for *GRM3* under a multiplicative model of inheritance.

4. Discussion

We performed the first association study of *GRM2* and *GRM3* with mood disorders and fluvoxamine treatment outcome in MDD in the

Table 2
Association analysis of *GRM2* and *GRM3* with mood disorders.

Gene	SNP ID[a]	Phenotype[b]	MAF[c]	*N*	Genotype distribution[d]			*P*-value[f]			Corrected *P*-value[f,g]	
					M/M	M/m	m/m	HWE[e]	Genotype	Allele	Genotype	Allele
GRM2	rs3821829	Controls	0.0468	802	731 (91.1)	67 (8.35)	4 (0.500)	0.0751				
	C>T	BP	0.0516	155	139 (89.7)	16 (10.3)	0 (0.00)	0.498	0.501	0.713		
		MDD	0.0477	325	295 (90.8)	29 (8.92)	1 (0.308)	0.750	0.869	0.924		
	rs12487957	Controls	0.333	802	346 (43.1)	378 (47.1)	78 (9.73)	0.0834				
	T>C	BP	0.281	155	80 (51.6)	63 (40.6)	12 (7.74)	0.934	0.148	0.0719		
		MDD	0.335	325	144 (44.3)	144 (44.3)	37 (11.4)	0.912	0.579	0.910		
	rs4687771	Controls	0.376	802	300 (37.4)	401 (50.0)	101 (12.6)	0.0632				
	T>A	BP	0.332	155	66 (42.6)	75 (48.4)	14 (9.03)	0.260	0.309	0.145		
		MDD	0.378	325	121 (37.2)	162 (49.8)	42 (12.9)	0.283	0.989	0.911		
GRM3 A	rs6465084	Controls	0.0842	802	673 (83.9)	123 (15.3)	6 (0.748)	0.884				
	A>G	BP	0.0903	155	128 (82.6)	26 (16.8)	1 (0.645)	0.796	0.896	0.722		
		MDD	0.0523	325	292 (89.8)	32 (9.85)	1 (0.308)	0.901	**0.0344**	**0.00928**	0.138	**0.0371**

[a] Major allele>minor allele.
[b] BP: bipolar disorder MDD: major depressive disorder.
[c] MAF: minor allele frequency.
[d] M: major allele, m: minor allele, The number in the parenthesis showed the percentage (%).
[e] Hardy–Weinberg equilibrium.
[f] Bold numbers represent significant *P*-value.
[g] Calculated by Bonferroni correction.

Table 3
Genotype and allele distributions of *GRM2* and *GRM3* in both definition groups.

Gene	SNP ID[a]	Clinical groups	MAF[b]	N	Genotype distribution[c]			P-value[d]		
					M/M	M/m	m/m	HWE	Genotype	Allele
GRM2	rs3821829	Responders	0.0667	60	52 (86.7)	8 (13.3)	0 (0.00)	0.580		
	C>T	Nonresponders	0.0263	57	54 (94.7)	3 (5.26)	0 (0.00)	0.838	N.A.	0.145
		Remission	0.0435	46	42 (91.3)	4 (8.70)	0 (0.00)	0.758		
		Nonremission	0.0493	71	64 (90.1)	7 (9.86)	0 (0.00)	0.662	N.A.	0.837
	rs12487957	Responders	0.350	60	25 (41.7)	28 (46.7)	7 (11.7)	0.843		
	T>C	Nonresponders	0.298	57	26 (45.6)	28 (49.1)	3 (5.26)	0.190	0.462	0.398
		Remission	0.370	46	19 (41.3)	20 (43.5)	7 (15.2)	0.650		
		Nonremission	0.296	71	32 (45.1)	36 (50.7)	3 (4.23)	0.0673	0.114	0.239
	rs4687771	Responders	0.383	60	22 (36.7)	30 (50.0)	8 (13.4)	0.656		
	T>A	Nonresponders	0.333	57	23 (40.4)	30 (52.6)	4 (7.02)	0.164	0.527	0.425
		Remission	0.391	46	17 (37.0)	22 (47.3)	7 (15.2)	0.979		
		Nonremission	0.338	71	28 (39.4)	38 (53.5)	5 (7.04)	0.0989	0.361	0.407
GRM3	rs6465084	Responders	0.0667	60	52 (86.7)	8 (13.4)	0 (0.00)	0.580		
	A>G	Nonresponders	0.0614	57	50 (87.7)	7 (12.3)	0 (0.00)	0.621	N.A.	0.869
		Remission	0.0652	46	40 (87.0)	6 (13.0)	0 (0.00)	0.636		
		Nonremission	0.0634	71	62 (87.3)	9 (12.7)	0 (0.00)	0.569	N.A.	0.955

[a] Major allele>minor allele.
[b] MAF: minor allele frequency.
[c] M: major allele, m: minor allele, The number in the parenthesis showed the percentage (%).
[d] Hardy–Weinberg equilibrium.

Japanese population. We detected a significant association between *GRM3* and MDD. In this study, we selected rs6465084 in *GRM3* as the SNP. Rs6465084 has been found to be associated with decreased verbal list learning and verbal fluency (Egan et al., 2004). In addition, Egan and colleagues reported that the rs6465084 A allele predicted decreased levels of *N*-acetylaspartate in the prefrontal cortex in an *in vivo* study, and suggested that the rs6465084 A allele reduced tissue glutamate levels and synaptic abundance (Egan et al., 2004). This influence of *GRM3* on prefrontal cortex and cognitive function suggests that abnormalities in glutamate neurotransmission may be involved in the pathophysiology of MDD by altering glutamate neurotransmission. Rs6465084 was shown to have MAFs $_{controls}$: 0.0842 and MAFs $_{MDD}$: 0.0523. Tochigi and colleagues reported that with this SNP, the MAFs in the Japanese population appear to be smaller than in Caucasians (Tochigi et al., 2006). Also, the MAFs in MDD patients were smaller than in controls. Our result is similar to the results of several studies in schizophrenia (Egan et al., 2004; Mossner et al., 2008). It might be that mood disorders and schizophrenia have common susceptibility genes. In support of this hypothesis, we show recent evidence (TSNAX-DISC1 gene)(Schosser et al., in press). Two studies have also suggested that rs6465084 was associated with the cognitive function in schizophrenic patients (Egan et al., 2004; Mossner et al., 2008). Therefore, further study will be required to investigate the relationship between rs6465084 and cognitive function in MDD patients. Recently, a whole genome association study (GWAS) reported an association between rs2237554 in *GRM3* and bipolar disorder (Sklar et al., 2008). Another GWAS reported that rs2189813, which was in LD with rs2237554 according to the HapMap database ($D'=1.00$ and $r^2=0.831$), was not associated with Japanese bipolar disorder patients (Hattori et al., in press)(release#23.a.phase2, Mar 2008, www.hapmap.org, population: Japanese Tokyo). Although we did not perform an association analysis of rs2237554, a replication study using larger samples than in the original studies will need to be carried out in the future. Also, Sartorius and colleagues reported that rs2228595 in *GRM3* predicted increased expression of the GRM3Delta4 splice variant (Sartorius et al., 2008). This SNP was shown to have "minor allele frequencies: 0.0330%" in the HapMap database (release#23.a.phase2, Mar 2008, www.hapmap.org, population: Japanese Tokyo). However, because our study had a small sample size, we considered it to be difficult to evaluate the association of rare variants from the viewpoint of power. Therefore, we did not include an association analysis of rs2228595.

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 for BP and the fluvoxamine therapeutic response in MDD samples, or unmatched age or gender samples. Because our samples for BP and the fluvoxamine therapeutic response in MDD were small, type II errors are possible in the results of these statistical association analyses. On average, the controls were much younger than the patients. This means that a number of young controls may go on to develop one of 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 bipolar disorder in the future (Bowden, 2001; Kishi et al., 2009; Kishi et al., 2008; Stensland et al., 2008). 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 patient who had been misdiagnosed, we promptly excluded the misdiagnosed case to maintain the precision of our sample. Second, we could not use an LD based strategy and perform a mutation search because *GRM3* has a massive gene structure. Therefore, in future studies it will be necessary to evaluate associations between other variants and mood disorders. It will be important to replicate and confirm these findings in other independent studies using larger samples.

5. Conclusion

In conclusion, we detected an association between only one marker (rs6465084) in GRM3 and Japanese MDD patients. However, because we did not perform an association analysis based on LD and a mutation scan of GRM3, a replication study using a larger sample and based on LD may be required for conclusive results.

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A functional polymorphism in estrogen receptor alpha gene is associated with Japanese methamphetamine induced psychosis

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ABSTRACT

Background: A recent study reported an association between rs2234693, which influences enhancer activity levels in estrogen receptor alpha gene (*ESR1*), and schizophrenia. This study reported that schizophrenic patients with the CC genotype have significantly lower *ESR1* mRNA levels in the prefrontal cortex than patients with other genotypes. The symptoms of methamphetamine induced psychosis are similar to those of paranoid type schizophrenia. Therefore, we conducted an association analysis of rs2234693 with Japanese methamphetamine induced psychosis patients. *Method:* Using rs2234693, we conducted a genetic association analysis of case-control samples (197 methamphetamine induced psychosis patients and 197 healthy controls). The age and sex of the control subjects did not differ from those of the methamphetamine induced psychosis patients. *Results:* We detected a significant association between *ESR1* and methamphetamine induced psychosis patients in allele/genotype-wise analysis. For further interpretation of these associations, we performed single marker analysis of subjects divided by sex. Rs2234693 was associated with male methamphetamine induced psychosis. *Discussion:* Our results suggest that rs2234693 in *ESR1* may play a role in the pathophysiology of Japanese methamphetamine induced psychosis patients.

1. Introduction

Methamphetamine (METH) use disorder is a common psychiatric disorder and a serious problem worldwide. METH stimulates the release of dopamine in the mesolimbic system (Munzar et al., 2004), and dopamine is in turn involved in the reinforcing action of many addictive drugs such as METH (Vocci et al., 2005). In addition, increased dopamine in the mesolimbic system is considered to produce psychotic symptoms such as hallucination and delusion (Laviolette, 2007). The symptoms of METH-induced psychosis are similar to those of paranoid type schizophrenia (Sato et al., 1992). Therefore, it may be that METH-induced psychosis and schizophrenia have common susceptibility genes. In support of this hypothesis, we reported that the V-act murine thymoma viral oncogene homologue 1 (AKT1) gene was associated with METH-induced psychosis (Ikeda et al., 2006) and schizophrenia (Ikeda et al., 2004) in the Japanese population.

A recent study reported an association between rs2234693, which influences enhancer activity levels of estrogen receptor alpha (ERalpha) in the ERalpha gene (*ESR1*) (Maruyama et al., 2000), and schizophrenia (Weickert et al., 2008). This study reported that schizophrenic patients with the CC genotype have significantly lower *ESR1* mRNA levels in the prefrontal cortex than patients with other genotypes (Weickert et al.,

Abbreviations: METH, methamphetamine; AKT1, V-act murine thymoma viral oncogene homologue 1; ERalpha, estrogen receptor alpha; *ESR1*, estrogen receptor alpha gene; SD, standard deviation; JGIDA, Japanese Genetics Initiative for Drug Abuse; HWE, Hardy–Weinberg equilibrium; SNP, single nucleotide polymorphism; mRNA, messenger ribonucleic acid; CD–CV hypothesis, common disease–common variants hypothesis.

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2008). Additionally, allele C of the *ESR1* SNP in intron 1 was thought to eliminate the transcription factor binding sites for activating enhancer binding protein 4 (transcriptional activator) in the sense strand and zinc finger protein (transcriptional repressor) in the antisense strand (Aoki et al., 1998; Fuks et al., 2001; Weickert et al., 2008). Therefore, Weickert and colleagues suggested that rs2234693 might be a functional SNP influencing *ESR1* expression regulation (Weickert et al., 2008). Estrogen has been shown to play an important role in protection from dopamine-induced toxicity caused by METH (Dluzen and McDermott, 2002; Gajjar et al., 2003; Liu and Dluzen, 2006).

From the above results, we suspected that estrogen receptor alpha might be related to the pathophysiology of METH-induced psychosis, and therefore conducted an association analysis of a functional SNP (rs2234693) in *ESR1* and METH-induced psychosis in the Japanese population.

2. Materials and methods

2.1. Subjects

The subjects in the association analysis were 197 METH-induced psychosis patients (164 males and 33 females; mean age ± standard deviation (SD) 37.6 ± 12.2 years) and 197 healthy controls (164 males and 33 females; 37.5 ± 12.2 years). The age and sex of the control subjects did not differ from those of the METH-induced psychosis patients. 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 unstructured interviews and a review of medical records. One hundred thirty-seven subjects with METH-induced psychosis also had dependence on drugs other than METH. Subjects with METH-induced psychosis were excluded if they had a clinical diagnosis of psychotic disorder, mood disorder, anxiety disorder or eating disorder. More detailed characterizations of these subjects have been published elsewhere (Kishi et al., 2008). All healthy controls, which included hospital staff, their families and medical students, were also psychiatrically screened based on unstructured interviews including current and past psychiatric history. None had severe medical complications such as liver cirrhosis, renal failure, heart failure or other Axis-I disorders according to DSM-IV. Written informed consent was obtained from each subject. This study was approved by the ethics committees at Fujita Health University, Nagoya University Graduate School of Medicine and each participating member of the Institute of the Japanese Genetics Initiative for Drug Abuse (JGIDA).

2.2. SNP genotyping

We used a TaqMan assay (Applied Biosystems, Foster City, CA, U.S.A.) for the SNP. One allelic probe was labeled with FAM dye and the other with fluorescent VIC dye. The plates were heated for 2 min at 50 °C and for 10 min at 95 °C, followed by 45 cycles at 95 °C for 15 s and 58 °C for 1 min. The primers used were forward 5′-TGCCTCATTATCAAAAGTGTCAAAT-3′ and reverse 5′-CCTGCTGCCTCATGTCATTGGCTAC-3′. Detailed information is available on request.

2.3. 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 chi-square test (SAS/Genetics, release 8.2, SAS Japan Inc, Tokyo, Japan). Bonferroni's correction was used to control inflation of the type I error rate.

The significance level for all statistical tests was 0.05.

We performed an explorative analysis of gender effects for the following reasons: (1) our subjects were unmatched for sex, especially those with METH-induced psychosis (164 males and 33 females); and (2) there is a possible sex difference in the pathophysiology of METH use disorder, since a sex difference has been reported for not only the use of METH but also the response to it (Brecht et al., 2004; Russell et al., 2008).

3. Results

Genotype frequencies of the SNP were in HWE (Table 1). We detected a significant association between rs2234693 and METH-induced psychosis in the allele/genotype-wise analyses (Table 1). For further interpretation of these associations, we performed single marker analysis of subjects divided by sex. Rs2234693 was associated with male METH-induced psychosis patients in the allele/genotype-wise analyses (Table 1). These results remained positive after correction for type I error due to multiple testing using Bonferroni's correction (Table 1).

4. Discussion

Our results showed an association between rs2234693 in *ESR1* and METH-induced psychosis in the Japanese population. Therefore, we reasoned that *ESR1* may play a role in the pathophysiology of METH-induced psychosis in the Japanese population. In addition, we performed an explorative analysis of gender effects. In this study, *ESR1* was associated with male METH-induced patients, who were the majority of our METH-induced psychosis samples. Similar to the study of Weickert and colleagues, there was a significantly higher percentage of the CC genotype among patients (percentage of CC genotype in total METH-induced psychosis and male METH-induced psychosis: 27.9% and 31.7%, respectively) than among controls (percentage of CC genotype in controls and male controls: 19.3% and 18.9%, respectively) (Weickert et al., 2008). This would seem to

Table 1
Association analysis of rs2234693 with methamphetamine induced psychosis and explorative analysis of gender subgroups.

Phenotype[a]	MAF[b]	N	Genotype distribution			P-value[c]			Corrected P-value[c,e]	
			TT	TC	CC	HWE[d]	Genotype	Allele	Genotype	Allele
Controls	0.414	197	72	87	38	0.208				
METH-induced psychosis	0.505	197	53	89	55	0.176	**0.0493**	**0.0101**		
Controls in males	0.421	164	57	76	31	0.528				
METH-induced psychosis in males	0.530	164	42	70	52	0.0668	**0.0199**	**0.00489**	**0.0398**	**0.00978**
Controls in females	0.379	33	15	11	7	0.0938				
METH-induced psychosis in females	0.379	33	11	19	3	0.199	0.114	1.00		

[a] METH-induced psychosis: methamphetamine induced psychosis.
[b] MAF: minor allele frequency.
[c] Bold represents significant P-value.
[d] HWE: Hardy–Weinberg equilibrium.
[e] Calculated using Bonferroni's correction.

support the hypothesis of common susceptibility genes for METH-induced psychosis and schizophrenia. It has also been reported that the CC genotype for rs2234693 has significantly lower expression of *ESR1* mRNA than the other genotype groups (T/C and T/T) in the prefrontal cortex of schizophrenics (Weickert et al., 2008). Two animal studies involving ovariectomized rats reported that long-term estradiol treatment may preserve striatal dopamine concentrations by decreasing the affinity of the dopamine transporter (Disshon et al., 1998; Ohtani et al., 2001). However, no human studies have been performed to date. We hypothesize that in these psychotic disorders decreased *ESR1* mRNA in the brain may weaken the estrogen response, resulting in increased dopamine-induced toxicity and psychotic symptoms. Several studies have suggested that treatment with estrogen can be an effective adjunctive therapy in schizophrenia. Our results suggest that estrogen therapy may be effective for not only schizophrenia but also for other psychotic disorders such as METH-induced psychosis (Kulkarni et al., 2008).

ESR1 was associated with male METH-induced patients in this study. It is known that there are sex differences in not only the response to METH but also in striatal dopamine release (Russell et al., 2008). It has also been shown that response to and use of METH differ with sex (Dluzen and Liu, 2008), and that estrogen plays an important role in protection from dopamine-induced toxicity caused by METH (Dluzen and McDermott, 2002; Gajjar et al., 2003; Liu and Dluzen, 2006). Other investigations have found an association of rs2234693 with risk of developing cognitive impairment in older women and major depressive disorder in females in the Chinese population (Yaffe et al., 2002). However, because our study included few female subjects, there is a possibility of type II errors in the results of our association analysis for these phenotypes. To overcome this limitation, a replication study using larger samples or samples from other populations will be required for conclusive results.

Because testing for HWE is commonly used for quality control in large-scale genotyping and is one of the few ways to identify systematic genotyping errors in unrelated individuals (Wittke-Thompson et al., 2005), we estimated HWE and confirmed the genotyping quality in this study. Genotype frequencies were in HWE for this SNP.

Other studies have reported associations of rs2234693 with endometrial cancer, breast cancer (van Duijnhoven et al., 2005), osteoporosis (Albagha et al., 2005; Albagha and Ralston, 2003; Ferrari and Rizzoli, 2005), stroke (Shearman et al., 2005), adiposity (Fox et al., 2005), atherosclerosis (Lehtimaki et al., 2002) and Alzheimer's disease (Mattila et al., 2000).

A few points of caution should be mentioned with respect to our results. First, the positive association we found may have been due to our small sample size. Ideal samples for this study are METH use disorder subjects with and without psychosis. Because only a few of our subjects had METH use disorder without psychosis, and we wanted to avoid statistical error, we did not perform association analysis in this group. Second, we did not include a mutation scan to detect rare variants. We designed the study based on the common disease–common variants hypothesis (CD–CV hypothesis) (Chakravarti, 1999). However, Weickert and colleagues have shown associations between schizophrenia, a common disease, and rare variants in *ESR1* (Weickert et al., 2008). To describe the genetic background of METH-induced psychosis by the common disease-rare variants hypothesis, further investigation will be required, such as medical resequencing using larger samples. However, statistical power is needed to evaluate the association of rare variants. To overcome these limitations, a replication study using larger samples or samples from other populations will be required for conclusive results.

5. Conclusion

Our results suggest that rs2234693 in *ESR1* may play a role in the pathophysiology of Japanese METH-induced psychosis.

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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,