P-values for each SNP marker, and the significance was determined at the 5% level using the chi-square test, as implemented in SPSS v13 (SPSS, Inc., Chicago, IL). All *P*-values were two-sided. Multimarker analysis was carried out by log-likelihood ratio tests for assessing haplotype-wise associations between schizophrenia and a combination of tagging SNPs with a permutation test for calculating empirical significance levels for differences between haplotype frequencies in case and control subsets.

Meta-Analysis

We performed a meta-analysis for rs138880, one of the two SNPs associated with schizophrenia in the previous study [Severinsen et al., 2006]. The other SNP, rs4468, was excluded because it was not polymorphic in our sample. Thus far, only one study has been published regarding an association analysis of the *BRD1* locus [Severinsen et al., 2006]. We used data from Severinsen's study and our study. First, the Q statistic test was performed to assess the possible heterogeneity in the combined studies. Second, a fixed effects model meta-analysis was conducted. The significance of the overall odds ratio (OR) was determined by the Z-test. The analysis was carried out on Comprehensive Meta-Analysis software (Version 2.2.046, Biostat, Englewood, NJ).

Lymphoblastoid Cell Lines (LCLs)

Peripheral blood was drawn into 7-ml plastic tubes containing sodium heparin, and lymphocytes were separated by a standard protocol. The cells were cultured in RPMI-1460 medium containing 20% fetal bovine serum, penicillin, and streptomycin, and filtered supernatant of a B95-8 cell culture infected with Epstein–Barr virus. Cyclosporine A was added until colonies were observed. After colony formation, the cells were passaged three times per week, without the addition of 10% fetal bovine serum and cyclosporine A. The cells were frozen in liquid nitrogen until needed, at which time they were thawed, passaged at least three times, and used within 4 weeks. We paid special attention while establishing and maintaining cell lines to exclude environmental confounders as much as possible.

Real-Time Quantitative Polymerase Chain Reaction (PCR) and Statistical Analysis

Total RNA of LCLs was extracted using RNeasy Plus Mini kit (50) (Qiagen, Valencia, CA). RNA yield and quality were assessed by measuring absorbance at 260 and 280 nm. Integrity and overall quality of the total RNA preparation were determined by native agarose gel electrophoresis (inspection of the 28S and 18S bands). Total RNA was used for cDNA synthesis by High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems Japan Ltd). Real-time quantitative PCR using TaqMan gene expression assays (Applied Biosystems Japan Ltd) was performed with ABI PRISM 7900HT (Applied Biosystems Japan Ltd). Amplification efficiency for each gene-specific primer pair was calculated based on the dilution series method [Livak and Schmittgen, 2001]. In each experiment, the r^2 value of the curve was more than 0.99. Measurement of the cycle threshold was performed in triplicate. The relative expression of *BRD1* was calculated by the modified $\Delta\Delta$ cycle threshold method as implemented in Relative Expression Software Tool 2008 (REST 2008) [Pfaffl et al., 2002]. The normalization factor was the geometric mean [Vandesompele et al., 2002] of the following genes: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*), beta-2-microglobulin (*B2M*), and ubiquitin C (*UBC*). These three genes were shown to have high expression stability in leukocytes [Vandesompele et al., 2002]. Bootstrapping techniques were used to provide 95% confidence intervals (CIs) for expression ratios without a normal or symmetrical distribution assumption.

RESULTS

Association Analysis

Regarding quality control, significant deviation from HWE was not observed. The genotypes of the duplicated samples showed complete concordance. Minor allele frequency for each tagging SNP in control samples generally showed a high concordance with that in HapMap database. Assuming a multiplicative model of inheritance and a disease prevalence of 1%, calculations showed that our sample had appropriate power (more than 80%) to detect gene-wide significant associations with genotype relative risk values from 1.24 to 1.55 (minor allele frequency values from 0.05 to 0.45). 3′ UTR SNP rs4468, which was associated with schizophrenia in a previous study, was not polymorphic in our Japanese sample, so we excluded rs4468 from subsequent analyses. Regarding the remaining 10 SNPs, no association was detected with schizophrenia in allele-/genotype-wise analyses or in the haplotype-wise analysis (two- to four-marker sliding window fashion; Table I). However, it should be noted that the rs138880 (associated SNP in previous article) showed the same trend in the Japanese population. In addition, haplotype showing the most significant association [Severinsen et al., 2006] was tested in the present study. We could not show a significant difference in the frequency of this haplotype between cases and controls (haplotype frequency in cases and controls: 0.0010 and 0.0010, respectively, $P = 0.99$).

Imputation of Ungenotyped SNPs

We used MACH to infer genotypes of 20 untyped SNPs. We provided genotypes for our own data (10 SNPs) as input together with haplotypes from the HapMap Japanese/Chinese population. The imputation method using MACH did not support an association between schizophrenia and the 20 SNPs in the *BRD1* locus (Table II).

Meta-Analysis

The SNP rs138880 that previously has been associated with schizophrenia showed the same trend in the Japanese population although it did not reach significance. The ORs for rs138880 reported in the Severinsen et al. [2006] and in this study were 1.73 and 1.14, respectively (Supplementary Table III). The pooled OR derived from the two studies (in total, 729 cases and 970 controls) was significant in the fixed model (pooled OR = 1.25, 95% CI = 1.03–1.52, $P = 0.02$; Supplementary Table III). Homogeneity analysis for the OR

TABLE I. Allele-Wise, Genotype-Wise, and Haplotype-Wise Analyses of 10 Tagging Single-Nucleotide Polymorphisms (SNPs)

	Allele frequency (proportion)				Single SNP		Haplotype wise		
	Case		Control						
dbSNP	M	m	M	m	Allele-wise	Genotype-wise	2-window[a]	3-window[a]	4-window[a]
rs138820	0.78	0.22	0.78	0.22	0.86	0.09			
							0.83		
rs4469	0.77	0.23	0.78	0.22	0.45	0.75		0.7	
							0.29		0.7
rs6009874	0.92	0.08	0.92	0.08	0.92	0.15		0.62	
							1.00		0.69
rs138840	0.92	0.08	0.92	0.08	0.82	0.92		1.00	
							1.00		0.47
rs138844	0.84	0.16	0.84	0.16	0.69	0.37		0.49	
							0.54		0.36
rs138850	0.59	0.41	0.59	0.41	0.82	0.25		0.46	
							0.52		0.44
rs138851	0.94	0.06	0.95	0.05	0.14	0.31		0.48	
							1.00		0.81
rs138863	0.94	0.06	0.95	0.05	0.15	0.38		0.71	
							0.68		1.00
rs2239848	0.85	0.15	0.86	0.14	0.79	0.72		1.00	
							1.00		
rs138880	0.86	0.14	0.87	0.13	0.22	0.09			

M, major allele; m, minor allele.
[a]Sliding window analysis, rare haplotype threshold 10%.

TABLE II. Allele-Wise Analysis of 20 Imputed Single-Nucleotide Polymorphisms

dbSNP	*P*-value	Quality[a]
rs138816	0.97	0.92
rs138821	0.91	0.96
rs2269626	0.72	0.95
rs138823	0.91	0.95
rs916418	0.96	0.99
rs916419	0.85	0.99
rs138827	0.82	0.99
rs138830	0.81	0.99
rs138834	0.87	0.99
rs138841	0.37	0.99
rs138843	0.86	1.00
rs138845	0.34	0.99
rs6009878	1.00	0.99
rs138853	0.14	1.00
rs138861	0.41	1.00
rs138866	0.27	0.99
rs138867	0.27	1.00
rs138870	0.27	0.99
rs138871	0.23	0.99
rs138884	0.23	1.00

[a]Quality is the average posterior probability for the most likely genotype.

revealed no significant evidence for heterogeneity of the OR ($Q = 2.98$, $df = 1$, $P = 0.084$).

Expression Analysis

The expression of *BRD1* mRNA was analyzed using LCLs from 29 cases and 30 controls. Cycle threshold values of *BRD1* and three internal controls (*B2M*, *UBC*, and *YWHAZ*) are shown in Supplementary Table IV. We could not detect any significant differences in *BRD1* mRNA levels between cases and controls ($P = 0.46$; Fig. 1).

DISCUSSION

The common disease–common variant hypothesis states that diseases that were evolutionarily neutral (i.e., had little or no effect on reproductive fitness), such as late-onset schizophrenia, during human history may be significantly influenced by common variants [Lander, 1996]. Therefore, if allelic variants at a disease susceptibility locus are responsible for the predisposition to a common complex disease, then allele-, genotype-, or haplotype-wise association tests will detect such variants (or tagging SNPs that are in linkage disequilibrium with the deleterious allele).

The first and only indication that the *BRD1*-related region harbors a variation that might influence susceptibility to schizophrenia was provided by Severinsen et al. [2006], who identified two fairly strong association signals between two SNPs (rs4468 and rs138880) and schizophrenia using a case–control sample from Scotland. The sample in this study consisted of 103 patients with

(i)

Gene	Type	95% C.I.	P(H1)
BRD1	Target	0.56 - 2.08	0.46

(ii)

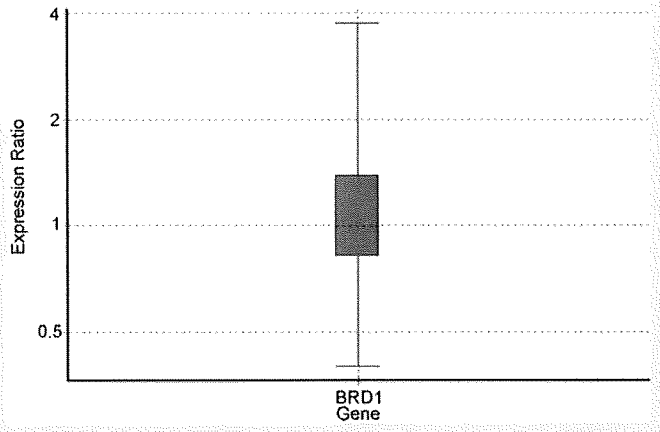

FIG. 1. Relative expression of bromodomain-containing 1 (BRD1) normalized to the geometric mean of three internal controls. i: The relative expression of BRD1 was normalized to the geometric mean of three internal controls (B2M, UBC, and YWHAZ). Bootstrapping techniques were used to provide 95% confidence intervals for expression ratios without normal or symmetric distribution assumption. The number of iterations was 10,000 in this analysis. *P* (H1) means the probability of the alternate hypothesis that the difference between sample and control groups is due only to chance. ii: Boxplot. Expression ratio is the relative expression of BRD1 in cases compared with controls (expression in control is equivalent to 1). Box represents the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

schizophrenia and 200 controls. Our study did not strongly support an association between schizophrenia and the *BRD1* locus although the only previously associated SNP included in our study (rs138880) showed the same trend, and the meta-analysis of this SNP using a fixed effects model was significant.

Psychiatric disorders are complex diseases that are characterized by the contribution of multiple susceptibility genes and environmental factors. Therefore, *BRD1* might be a population-specific factor for schizophrenia. However, this conclusion should be made only with the following considerations. First, it is possible that our study was still underpowered to reliably detect common low-risk variants. This may be related to etiological heterogeneity or inaccurate diagnoses in schizophrenia, which would attenuate the genetic relative risk. Second, only the hypothesis of an association with common SNPs of *BRD1* has been tested, both here and in the previous study; therefore, future studies using resequencing methods to detect rare variants in the *BRD1* locus will be needed for a complete understanding of relationship between this genetic locus and schizophrenia. Third, even though the Japanese population is relatively homogeneous [Haga et al., 2002], small population stratifications may have affected our findings. A recent analysis with the use of approximately 140,000 SNPs in 7003 Japanese individuals has shown that local regions within the main island of Japan are genetically differentiated in spite of frequent human migration within Japan in modern times [Yamaguchi-Kabata et al., 2008]. However, we believe that the impact of population stratification on our study is negligible, as our samples were collected in a relatively narrow region in the middle of the main island of Japan. Fourth, regarding the Japanese and the Caucasian populations, comparative linkage disequilibrium analysis of the HapMap data showed a different block structure around the *BRD1* locus [Gabriel et al., 2002]. Compared with the Caucasian population, linkage disequilibrium (LD) blocks in the Japanese population are shorter, and the block structure is coarser, having lower r^2 values. This might influence interpopulation transferability of tagging SNPs in the *BRD1* locus and result in a failure to detect an association with schizophrenia in the Japanese population. Interestingly, selective sweep analysis has provided evidence of recent positive selection on genes associated with schizophrenia, and *BRD1* gene was reported to have been affected by positive selection in Caucasian but not in Asian population [Crespi et al., 2007]. This indicates that the positive selection specific to the Caucasian population might produce the difference in LD structure in *BRD1* locus.

We could not detect significant differences in *BRD1* mRNA levels between cases and controls in the expression analysis. These results are consistent with the findings in the association study. However, there were several limitations in the expression assays. Using non-neuronal samples such as LCLs is based on the assumption that heritable mechanisms associated with the risk of schizophrenia have systemic effects and result in changes to gene expression in various tissues. To validate the use of gene expression data in a more accessible tissue as a surrogate for gene expression in the central nervous system, Sullivan et al. [2006] evaluated the comparability of transcriptional profiling of a variety of human tissues with Affymetrix U133A microarray augmented with a custom microarray. Their analyses suggested that careful use of peripheral gene expression may be a useful surrogate for gene expression in the central nervous system.

In conclusion, we could not strongly show that common SNPs in the *BRD1* gene account for a substantial proportion of the genetic risk for schizophrenia in the Japanese population, although small effects of population stratification or differences in LD structure could not be ruled out. Considering the significance in the meta-analysis for the only previously associated SNP included in our study, further investigations are needed for conclusive results.

ACKNOWLEDGMENTS

We sincerely thank the patients and healthy volunteers for their participation in this study. We also thank Dr. R. Ishihara for the technical assistance. This work was supported in part by research grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Ministry of Health of Japan, Labor and Welfare, Grant-in-Aid for Scientific Research on Pathomechanisms of Brain Disorders from the Ministry of Education, Culture, Sports, Science and Technology of Japan, MEXT ACADEMIC

FRONTEIER, the Japan Health Sciences Foundation (Research on Health Sciences focusing on Drug Innovation), and the Core Research for Evolutional Science and Technology. These groups had no role in study design, in data collection, analysis and interpretation, in the writing, or in the decision to submit the article for publication. Authors Itaru Kushima, Branko Aleksic, and Masashi Ikeda designed the study, wrote the protocol, and drafted the manuscript. Authors Norio Ozaki and Nakao Iwata 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.

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Neuropsychopharmacology (2010) 35, 1155–1164

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Association of the *HSPG2* Gene with Neuroleptic-Induced Tardive Dyskinesia

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Tardive dyskinesia (TD) is characterized by repetitive, involuntary, and purposeless movements that develop in patients treated with long-term dopaminergic antagonists, usually antipsychotics. By a genome-wide association screening of TD in 50 Japanese schizophrenia patients with treatment-resistant TD and 50 Japanese schizophrenia patients without TD (non-TD group) and subsequent confirmation in independent samples of 36 treatment-resistant TD and 136 non-TD subjects, we identified association of a single nucleotide polymorphism, rs2445142, (allelic $p = 2 \times 10^{-5}$) in the *HSPG2* (heparan sulfate proteoglycan 2, perlecan) gene with TD. The risk allele was significantly associated with higher expression of *HSPG2* in postmortem human prefrontal brain ($p < 0.01$). Administration of daily injection of haloperidol (HDL) for 50 weeks significantly reduced *Hspg2* expression in mouse brains ($p < 0.001$). Vacuous chewing movements (VCMs) induced by 7-week injection of haloperidol–reserpine were significantly infrequent in adult *Hspg2* hetero-knockout mice compared with wild-type littermates ($p < 0.001$). Treatment by the acetylcholinesterase inhibitor, physostigmine, was significantly effective for reduction of VCMs in wild-type mice but not in *Hspg2* hetero-knockout mice. These findings suggest that the *HSPG2* gene is involved in neuroleptic-induced TD and higher expression of *HSPG2*, probably even after antipsychotic treatment, and may be associated with TD susceptibility.
Neuropsychopharmacology (2010) **35,** 1155–1164; doi:10.1038/npp.2009.220; published online 13 January 2010

Keywords: acetylcholine; neurogenetics; schizophrenia/antipsychotics; pharmacogenetics/pharmacogenomics; tardive dyskinesia

INTRODUCTION

Antipsychotic-induced tardive dyskinesia (TD) is an involuntary movement disorder that develops in patients who are undergoing long-term treatment with antipsychotic medications. The clinical symptoms most commonly involve orobuccal, lingual, and facial muscles, especially in older individuals. The lingual involvement in the form of fine vermicular movements of the tongue while it is sitting at the base of the oral cavity is a common early feature (Sachdev, 2000). In more severe cases, the movements may involve trunk and limbs (Tarsy and Baldessarini, 2006). Such movements lower the quality of life (QOL) of patients (Gerlach, 2002). Therefore, predicting those patients who are vulnerable to TD remains a high priority for psychiatrists in selecting the best medication for a given individual. Introduction of second-generation atypical antipsychotics has reduced the occurrence of TD to approximately 1% annually compared with the 5% frequency with typical agents (de Leon, 2007; Remington, 2007). Owing to the lack of effective treatments for TD, however, therapeutic management of TD can be problematic for schizophrenia patients receiving antipsychotic medications, especially for those patients who develop severe treatment-resistant TD. Therefore, the strategies to prevent TD are often discussed

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Received 20 May 2009; revised 6 November 2009; accepted 23 November 2009

in the context of safety and use of antipsychotic drugs (Inada *et al*, 2008).

The etiology of TD is complex and remains unclear. Age, gender, and ethnicity are all the suggested risk factors for TD. Smoking, drinking, and using street drugs may also increase the risk of TD (Menza *et al*, 1991). There is some evidence for a genetic component to TD (Muller *et al*, 2004) and molecular genetic studies of TD were conducted to identify genes related to TD (Malhotra *et al*, 2004).

The pathophysiology of TD is not completely understood. The causative role of antipsychotic and other dopamine antagonists resulted in the proposal of the dopamine supersensitivity hypothesis of TD (Klawans *et al*, 1980). However, as the hypothesis explains only some aspects of TD, many other pathophysiological models including changes in other neurotransmitter signaling systems that are affected by neuroleptics have been considered. They include gamma-aminobutyric acid (Gerlach and Casey, 1988), norepinephrine (Saito *et al*, 1986), serotonin (Haleem, 2006), and acetylcholine (Ach) (Tammenmaa *et al*, 2002).

The advent of single nucleotide polymorphism (SNP) chips for genome-wide association analysis has made screening of susceptibility genes for TD possible. We carried out a genome-wide association study of treatment-resistant TD in schizophrenia patients and reported that SNPs associated with TD were aggregated significantly in genes belonging to the gamma-aminobutyric acid receptor signaling pathway (Inada *et al*, 2008). In this study, we analyzed the *HSPG2* gene, which includes SNPs that showed the most significant association with TD in our genome-wide association study.

MATERIAL AND METHODS

Ethical Considerations

This study was initiated after approval by the ethics committee of each institution. Written informed consent was obtained from all patients after adequate explanation of the study.

Human Subjects

Human subjects in this study were 86 Japanese schizophrenia patients with TD and 186 Japanese schizophrenia patients without TD, who have been described elsewhere (Inada *et al*, 2008). Briefly, subjects were identified at psychiatric hospitals located around Tokyo and Nagoya areas of Japan. All patients satisfied the diagnostic criteria of DSM-IV (Association, 1994) for schizophrenia. All subjects and their parents were of Japanese descent. All subjects had been receiving antipsychotic therapy for at least 1 year and their TD status was monitored for at least 1 year. TD was assessed according to the Japanese version of the Abnormal Involuntary Movement Scale. TD was diagnosed according to the criteria proposed by Schooler and Kane (Schooler and Kane, 1982). Once TD was identified, the patients were followed up and received standard therapeutic regimens for TD to minimize TD symptoms. If TD persisted even after 1 year of therapy, patients were considered potential treatment-resistant TD patients. Treatment-resistant TD patients were defined as those patients with dyskinetic movements that persisted for more than 1 year and did not improve even after 1 year of appropriate treatment after guideline-recommended therapeutic regimens for TD. We hypothesized that treatment-resistant TD, a severe form of TD, was suitable for detection of genetic association with TD. Only treatment-resistant TD patients were included as those affected with TD in this study.

Genotyping, Resequencing, and Statistics

Association screening was performed using the Illumina Sentrix Human-1 Genotyping 109k BeadChip according to the manufacturer's instructions (Illumina, San Diego CA, USA). All DNA samples were subjected to rigorous quality control to check for fragmentation and amplification. Approximately 750 ng of genomic DNA was used in each sample. Normalized bead intensity data obtained for each sample was entered into the Illumina BeadStudio 3.0 software, which converted fluorescence intensities into SNP genotypes. A GenCall Score of 0.85 was used as a minimum threshold for per-sample genotyping completeness. The mean call rate across all samples was 97%. After removing SNPs with a low genotyping rate ($p<0.95$: $n=3952$), SNPs deviating from the Hardy–Weinberg equilibrium ($p<0.001$: $n=135$), SNPs with low minor allele frequency (MAF<0.05: $n=2762$), and SNPs located outside exons and introns, we screened for SNPs associated with TD using 40 573 SNPs. SNPs located within 10 kb from the 5′ and 3′ ends of known genes were included. SNPs in the linkage disequilibrium (LD) of $r^2>0.8$ with other SNPs were excluded. The call rate was at least 99.4% for the 40 573 SNPs. The concordance rate was evaluated by comparisons of genotypes in the 100 screening samples and this gave concordance of over 98% for each sample. Genotyping using TaqMan probes (Applied Biosystems, Foster City, CA, USA) was carried out twice for each SNP, and genotype concordance was 99.7%. Genotyping completeness was >0.99. We treated these uncalled or discrepant genotypes as missing genotypes.

To screen for novel polymorphisms, we used direct sequencing with a Big Dye Terminator Cycle Sequencing kit and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). All exons and the exon–intron junctions of the *HSPG2* gene were amplified from the genomic DNAs of the 86 TD group patients. The sequences of primers for mutation screening are available on request.

For a more detailed analysis of the associations between SNPs in the *HSPG2* gene and TD, the tag SNPs in the gene were selected using the Haploview program (http://www.broad.mit.edu/mpg/haploview/) with the condition of an r^2 threshold of 0.8 and a minor allele frequency of 0.1, and genotyped by the TaqMan method. Allelic discrimination was performed using the ABI PRISM 7900HT Sequence Detection System using SDS 2.0 software (Applied Biosystems, Foster City, CA, USA).

Allelic associations between SNPs and TD, and departure from the Hardy–Weinberg equilibrium were evaluated by χ^2 test or Fisher's exact test. Bonferroni's correction for multiple comparisons was applied.

Human Postmortem Brains

Brain specimens were from individuals of European (Australian) and Japanese descent. The Australian sample comprised 10 schizophrenic patients and 10 age- and gender-matched controls. The diagnosis of schizophrenia was made according to the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV criteria (American Psychiatric; Association (1994)) by a psychiatrist and a senior psychologist. Control subjects had no known history of psychiatric illness. Tissue blocks were cut from gray matter in an area of the prefrontal cortex referred to as Brodmann's area 9 (BA9). Japanese samples of BA9 gray matter from Japanese brain specimens consisted of six schizophrenic patients and 11 age- and gender-matched controls. In addition, postmortem brains of 37 deceased Japanese patients with schizophrenia were also analyzed. The Japanese subjects met the DSM-III-R criteria for schizophrenia. Details of the condition of the postmortem brains have been described elsewhere (Ishiguro *et al*, 2008; Koga *et al*, 2009).

Analysis of *Hspg2* Transcription in Human Brain Tissue

Total RNA was extracted from human brain tissues with ISOGEN Reagent (Nippon Gene, Tokyo, Japan). The RNA quality was checked using a Nanodrop ND-1000 spectrophotometer (LMS, Tokyo, Japan) to have an OD 260/280 ratio of 1.8–2 and an OD 260/230 of 1.8 or greater. Expression of the *HSPG2* genes was analyzed by the TaqMan real-time polymerase chain reaction system (Applied Biosystems, Foster City, CA). From RNA, cDNA was synthesized with Revertra Ace (Toyobo, Tokyo, Japan) and oligo dT primers. Expression of the *HSPG2* gene was analyzed with an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems), with the TaqMan gene expression assays for *HSPG2* (Hs01078535_m1), and normalized to the expression of Human GAPDH Control Reagents (Applied Biosystems).

Genotype effects on *HSPG2* expression were analyzed in Australian subjects and replicated in Japanese subjects using analysis of variance followed by Tukey's *post hoc* tests by JMP software version 7.0.1 (SAS Institute, Cary, NC, USA) was used.

Animals

Animals were same-sex housed before behavior testing. The same animals were used for all behavior tests.

Four-week-old C57BL/6J male mice (weight: 20–25 g) treated with haloperidol (HDL) or vehicle-saline and 7-week-old male mice (wild type: 8; *Hspg2*$^{+/-}$: 7) with orofacial dyskinesia were housed under 10 h : 14 h light/dark conditions with normal food and water *ad libitum*, with mice housed separately in groups of 4 or 5 mice.

The generation of *Hspg2* knockout mice and the phenotypes of the mice have been described elsewhere (Arikawa-Hirasawa *et al*, 1999). As *Hspg2* null mice are embryonic lethal, timed matings between heterozygotes were carried out to generate homozygous and wild-type mice in this study.

All animal protocols were approved by the Animal Care and Use committee of University of Tsukuba.

Drugs

Reserpine (methyl reserpate 3,4,5-trimethoxycinnamic acid ester; Wako, Osaka, Japan) and HDL (Wako, Osaka, Japan) were diluted in glacial acetic acid and then diluted in distilled water. Physostigmine (Wako), a reversible cholinesterase inhibitor, was diluted in saline. All solutions were treated subcutaneously in volumes not exceeding 10 ml/kg body weight.

HDL Treatment

To examine the effects of antipsychotic treatments on gene expression, we made two groups: an acute treatment group: 4-week-old C57BL/6J male mice were treated with intraperitoneal injection (i.p.) of 1.0 mg/kg HDL ($n = 10$) or vehicle-saline ($n = 10$) once each day for 4 weeks; and a long-term treatment group: 4-week-old C57BL/6J male mice were treated with intraperitoneal injection of 1.0 mg/kg HDL ($n = 10$) or vehicle-saline ($n = 10$) once each day for 50 weeks. Mice were killed 4 h after the last injection to obtain brain tissues.

Induction of Vacuous Chewing Movements

Mice were treated with i.p. of 2 mg/kg HDL and 0.3 mg/kg reserpine every day for 7 weeks to induce the putative TD analogue vacuous chewing movements (VCMs) (Araujo *et al*, 2004; Burger *et al*, 2005; Naidu *et al*, 2003). Before injection and 4 hours after the injection on the 47th day, locomotor activity test and rotarod test were carried out. On the 48th and 49th days, 1, 2, 3, 4, and 24 h after the last injection, the animals were observed for quantification of VCMs for 2 days. On the 50th day, to verify the effects of physostigmine on VCMs, mice were injected with 0.1 mg/kg physostigmine. At 1, 2, 3, 4, and 24 h after the injection of physostigmine, the animals were observed for quantification of VCMs. On the 51st and 52nd day, mice were treated with 2 mg/kg HDL and 0.3 mg/kg reserpine and then observed for quantification of VCMs. On the 53rd day, mice were treated with vehicle-saline, and 1, 2, 3, 4, and 24 h after the injection, the animals were observed for quantification of VCMs.

Analysis of *Hspg2* Transcription in Brain Tissue of Mice

The prefrontal cortex, midbrain, hippocampus, thalamus, and striatum were taken by dissection, and total RNA was extracted with an RNeasy kit (Qiagen, K.K., Tokyo, Japan). After cDNA synthesis from total RNA samples, the transcription level of cDNA samples was analyzed by a TaqMan Expression assay for *Hspg2* (Mm00464581_m1; Applied Biosystems) and normalized to that of rodent *Gapdh* with Rodent *Gapdh* Control Reagents (Applied Biosystems). The average relative expression levels of five regions were compared with the saline groups by Student's *t*-test.

Table 1 Allelic *p*-Values of SNPs for Association with TD in Screening and Replication Samples

SNP	Chromosome	Location	Gene	Position relative to gene	Allele frequency			Uncorrected allelic *p*	
					TD group	Non-TD group	Screening	Replication	Combined
rs7529452	chr1	1p36.22	*PLOD1*	coding	0.396	0.381	**0.001**	**0.05**	
rs2445142	chr1	1p36.12	*HSPG2*	intron	0.579	0.380	**0.001**	**0.002**	**0.00002**
rs1934712	chr1	1p21.1	*COL11A1*	flanking_3UTR	0.435	0.343	**0.0007**	0.98	
rs2306444	chr1	1p12	*MAN1A2*	intron	0.482	0.392	**0.0005**	0.59	
rs869807	chr1	1p12	*TBX15*	flanking_3UTR	0.282	0.185	**0.0009**	0.97	
rs6668395	chr1	1q41	*DUSP10*	flanking_5UTR	0.418	0.535	**0.001**	0.59	
rs6426327	chr1	1q44	*SMYD3*	intron	0.412	0.324	**0.00002**	0.20	
rs4558632	chr2	2p21	*EML4*	flanking_5UTR	0.253	0.171	**0.0003**	0.82	
rs6714424	chr2	2p16.2	*ASB3*	flanking_5UTR	0.212	0.120	**0.0007**	0.76	
rs2060279	chr2	2p12	*LRRTM4*	flanking_5UTR	0.685	0.777	**0.0005**	0.52	
rs11694702	chr2	2q13	*BUB1*	flanking_5UTR	0.329	0.241	**0.001**	0.76	
rs1873201	chr2	2q24.3	*KCNH7*	flanking_5UTR	0.395	0.301	**0.002**	0.78	
rs11688866	chr2	2q31.3	*UBE2E3*	flanking_5UTR	0.452	0.581	**0.0005**	0.45	
rs3749279	chr3	3p22.3	*STAC*	intron	0.202	0.099	**0.001**	0.59	
rs6443468	chr3	3q26.32	*TBL1XR1*	flanking_5UTR	0.373	0.511	**0.001**	0.26	
rs13115988	chr4	4q22.1	*LOC285513*	3UTR	0.694	0.645	**0.001**	0.32	
rs700237	chr5	5p13.1	*C9*	flanking_5UTR	0.898	0.830	**0.0009**	0.80	
rs832582	chr5	5q11.2	*MGC33648*	flanking_5UTR	0.641	0.543	**0.001**	0.37	0.03
rs13153252	chr5	5q14.3	*EDIL3*	intron	0.375	0.486	**0.0002**	0.94	
rs6594324	chr5	5q21.3	*FER*	flanking_5UTR	0.692	0.578	**0.001**	0.37	
rs915125	chr6	6q14.1	*FAM46A*	flanking_5UTR	0.207	0.262	**0.0009**	0.41	
rs2691180	chr6	6q21	*CDC2L6*	intron	0.789	0.890	**0.0007**	0.41	
rs9376506	chr6	6q24.1	*CITED2*	flanking_5UTR	0.564	0.457	**0.001**	0.71	
rs1832445	chr6	6q24.1	*FLJ39824*	flanking_3UTR	0.494	0.604	**0.001**	0.94	
rs3735478	chr7	7p13	*DKFZp761I2123*	coding	0.058	0.145	**0.001**	0.32	0.006
rs1047053	chr7	7q36.2	*DPP6*	3UTR	0.657	0.758	**0.0005**	0.3	0.02
rs2583086	chr8	8q13.2	*SULF1*	intron	0.169	0.255	**0.001**	0.95	
rs4738269	chr8	8q13.3	*KCNB2*	intron	0.571	0.396	**0.0007**	**0.04**	**0.0002**
rs2927111	chr8	8q23.1	*STARS*	flanking_5UTR	0.369	0.487	**0.0004**	0.4	0.01
rs3019982	chr8	8q23.1	*ANGPT1*	flanking_3UTR	0.612	0.479	**0.0002**	0.18	0.004
rs4242345	chr8	8q24.13	*ANXA13*	flanking_3UTR	0.659	0.764	**0.00004**	0.56	
rs1413299	chr9	9q22.33	*COL15A1*	intron	0.682	0.746	**0.001**	0.73	
rs2274359	chr10	10p15.3	*RBM17*	intron	0.929	0.834	**0.0005**	0.51	
rs1932596	chr10	10q21.1	*PCDH15*	intron	0.628	0.543	**0.0007**	0.51	
rs1058198	chr10	10q22.3	*DLG5*	coding	0.152	0.290	**0.0006**	0.22	0.0007
rs10748816	chr10	10q24.32	*ELOVL3*	intron	0.494	0.634	**0.00008**	0.13	0.002
rs2246775	chr10	10q24.32	*GBF1*	intron	0.646	0.747	**0.0009**	0.66	
rs765934	chr10	10q26.3	*MGMT*	flanking_5UTR	0.732	0.642	**0.0012**	0.74	
rs886292	chr11	11p15.1	*ABCC8*	intron	0.825	0.696	**0.0005**	**0.02**	0.0015
rs286925	chr11	11p13	*EHF*	5UTR	0.542	0.611	**0.0005**	0.75	
rs568758	chr11	11q13.4	*SPCS2*	intron	0.738	0.818	**0.0009**	0.82	
rs624786	chr11	11q13.4	*NEU3*	flanking_5UTR	0.735	0.812	**0.0015**	0.83	
rs1444590	chr12	12q13.11	*SLC38A1*	intron	0.789	0.839	**0.0005**	0.39	
rs1154664	chr12	12q24.32	*KIAA1906*	flanking_3UTR	0.688	0.590	**0.0002**	0.80	
rs1924174	chr13	13q33.3	*LIG4*	flanking_3UTR	0.282	0.195	**0.0013**	0.19	0.04
rs1189827	chr14	14q22.3	*SEC10L1*	flanking_3UTR	0.741	0.663	**0.0007**	0.45	
rs11625123	chr14	14q32.12	*ITPK1*	intron	0.124	0.225	**0.0009**	0.45	0.007
rs10140345	chr14	14q32.2	*VRK1*	flanking_3UTR	0.300	0.273	**0.0011**	0.09	

Table 1 Continued

SNP	Chromosome	Location	Gene	Position relative to gene	Allele frequency			Uncorrected allelic *p*	
					TD group	Non-TD group	Screening	Replication	Combined
rs2061051	chr15	15q12	*GABRG3*	intron	0.206	0.350	**0.0014**	**0.04**	**0.0006**
rs3764211	chr15	15q13.1	*APBA2*	flanking_3UTR	0.726	0.815	**0.0013**	0.12	0.005
rs1036673	chr15	15q24.1	*PML*	3UTR	0.721	0.592	**0.0006**	0.64	
rs3809729	chr17	17p12	*DNAH9*	flanking_5UTR	0.867	0.869	**0.0007**	0.11	
rs4630608	chr17	17p11.2	*FBXW10*	intron	0.250	0.274	**0.0010**	**0.04**	
rs2287352	chr17	17q12	*ACACA*	flanking_5UTR	0.247	0.306	**0.0014**	0.85	
rs3744165	chr17	17q25.3	*FLJ13841*	5UTR	0.093	0.130	**0.0010**	0.15	
rs474122	chr18	18p11.31	*DLGAP1*	flanking_5UTR	0.404	0.330	**0.0002**	0.38	
rs12460403	chr19	19p13.3	*HMG20B*	flanking_3UTR	0.285	0.194	**0.0011**	0.55	
rs437168	chr19	19q13.12	*NPHS1*	coding	0.223	0.139	**0.0007**	0.9	
rs10419669	chr19	19q13.31	*CBLC*	intron	0.094	0.179	**0.0003**	0.84	
rs8112223	chr19	19q13.41	*HAS1*	flanking_5UTR	0.314	0.219	**0.0003**	0.43	
rs2328500	chr20	20p11.23	*C20orf26*	intron	0.376	0.324	**0.001**	0.12	
rs7281019	chr21	21q22.11	*TCP10L*	intron	0.924	0.862	**0.00008**	0.57	0.04
rs2056965	chr22	22q12.3	*LOC91464*	flanking_5UTR	0.422	0.348	**0.0002**	0.49	

Abbreviations: SNP, single nucleotide polymorphism; TD, tardive dyskinesia.
p-Values with bold emphasis indicate $p<0.05$ in 1st *p* and 2nd *p*, and combined $p<$ 1st *p*.

Evaluation of VCMs

Mice were placed individually in observation cages ($16 \times 17 \times 19\,cm^3$) without food. Hand-operated counters were used to quantify VCMs continuously for 5 min. VCMs were referred to as single mouth openings in the vertical plane not directed toward physical material. If VCMs occurred during a period of grooming, they were not taken into account. Mirrors were placed under the floor and behind the back wall of the cage to permit observation of oral movements when the animal faced away from the observer. The observations were made by two observers who were blind to the animal's group assignment. The observation criteria were not subjective, because an excellent inter-observer agreement was found in a previous pilot experiment (Pearson's correlation = 0.98). All behavioral experiments were conducted between 1000 and 1800hours.

Locomotor Activity

The locomotor activity test was conducted between 1200 and 1700 hours in a dimly lit testing room. Mice were habituated to the room for at least 30 min before testing. The locomotor activity test was videotaped with a Sony Digital Video Camera (Sony, Tokyo, Japan). The behavioral testing apparatus was a black Plexiglas rectangular box (41 cm long × 22 cm wide × 20.5 cm tall) and activity was recorded for 20 min. The total distance traveled (locomotion) was scored.

Rotarod Test

The rotarod test was conducted between 1200 and 1700 hours in a dimly lit testing room. All mice were brought to the testing room in their home cages and were allowed to sit undisturbed in the testing room for at least 5 min before the start of behavioral testing. Motor performance was assessed by rotarod (Med Associates, St Albans, VT). A 1-min training session was given to each mouse on the rotarod (diameter 8 cm, 7 rpm) 5 min before the first measurement. Motor performance (time until the first fall) was registered during a 2-min session.

Statistical Analysis for Behavioral Data and Gene Expression in Animal Experiments

Effects of genotype, drug treatment, and time were analyzed using analysis of variance) followed by Tukey's *post hoc* tests or using Student's *t*-test. Individual differences of the number of VCMs between before and after injection of physostigmine and saline were tested by nonparametric test for one sample test of mean = 0.

RESULTS

Association Study

We screened for SNPs associated with TD using 40 573 tag SNPs on the Sentrix® Human-1 Genotyping BeadChip (Illumina) to identify loci associated with susceptibility to TD in 50 TD and 50 non-TD subjects (Inada *et al*, 2008). The potential impact of population structure on this association study was evaluated by using the genome-wide χ^2 inflation factor, λ, as a genomic control (Devlin and Roeder, 1999; Devlin *et al*, 2001). The estimated value of λ was 1.04, by which genome-wide association *p*-values were corrected. The lowest uncorrected allelic *p*-value for association with TD was 1×10^{-5}. Therefore, no SNP was

significantly associated with TD after Bonferroni correction. An attempt was made to replicate the association of 63 SNPs, which were allelic p-values <0.002 and located within 10 kb from known genes with the TaqMan genotyping assay (Table 1). A potential association was found for four SNPs (allelic $p<0.05$) (Table 1). However, no significant association was found after correction for multiple testing of 63 SNPs in the replication sample only. Among these four SNPs, an association between *GABRG3* SNP and TD has already been reported (Inada *et al*, 2008). The lowest allelic p-value for the association was found for rs2445142 ($p=2\times10^{-5}$) when the initial genome-wide sample and replication sample were combined. The SNP is located in the *HSPG2* gene.

Next, we tested associations of 24 tag SNPs including rs2445142 in the *HSPG2* gene with TD and found a nominal significant association for five SNPs in addition to rs2445142 (Table 2). Other than rs2445142, we found a significant association of rs2124368 located in intron 43 of the *HSPG2* gene with TD even after applying Bonferroni's correction for multiple testing (uncorrected allelic $p=0.0003$, corrected $p=0.007$). The SNP rs2124368 was not in LD with rs2445142, which was located in intron 1 ($D'=0.13$, $r^2=0.01$).

Subsequently, we genotyped the SNPs of rs2501255 (intron 1), rs2501257 (intron 1), rs897474 (intron 3), rs2254357 (exon 6), rs2254358 (exon 6), and rs2497632 (intron 9), because of the expected complete LD between these SNPs and rs2445142 based on the HapMap database. We confirmed that they were in complete LD with rs2445142 ($r^2=1.00$). These SNPs were located in introns 1–9 of the *HSPG2* gene. Age, sex, and age of onset were not associated with these SNPs. Acute extrapyramidal symptoms were associated with rs2445142 and the six SNPs in complete LD with rs2445142 (allelic $p=0.00002$). Resequencing all exons of the *HSPG2* gene in patients with TD did not reveal novel SNPs. Finally, we genotyped missense SNPs of rs3736360, rs2229493, s2291827, rs2228349, rs2229491, rs2229490, rs2229489, rs2229475, rs897471, rs2229481, and rs989994, which were listed in dbSNP and did not find significant associations of these SNPs with TD (data not shown). Thus, we tested a total of 103 SNPs, including 41 SNPs in the *HSPG2* gene, in our total subjects of 86 TD and 136 non-TD patients.

Association Between *Hspg2* Expression Levels in the Postmortem Prefrontal Cortex and Rs2445142

The transcription level in the postmortem prefrontal cortex, as measured by TaqMan real-time polymerase chain reaction, was not significantly different by diagnosis, age, sex, postmortem intervals, or pH of brain samples. A significant genotype effect on *HSPG2* gene expression levels was observed in 20 Australian subjects ($F(2,17)=4.9$, $p=0.02$) and replicated in 54 Japanese subjects ($F(2,51)=3.5$, $p=0.04$). The association was significant in the combined subjects ($F(2,71)=7.6$, $p=0.001$). Tukey's *post hoc* tests showed that *HSPG2* expression levels were significantly higher in the subjects with the GG genotype than in those with the CC genotype (Figure 1). Unfortunately, information about TD in the brains we analyzed was not available.

Table 2 Allelic p-Values of Tag SNPs in the *HSPG2* Gene for Association with TD

	Location	Allele frequency*			
		Allele	TD group	Non-TD group	Allelic p
rs3736360	exon 96 (N4331S)	A/G	0.19	0.20	0.8715
rs3767137	intron 77	A/G	0.23	0.19	0.2759
rs10917053	intron 71	A/G	1.00	0.99	0.3308
rs7355045	intron 64	G/A	0.84	0.81	0.4235
rs2290501	intron 60	C/A	0.22	0.21	0.7134
rs1563370	intron 52	A/G	0.35	0.27	0.0687
rs2229475	exon 47 (I1967V)	G/A	0.01	0.01	0.9477
rs2305562	intron 43	A/G	0.61	0.49	0.0117
rs4654991	intron 42	G/A	0.39	0.36	0.5605
rs2124368	intron 42	G/A	0.77	0.60	0.0003
rs897472	intron 36	C/A	0.09	0.05	0.1098
rs897471	exon 36 (V1503A)	A/G	0.88	0.87	0.7005
rs2229478	exon 8 (L248L)	A/G	0.53	0.42	0.0273
rs3767141	intron 6	G/A	0.66	0.60	0.1811
rs2445142	intron 1	G/C	0.58	0.38	0.00002
rs878949	intron 1	A/G	0.22	0.20	0.5867
rs1545593	intron 1	C/A	0.41	0.30	0.0122
rs1002480	intron 1	G/C	0.41	0.32	0.0368
rs6698486	intron 1	G/A	0.46	0.38	0.0754
rs10799719	intron 1	G/A	0.80	0.75	0.1789
rs9426785	intron 1	A/G	0.57	0.55	0.7389
rs4654773	intron 1	A/G	0.45	0.45	0.9165
rs11587857	intron 1	G/A	0.50	0.46	0.3465
rs4233280	5′ flanking	A/G	0.07	0.03	0.0588

Abbreviations: SNP, single nucleotide polymorphism; TD, tardive dyskinesia
*The frequency of the first allele.

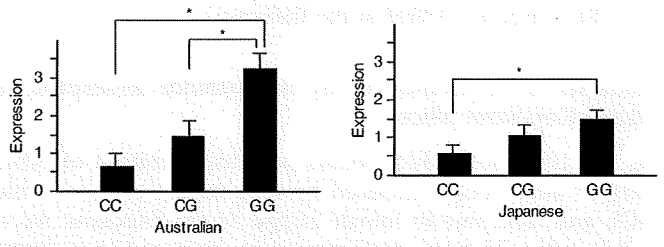

Figure 1 *HSPG2* expression levels in the postmortem prefrontal region by genotype. The vertical scores show the average (SEM) of relative expression levels in each of the three genotype groups, compared with the mean gene expression in the total samples. *Indicates $p<0.05$ by Tukey's *post hoc* tests.

Hspg2 Gene Expression in the Mouse Brains by HDL Treatment

Hspg2 expression levels were evaluated in the mouse brain after treatment with the antipsychotic drug, HDL. The expression of *Hspg2* levels did not alter after a 4-week treatment of HDL except for the striatum where *Hspg2* was expressed significantly higher than after the saline

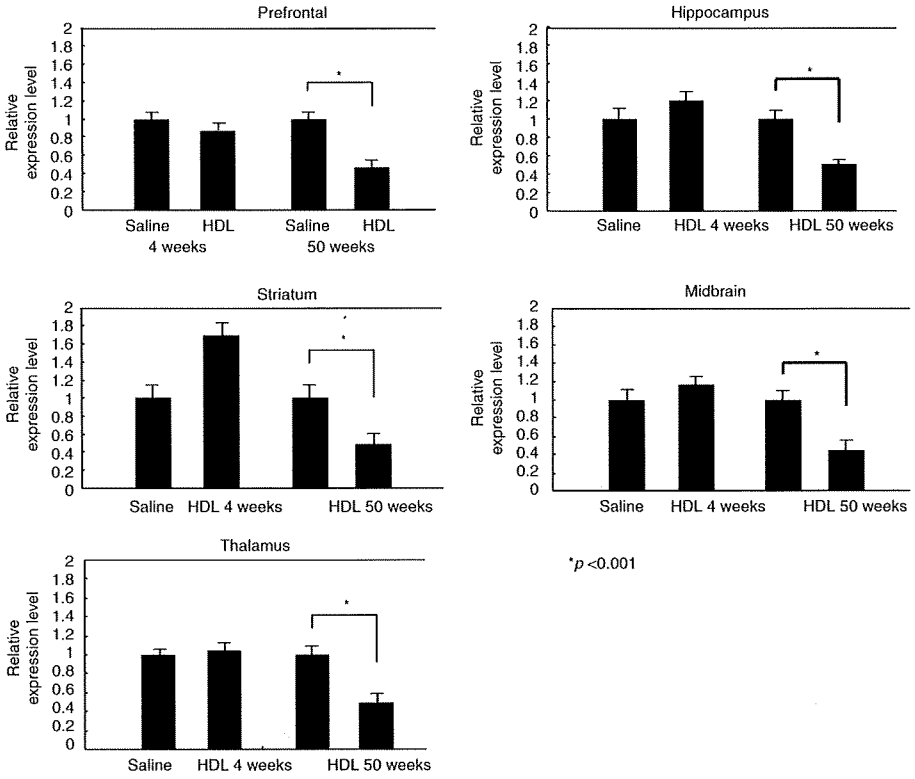

Figure 2 Effects of haloperidol (HDL) on *HSPG2* gene expression in the mouse brains. Relative expression levels of *Hspg2* from the prefrontal cortex, midbrain, hippocampus, thalamus, and striatum in the mouse brains after treatment with HDL for 4 weeks ($n = 10$) and HDL for 50 weeks ($n = 10$) were compared with the saline groups for 4 weeks ($n = 10$) and 10 weeks ($n = 10$) by Student's *t*-test.

treatment. Significantly lower expression of *Hspg2* was observed in all brain regions after a 50-week treatment with HDL than after a 50-week treatment with saline (Figure 2) ($F(1, 18) = 42.9$, $p < 0.0001$ at the prefrontal cortex; $F(1, 18) = 20.1$, $p = 0.0003$ at the hippocampus; $F(1, 18) = 15.9$, $p = 0.0009$ at the striatum; $F(1, 17 = 19.3$, $p = 0.0004$ at the midbrain; $F(1, 18) = 16.5$, $p = 0.0007$ at the thalamus).

Analysis of VCMs Induced by Haloperidol–Reserpine in *Hspg2* Knockout Mice

As we could not induce VCMs by administration of HDL only to mice, VCMs induced by long-term treatment with HDL and reserpine in female *Hspg2* hetero-knockout mice and female wild-type gene litters were measured to evaluate the relationship between expression levels of *Hspg2* and TD (Figure 3a). *Hspg2*-null knockout mice were embryonic lethal. The relative expression levels of *Hspg2* in *Hspg2* hetero-knockout mouse brains were almost half of that in the wild littermates (data not shown). Body weight, locomotor activities, and performance in the rotarod test before and after 48 days of administration of HDL and reserpine were not significantly different between *Hspg2* hetero-knockout and wild litters (data not shown). There was a significant effect of genotype ($F(1, 545) = 36.8$, $p < 0.0001$), post-treatment time ($F(4, 495) = 6.15$, $p < 0.0001$), and treatment ($F(3, 543) = 5.7$, $p = 0.0008$) for the number of VCMs for 5 min. *Post hoc* analysis showed that the number of VCMs were significantly lower in hetero-knockout mice than in wild-type mice after the last injection of HDL and reserpine after 48 or 49 consecutive days of administration of HDL and reserpine, and subsequent injection of physostigmine on the 50th day, or saline on the 53rd day (Figure 3b). The response of VCMs to physostigmine was subsequently evaluated (Figure 3c). There was a significant effect of genotype ($F(1, 128) = 36.9$, $p < 0.0001$), but not post-treatment time ($F(4, 125) = 1.03$, $p = 0.39$) for individual differences in the number of VCMs between pre-injection and post-treatment time. As for saline treatment, there was no significant effect of genotype ($F(1, 118) = 0.13$, $p = 0.72$) and post-treatment time ($F(4, 115) = 0.31$, $p = 0.87$). The numbers of VCMs were significantly reduced by injection of physostigmine compared with those before the injection at 24 h after HDL and reserpine injection in the wild-type mice but the differences in the numbers of VCMs before and after injection of physostigmine were not significant in hetero-knockout mice. The number of VCMs did not significantly alter after injection of saline in hetero-knockout mice and wild-type mice.

DISCUSSION

From a genome-wide association analysis, this study identified the role of *HSPG2* in neuroleptic-induced TD. The association was not significant in the initial screening and second confirmation after correction for multiple testing. However, screening with the tag SNPs for *HSPG2*,

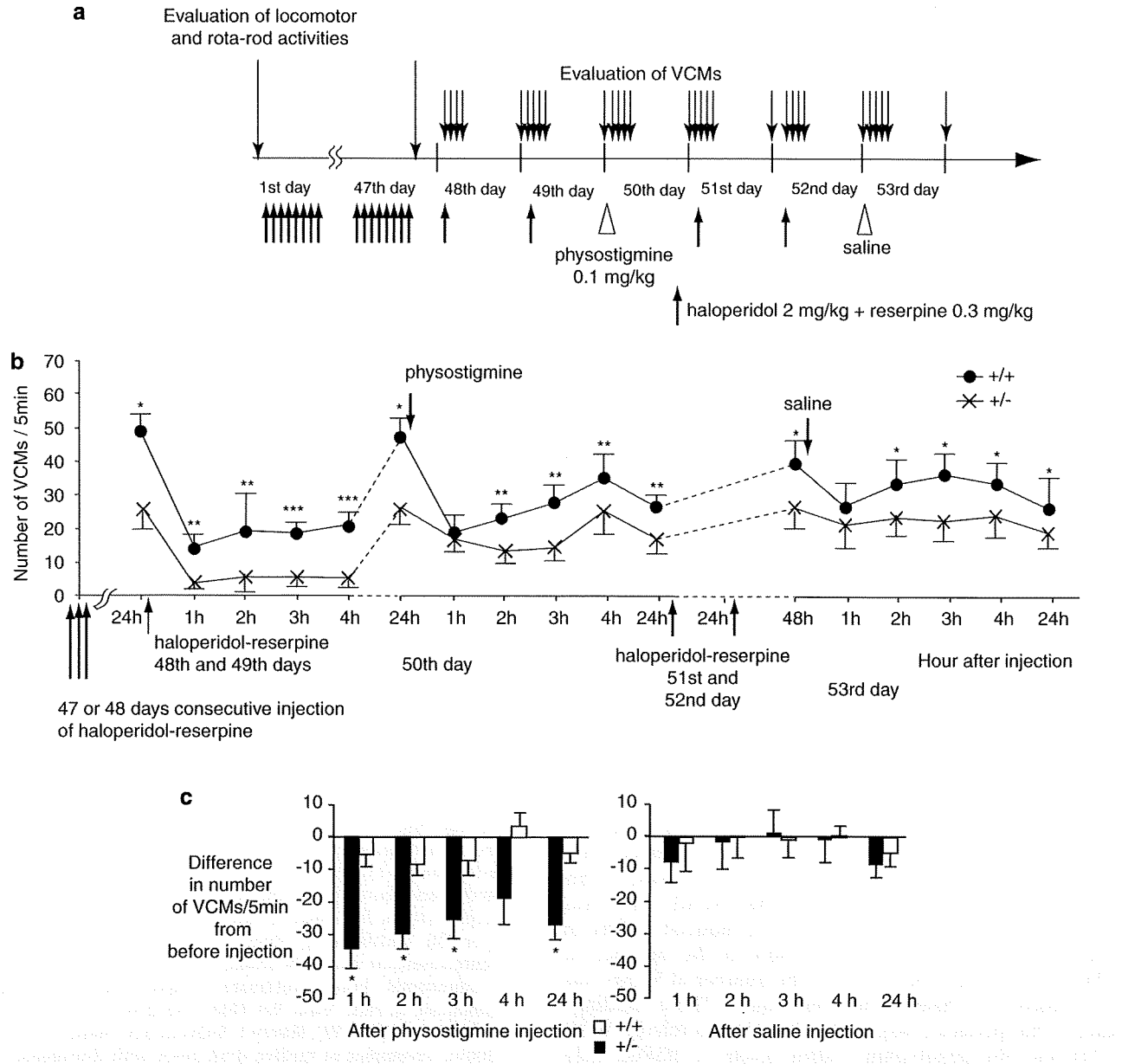

Figure 3 Analysis of vacuous chewing movements (VCMs) induced by haloperidol–reserpine in *Hspg2* knockout mice. (a) Schematic presentation of schedule of injections and measurements. (b) The average number (SEM) of VCMs for 5 min after injection. The abscissa axis shows the time after injection of HDL and reserpine, physostigmine, or saline. Significant difference between *Hspg2* hetero-knockout and wild-type mice is shown as * at $p<0.05$, ** at $p<0.01$, and *** at $p<0.001$. (c) Reduction of the number of VCMs after physostigmine or saline injection. Individual differences of the number of VCMs before injection (50th day for physostigmine or 53rd day for saline) to each time after injection grouped by the genotype are shown. Significant difference from mean = 0 is shown as * at $p<0.001$.

where the SNP (rs2445142) with the smallest *p*-value for association with TD in our genome-wide association study was located, identified one SNP (rs2124368) associated with TD even after correction for multiple testing. These two SNPs, which were found to be associated with TD, one identified by a genome-wide screening and another identified by screening with the tag SNPs, were not in LD. However, it is not obvious whether the finding for genetic association with TD of these SNPs in the *HSPG2* gene can be interpreted as significant, because of two steps of genome-wide association analyses before the step of screening of tag SNPs. Furthermore, the Human-1 BeadChip used in our initial screening is far from a complete genome coverage. This may affect the credibility of the results. Confirmation of associations in other populations is necessary.

The SNP rs2445142 that showed the lowest association *p*-value in this study was associated with the expression levels of *HSPG2* in the human postmortem prefrontal cortex. The risk allele was associated with increased expression of *HSPG2*. The SNP rs2445142 is located in intron 1 of the *HSPG2* gene and is in complete LD with at least six SNPs located from introns 1–9. Among the SNPs associated with TD found in this study, the program TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) predicts alteration of the transcription factor, LYF-1, binding affinity between the T and C alleles of rs897474 in intron 3.

Synonymous SNPs, rs2254357 (exon 6), and rs2254358 (exon 6) that were associated with TD might affect mRNA decay rates. Unfortunately, the mechanism of the association between these SNPs and HSPG2 expression levels could not be elucidated in this study.

From findings in human postmortem brain samples, we speculated that increased expression of *HSPG2* is a risk factor for TD and interpreted that decreased expression of *Hspg2* in mouse brains after chronic administration of HDL was a compensatory or adaptive response to neuroleptic drugs. We, therefore, hypothesized that decreased expression level of *HSPG2* is protective for TD. We examined our hypothesis using hetero-knockout mice and confirmed it after finding lower numbers of VCMs in hetero-knockout mice than in the wild-type littermates after chronic administration of HDL and reserpine. We carried out the experiment using only female mice; therefore, we do not have the data on the sex difference.

The mechanism behind our hypothesis that increased expression levels of *HSPG2* may induce a susceptibility to neuroleptic-induced TD is not known at present. A potential efficacy of cholinergic drugs in the treatment of TD has been reported (Caroff *et al*, 2001; Tammenmaa *et al*, 2004). AChE terminates neurotransmission at cholinergic synapses by hydrolyzing acetylcholine. At the neuromuscular junction, AChE is in the basal lamina, where AChE tetramers bind the collagen ColQ, which interacts in turn with the dystroglycan complex through perlecan (Peng *et al*, 1999). Perlecan is an essential component of the ColQ–AChE localization in neuromuscular junction (Rotundo *et al*, 2005). At central synapses, AChE tetramers bind directly to the PRiMA (Perrier *et al*, 2002). Although ColQ also anchors AChE in brain and heart in addition to skeletal muscle (Feng *et al*, 1999), the role of perlecan in acetylcholine receptor signaling in central synapses is unclear. In this study, we tested the effect of the AChE inhibitor, physostigmine, on HDL- and reserpon-induced VCMs in mice. We found significant reduction in the number of VCMs only in wild-type mice and the number of VCMs was not reduced in hetero-knockout mice. These findings indicate that perlecan may be involved in the role of AChE in TD and the genotyping and/or levels of *HSPG2* may provide useful information about the effectiveness of treatment of TD with AChE.

The other important molecule to which perlecan and TD may be related is FGF2. Perlecan promotes FGF2–FGFR1 binding (Whitelock *et al*, 1996) and HSPGs including perlecan were upregulated by responding to injury and may have a role in intracellular trafficking of FGF2 in neurons and glia in the adult rat cerebral cortex (Leadbeater *et al*, 2006). Clozapine increases FGF2 expression and, on the basis of the neuroprotective activity of FGF2, a potential use of clozapine in TD was proposed (Riva *et al*, 1999).

Perlecan is expressed at the capillary endothelial cells in the brain and perlecan at the blood–brain barrier (BBB) may have a role in maintaining the blood–brain barrier function because of acceptance of the FGF2 secreted from astrocytes (Deguchi *et al*, 2002). It is reported that neuroleptics, such as HDL and chlorpromazine, alter the blood–brain barrier function and increase brain iron levels, which affect neuroleptic-induced dopamine receptor supersensitivity (Ben-Shachar *et al*, 1993).

Although the exact mechanisms of the association between HSPG2 and TD are unclear, this study identified the role of *HSPG2* in neuroleptic-induced TD.

ACKNOWLEDGEMENTS

This study was supported by grants from the Mitsubishi Pharma Research Foundation and Kakenhi 20023006 and 20390098. Australian human brain tissues were received from the NSW Tissue Resource Centre, which is supported by The University of Sydney, Neuroscience Institute of Schizophrenia and Allied Disorders, National Institute of Alcohol Abuse and Alcoholism, and the NSW Department of Health.

DISCLOSURE

The authors declare that no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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Original Paper

Neuropsychobiology

Neuropsychobiology 2010;61:57–63
DOI: 10.1159/000265130

Received: November 28, 2008
Accepted after revision: June 10, 2009
Published online: December 12, 2009

Genetic Association Analysis of Functional Polymorphisms in Neuronal Nitric Oxide Synthase 1 Gene *(NOS1)* and Mood Disorders and Fluvoxamine Response in Major Depressive Disorder in the Japanese Population

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Key Words
Bipolar disorder · Major depressive disorder · Fluvoxamine · Neuronal nitric oxide synthase 1 gene · Functional single nucleotide polymorphisms

Abstract
Background/Aim: Nitric oxide has been reported to play a role in neural transmitter release and N-methyl-D-aspartate receptor activation, as well as to be related to oxidative stress. Abnormalities in both of these mechanisms are thought to be involved in the pathophysiology of mood disorders including major depressive disorder (MDD) and bipolar disorder (BP). In addition, several lines of evidence support an association between abnormalities in neuronal nitric oxide synthases (nNOS) and mood disorders. Therefore, we studied the association of nNOS gene *(NOS1)* and mood disorders and the efficacy of fluvoxamine treatment in Japanese MDD patients. ***Materials and Methods:*** Using a single nucleotide polymorphism (SNP; rs41279104, also called ex1c), we conducted a genetic association analysis of case-control samples (325 MDD patients, 154 BP patients and 807 controls) in the Japanese population. In addition, we performed an association analysis between *NOS1* and the efficacy of fluvoxamine treatment in 117 MDD patients. We defined a clinical response as a decrease of more than 50% in baseline SIGH-D (Structured Interview Guide for the Hamilton Rating Scale for Depression) score within 8 weeks, and clinical remission as an SIGH-D score of less than 7 at 8 weeks. ***Results:*** We did not detect a significant association between *NOS1* and MDD, BP or the fluvoxamine therapeutic response in MDD in allele/genotype-wise analysis. ***Conclusions:*** We did not detect an association between only one marker (rs41279104) in *NOS1* and Japanese mood disorder patients and fluvoxamine response, but sample sizes were probably too small to allow a meaningful test. Moreover, because we did not perform an association analysis based on linkage disequilibrium and a mutation scan of *NOS1*, a replication of the study using a larger sample and based on linkage disequilibrium may be required for conclusive results.

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Introduction

Nitric oxide (NO) is involved in a variety of mechanisms, such as neurotransmitter release [1, 2], N-methyl-D-aspartate receptor activation [2, 3], and oxidative stress in the brain [4]. Abnormalities in these mechanisms are thought to be involved in the pathophysiology of mood disorders, including major depressive disorder (MDD) and bipolar disorder (BP) [1, 4–6]. Some studies reported a decreased plasma NO level in MDD patients and increased plasma NO level in BP type I patients compared with controls [7–9]. Selective serotonin reuptake inhibitors such as fluvoxamine and paroxetine, which are major therapeutic agents for MDD, inhibit serotonin transporters in the presynaptic neuron, thereby increasing the extracellular serotonin level. This mechanism is believed to relieve depressive symptoms. NO affects the reuptake of serotonin [10, 11]. Finkel et al. [12] reported reduced plasma NO levels in patients with MDD complicated by ischemic heart disease following paroxetine treatment. Also, preclinical antidepressant effects were reported for tramadol, which is an analgesic that has a low affinity for opioid receptors and inhibits the reuptake of norepinephrine and serotonin [1]. Tramadol showed antidepressant-like effects in the forced swimming test using rats. These authors suggested that inhibition of the L-arginine-NO-cGMP pathway was involved in this antidepressant-like effect [13]. Considering these results, NO appears to be a good candidate for the pathophysiology of mood disorders and the therapeutic target in MDD [1, 6, 14].

NO is synthesized from L-arginine by a family of isoformic enzymes known as nitric oxide synthases (NOS). Three isoforms of NOS have been identified: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) [1]. Several lines of evidence support an association between abnormalities in nNOS and mood disorders. Firstly, levels of nNOS immunoreactivity in the noradrenergic locus coeruleus and suprachiasmatic nucleus were lower in MDD patients than in control subjects [7, 15]. Secondly, in an animal study using mice, 7-nitroindazole, a selective inhibitor of nNOS, was shown to produce antidepressant-like and anxiolytic-like effects in a forced swimming test [16]. Zhou et al. [17] reported that nNOS-mutant mice did not show these effects in the forced swimming test. Therefore, these authors suggested that inhibiting nNOS signaling in the brain might be a novel approach for treating depressive symptoms. Thirdly, genetic association analyses showed that the nNOS gene *(NOS1)* was associated with schizophrenia [18–21]. In addition, Kawohl et al. [22] observed an association between *NOS1* and lower loudness dependence of auditory evoked potentials (LDAEP) in healthy control subjects. Since LDAEP has been indicated as a reliable noninvasive indicator of central serotonin function [23], *NOS1* is thought to influence serotonergic neural transmission. Fourthly, a recent postmortem study showed increased nNOS expression in the hippocampal CA1 and subiculum in mood disorder patients [24]. Finally, *NOS1* (OMIM *163731, 29 exons in a genomic region spanning 149.404 kb) is located on 12q24. This genomic region was shown to be closely related to susceptibility to BP [25] and MDD [26, 27]. Therefore, we studied the association of functional polymorphisms in *NOS1* and mood disorders and the efficacy of fluvoxamine treatment in Japanese MDD patients.

Materials and Methods

Subjects

The subjects in the association analysis were 325 MDD patients (160 males and 165 females; mean age ± standard deviation 47.3 ± 14.9 years), 154 BP patients (80 males and 74 females; 95 patients with bipolar I disorder and 59 patients with bipolar II disorder; mean age ± standard deviation 47.9 ± 14.2 years) and 807 healthy controls (354 males and 453 females; mean age ± standard deviation 37.2 ± 15.9 years). Of the 325 MDD patients, 117 (58 males and 59 females; mean age ± standard deviation 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 the Hamilton Rating Scale for Depression (SIGH-D). The MDD patients other than these 117 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. Fluvoxamine was taken 2 or 3 times a day for 8 weeks. The initial total dose in 1 day was 50–100 mg. Fluvoxamine was 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. 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 cirrhosis, renal failure, heart failure, or other axis I disorders according to DSM-IV.

Data Collection

The scores of the 117 MDD patients in this study on the 17 items of the SIGH-D were 12 or higher [28]. We defined a clinical response as a decrease of more than 50% in baseline SIGH-D score within 8 weeks, and clinical remission as an SIGH-D score of less than 7 at 8 weeks. Detailed information on data collection had been described in a previous paper [29]. The clinical characteristics of the patients in this study, classified according to these def-

Table 1. Clinical characteristics of the patients in both definition groups

	Total	Male	Female	Age (mean ± SD), years	Baseline SIGH-D score (mean ± SD)	Fluvoxamine dose at 8 weeks (mean ± SD) mg/day	Number of previous episodes (mean ± SD)
Overall	117	58	59	44.8 ± 16.7	20.1 ± 5.84	122 ± 41.0	1.38 ± 0.656
Clinical response group[1]							
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[2]							
Remitters	45	22	23	43.7 ± 15.9	19.6 ± 5.06	113 ± 43.9	1.37 ± 0.598
Nonremitters	72	38	34	44.8 ± 17.3	20.5 ± 6.27	128 ± 39.4	1.38 ± 0.711
p value		0.682		0.754	0.382	0.076	0.903

[1] Clinical response was defined as a 50% or greater decrease in the baseline SIGH-D score.
[2] Clinical remission was defined as a final SIGH-D score of less than 7.

initions, can be seen in table 1. We did not include the plasma concentration of fluvoxamine.

Single Nucleotide Polymorphism Selection

We selected rs41279104 (also called 'ex1c') in *NOS1*, which is associated with prefrontal brain functioning, for use in the following association analysis [20]. In addition, Saur et al. [30] reported that the presence of allele A of *NOS1* in exon 1c reduced the transcriptional efficiency of the *NOS1* exon 1c by approximately 30%.

Single Nucleotide Polymorphism Genotyping

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

Statistical Analysis

Genotype deviation from the Hardy-Weinberg equilibrium (HWE) was evaluated by the χ^2 test (SAS/Genetics, release 8.2; SAS Japan Inc., Tokyo, Japan). Marker-trait association analysis was used to evaluate the allele- and genotype-wise association with the χ^2 test (SAS/Genetics, release 8.2; SAS Japan Inc.). In addition, we included another test for the association between the percent decrease in the SIGH-D score from baseline to the end of the period of observation and rs41279104 in *NOS1* genotype data, with analysis of covariance (ANCOVA) using the least-square method. Gender, age at the time of recruitment, fluvoxamine dose at 8 weeks and SIGH-D total score at baseline were covariates used in the analysis to better model the effect of genotype on the percent decrease in the SIGH-D score from baseline to the end of the period of observation. The statistical package JMP for Windows was used for ANCOVA (JMP 5.0. 1J; SAS Japan Inc.).

Power calculation was performed using the genetic power calculator [31]. The significance level for statistical tests was 0.05. Bonferroni correction was used to control inflation of the type I error rate.

Results

Genotype frequencies were in HWE for this SNP. We did not find an association between this SNP and Japanese BP or MDD in any of the analyses (table 2). It is known that there are sex differences in the pathophysiology of mood disorders [32–34]. To further investigate these associations, we performed an explorative single-marker analysis of subjects divided by sex. However, no association was detected in either sex in MDD or BP patients (online supplementary table 1 and 2, www.karger.com/doi/10.1159/000265130). With regard to the clinical characteristics of patients, only one difference was detected between responders and nonresponders in baseline SIGH-D scores (p = 0.0102) (table 1). One patient each was prescribed alprazolam, loflazepate and etizolam. Two patients each were prescribed lorazepam, brotizolam, flunitrazepam and zopiclone. We did not find any association between *NOS1* and the fluvoxamine therapeutic response in MDD in allele- and genotype-wise analysis (table 3). Also, ANCOVA was performed to test the effect of the tagging SNP genotype on the percent decrease in the SIGH-D score from baseline to the end of the period of observation when MDD patients were treated with fluvoxamine. There were no statistically significant differences in the percent decrease in the SIGH-D score from baseline to the end of the period of observation in which there was a fluvoxamine response to rs41279104 in *NOS1* genotype (p = 0.413).

Table 2. Functional SNP in *NOS1* and mood disorders

SNP ID	Phenotype	MAF	Number	Genotype distribution			p value		
				C/C	C/T	T/T	HWE	genotype	allele
rs41279104 (ex1c)	Controls	0.177	807	549	230	28	0.521		
	MDD	0.167	325	226	90	9	0.991	0.787	0.531
	BP	0.185	154	101	49	4	0.496	0.638	0.741

MAF = Minor allele frequency.

Table 3. Genotype and allele distributions of functional SNP in *NOS1* in both definition groups

SNP ID	Clinical groups	MAF	Number	Genotype distribution			p value		
				C/C	C/T	T/T	HWE	genotype	allele
rs41279104 (ex1c)	responders	0.158	60	41	19	0	0.145	0.109	0.176
	nonresponders	0.228	57	35	18	4	0.426		
	remission	0.155	45	31	14	0	0.216	0.262	0.259
	nonremission	0.215	72	45	23	4	0.644		

MAF = Minor allele frequency.

In a power analysis, we obtained more than 80% power for the detection of an association when we set the genotype relative risk for *NOS1* at 1.95 in MDD, 3.47 in BP and 1.42–1.95 in MDD patients treated with fluvoxamine, under a multiplicative model of inheritance.

Discussion

We performed the first association study of the fluvoxamine therapeutic response in Japanese MDD patients. In addition, we included an association analysis of *NOS1* with Japanese MDD and BP. However, we did not detect a significant association in any of the analyses. We selected rs41279104 (also called 'ex1c') in *NOS1*, which is associated with prefrontal brain functioning [20]. In addition, Saur et al. [30] reported that the presence of allele A of *NOS1* in exon 1c reduced the transcriptional efficiency of the *NOS1* exon 1c by approximately 30%. Also, we performed another test for the differences in the percent decrease in the SIGH-D score from baseline to the end of the period of observation. In this test, we used the SIGH-D score among the data for rs41279104 in *NOS1* genotype that were evaluated by ANCOVA after adjustment for sex, age at the time of recruitment, fluvoxamine dose at 8 weeks and SIGH-D total score at baseline. No association was found. rs41279104 influences expression levels of *NOS1* [30] and is associated with prefrontal brain functioning [20] and lower LDAEP in healthy control subjects [22]. Similar to our study, several other investigations have found no association between rs2682826 (also called 'C276T') in *NOS1* and BP or MDD using case-control samples [35, 36]. rs2682826 in exon 29 is located 276 bp downstream from the translation termination site [37]. We did not include an association analysis with rs2682826, because this SNP does not influence biological function. On the other hand, Reif et al. [38] reported an association between the eNOS gene *(NOS3)* and BP patients. The levels of platelet eNOS activity in MDD patients were lower than in control subjects. In an animal study, eNOS gene knockout mice showed better and faster learning in a learned helplessness paradigm that reflected cognitive function. Also, aminoguanidine, an iNOS inhibitor, showed antidepressant-like effects in murine models of depression, such as the forced swimming test [39]. Therefore, we considered the iNOS gene *(NOS2)* and *NOS3* to be good candidates for the pathophysiology of psychiatric disorders such as schizophre-

nia, MDD and BP. However, whole-genome association studies showed an association between *NOS1*, *NOS2* and *NOS3* and BP [40–42]. Recently, a whole-genome association study reported an association between rs6490121 in *NOS1* and schizophrenia [43]. Other association studies detected positive associations between *NOS1* and schizophrenia [18–21]. Lee and Kim [44] reported that plasma levels of NO metabolite were significantly lower in schizophrenics than in normal controls, both before and after treatment with risperidone, and that there were improvements in psychiatric symptoms associated with plasma levels of NO after schizophrenics were treated with rispridone. Because *NOS1* is one of the susceptibility factors for the major pharmacological therapeutic targets of atypical antipsychotics, a case-control study on the pharmacogenomics of psychotic disorders (antipsychotic response) with *NOS1* will also need to be conducted in the future.

Rujescu and colleagues [2] reported that ra2682826, rs1353939 and rs693534 in *NOS1* were associated with Caucasian suicide completers and suicide attempters, including people with mood disorders. Although we did not perform an association analysis of these SNPs, a replication study using larger samples than in the original studies will need to be carried out in the future.

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 samples unmatched for age, or small sample sizes, especially in BP and MDD patients treated with fluvoxamine. 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. Also, our control subjects did not undergo structured interviews. There may also have been a problem of sampling bias with mood disorders; for example, some patients diagnosed with MDD may develop BP in the future [45–47]. Second, in this study, we did not apply the 'gene-wide' approach recommended by Ikeda et al. [48] to consider population differences. In addition, 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. In addition, the analysis of copy number variations, acetylation and methylation rates in *NR1D1* was not performed in our study. Last, we did not include the plasma concentration of fluvoxamine. The daily fluvoxamine dosage was higher in nonresponders and nonremitters than in responders and remitters, respectively, though this should be self-evident for a study design incorporating fixed-flexible dosing. However, these effects should be minimal since no correlation between plasma fluvoxamine concentration and clinical response has been reported [49].

In conclusion, we did not detect an association between only one marker (rs41279104) in *NOS1* and Japanese mood disorder patients and fluvoxamine response, but sample sizes were probably too small to allow a meaningful test. Moreover, because we did not perform an association analysis based on linkage disequilibrium and a mutation scan of *NOS1*, a replication of the study using a larger sample and based on linkage disequilibrium may be required for conclusive results.

Acknowledgements

We thank Ms. M. Miyata and Ms. S. Ishihara for their technical support. This work was supported in part by research grants from the Ministry of Education, Culture, Sports, Science and Technology, the Ministry of Health, Labor and Welfare, and the Japan Health Sciences Foundation (Research on Health Sciences focusing on Drug Innovation).

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