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ORIGINAL ARTICLE

Kazuyuki Tadokoro · Ryota Hashimoto · Masahiko Tatsumi ·
Asako Kosuga · Kunitoshi Kamijima · Hiroshi Kunugi

The Gem interacting protein (GMIP) gene is associated with major depressive disorder

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Abstract Major depressive disorder (MDD) is a mood disorder with a significant heritable component. Structural neuronal impairment has been considered to be implicated in MDD, as it leads to brain morphological alterations such as hippocampal atrophy. The Gem interacting protein, GMIP, is a novel Rho GTPase-activating protein known to play important roles in neurite growth and axonal guidance. We examined the GMIP gene for possible association in a Japanese sample of 164 patients with MDD and 164 controls matched for sex. We found a significant association with MDD for one single nucleotide polymorphism (SNP) (−525G/A) located on the 5′-upstream region of the GMIP gene (p=0.039, odds ratio 1.66, 95% CI 1.05–2.69) and stronger evidence for association in a multimarker haplotype analysis (p=0.004). We then performed a promoter-luciferase reporter assay; the promoter activity for −525A allele, which was in excess in the MDD patients, was significantly decreased compared with the −525G allele in transient transfection experiments using three types of cell lines. Our results suggest that genetic variations in the GMIP gene can confer susceptibility to MDD, and the associated promoter SNP might play a role in the transcriptional regulation of the GMIP gene. Further study needs to be undertaken to validate the association between the GMIP gene and MDD.

Keywords Gem interacting protein (GMIP) · Major depressive disorder · Rho GTPase-activating protein · Single nucleotide polymorphisms (SNPs) · Luciferase reporter assay

K. Tadokoro · R. Hashimoto (✉) · H. Kunugi
Department of Mental Disorder Research,
National Institute of Neuroscience,
National Center of Neurology and Psychiatry, Japan,
4-1-1, Ogawahigashicho, Kodaira,
Tokyo, 187-8502, Japan
e-mail: rhashimo@ncnp.go.jp
Tel.: +81-42-3412712
Fax: +81-42-3461744

M. Tatsumi
Yokohama Shinryo Clinic,
Yokohama,
Kanagawa, 221-0385, Japan

M. Tatsumi · K. Kamijima
Department of Psychiatry,
Showa University School of Medicine,
Tokyo, 142-8666, Japan

A. Kosuga
Showa University Karasuyama Hospital,
Tokyo, 157-8577, Japan

Introduction

Major depressive disorder (MDD) is a mood disorder that strikes a large proportion of the population. It is a complex disorder with unknown etiology, likely the result of the interplay between vulnerability genes and environmental stressors [4]. MDD has traditionally been considered to have a neurochemical basis, but recent studies have associated this complex disorder with volume reductions in brain structures and in the numbers and/or sizes of glia and neurons in discrete brain areas [12]. Although the precise cellular mechanisms underlying these morphometric changes are unknown, the increasing data indicate that MDD are associated with impairments of structural plasticity.

The Rho family of GTP-binding proteins act as a key regulator for developing neuronal structure, e.g., neurite and growth cone formation [14]. These proteins cycle between active GTP-bound and inactive GDP-bound forms. The activation of the GTP-bound form is regulated by GTPase-activating proteins, which stimulate GTP hydrolysis, leading to inactivation [6]. The Gem interacting protein gene, GMIP, was identified as a novel GTPase-activating protein, which binds to Gem, a member of the Ras superfamily of GTPases [1]. The human GMIP gene is localized to chromosome 19p11–12 and consists of 21 exons and 20 introns, spanning 14.13 kb. The GMIP mRNA of 3,840 bp, which is expressed ubiquitously, gives rise to a protein of 970 amino acids [1]. GMIP interacts with Gem through its N-terminal half and has a

Rho GTPase-activating protein domain in its C-terminal half. GMIP is able to inhibit RhoA function, leading to the actin cytoskeletal reorganization in vivo. Therefore, genetic variability of the GMIP gene is of considerable interest in the evaluation of the risk of MDD. To our knowledge, however, there is no study examining the possible association between the GMIP gene and MDD. In the present study, we performed an association study with single nucleotide polymorphisms (SNPs) in the region of the GMIP gene in a Japanese population of MDD cases and control subjects and analyzed the function of disease-associated SNP on transcriptional regulation.

Materials and methods

Subjects

Subjects were 164 patients [59 males, mean age of 49.5 years (SD 12.7)] with MDD and 164 healthy controls matched for sex [59 males, 47.4 years (SD 9.5)]. The mean ages in males are 47.2 years (SD 11.4), from 24 to 76 years old in MDD patients, and 45.1 years (SD 6.2), from 38 to 62 years old in controls; the mean ages in females are 50.8 years (SD 13.2), from 17 to 82 years old in MDD patients, and 48.6 years (SD 10.7), from 31 to 76 years old in controls. All the subjects were biologically unrelated Japanese and recruited from the same geographical area (the Western part of Tokyo Metropolitan). Consensus diagnosis was made for each patient by at least two psychiatrists according to the *Diagnostic and Statistical Manual of Mental Disorders, 4th edition* (DSM-IV) criteria on the basis of unstructured interviews and information from medical records. Among the 164 patients, 96 (59%) individuals had recurrent depressive episodes, and the remainder had a single episode. Eighty (49%) subjects had a history of admission to a psychiatric hospital, and 46 (28%) had a history of attempted suicide. The mean age of onset was 42.2 years (SD 12.7). The controls were healthy volunteers recruited from hospital staffs. They were interviewed, and those individuals who had a current or past history of psychiatric treatment were not included in the study. Subjects with significant medical problems, history of head trauma, neurosurgery, and alcohol or substance abuse were excluded. After a description of the study, a written informed consent was obtained from every subject. The study protocol was approved by institutional ethical committees.

SNP genotyping

Venous blood was drawn from the subjects, and genomic DNA was extracted from whole blood according to the standard procedures. We searched and selected validated SNPs in allele frequency in the genomic region of the GMIP gene-distributed 3- to 6-kb interval in silico. Four SNPs (SNP1, rs3794996; SNP2, rs2043293; SNP3, rs2304129; and SNP4, rs880090) in the GMIP gene and two SNPs outside of the genomic region of GIMP (SNP5, rs2288865 and SNP6, rs1054685) (Fig. 1) were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay previously described [9, 15]. Briefly, primers and probes for the detection of the SNPs are: SNP1: forward primer 5′-TCCCTGGTGTGCTGTAATTGG-3′, reverse primer 5′-CCCTTCCGTGACCCTCAAAG-3′, probe 1 5′-VIC-CCTTGAACCTGATCCAG-MGB-3′, and probe 2 5′-FAM-CTTGAGCCTGATCCAG-MGB-3′; SNP2: forward primer 5′- GGGAGCTGTGAGGCTGTAG-3′, reverse primer 5′-TGCTATGGATGTCTTCCCTAAGC-3′, probe 1 5′-VIC-CCAGACCAGCCCAGTG-MGB-3′, and probe 2 5′-FAM-CCAGACCAGACCAGTG-MGB-3′; SNP3: forward primer 5′-GCGGGACGGAGTGTGA-3′, reverse primer 5′-TCAAGGGTCAGCAAAGGTCATTT-3′, probe 1 5′-VIC-TCATTAACCCAGGCCACA-MGB-3′, and probe 2 5′-CATTAACCGAGGCCACA-MGB-3′; SNP4: forward primer 5′-ACCACCCTGGCACCTTAAATAAG-3′, reverse primer 5′-TGGTGGGAGGTAGGGATATAT GG-3′, probe 1 5′-VIC-TCCGTGTTCACAATC-MGB-3′, and probe 2 5′-FAM-TCCGTCTTCACAATC-MGB-3′; SNP5: forward primer 5′-CCTCGCATGTGCCCACTA-3′, reverse primer 5′-GCATTTCCTGAGCTCTGACCAT-3′, probe 1 5′-VIC-ATGGAAGGCCCTGTTC-MGB-3′, and probe 2 5′-FAM-AATGGAAGGTCCTGTTC-MGB-3′; and SNP6: forward primer 5′-AGGTTTGTGGCTCCTTG CA-3′, reverse primer 5′-CCCCGTGGACTGCTTCAA-3′, probe 1 5′-VIC-CTGTCCCCGATAGAA-MGB-3′, and probe 2 5′-FAM-CTGTCCCCAATAGAA-MGB-3. PCR cycling conditions were at 95°C for 10 min, 45 cycles of 92°C for 15 s, and 60°C for 1 min.

Polymorphism screening in the 5′-upstream region of the GMIP gene

To detect sequence variations in the 5′-upstream region of the human GMIP gene, we initially sequenced DNA samples from 24 control subjects that have a 95% power

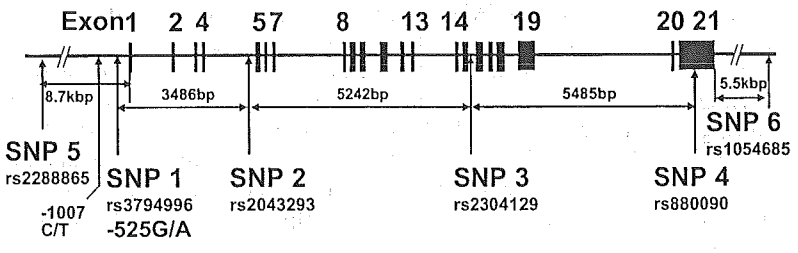

Fig. 1 Genomic structure and location of SNPs for human GMIP gene. Exons are denoted by *bold vertical lines in black*. The rs number of each SNP is the NCBI SNP cluster ID from the dbSNP database. The distances of the adjunct SNPs are also shown

to detect polymorphisms with a frequency of more than 5% [11]. Using three sets of GMIP-specific primers (forward primer 1 5′-CATCACAAGGTCAGGAGATCGA-3′, reverse primer 1 5′-CAGTGGAATTTTGGGGCTGGAA-3′, forward primer 2 5′-GGAAACCTGGCTTGGCTCT TA-3′, reverse primer 2 5′-GGCCTGATATTCTGTCCGA TTC-3′, forward primer 3 5′-CAAACCTCCACTCCCTA CCT-3′, and reverse primer 3 5′-GTCCTTCCCATTCAGG AACT-3′), designed on the basis of the published GMIP mRNA sequences (accession number AF132541), we amplified, by PCR overlapping, fragments of the putative promoter region and exon 1 (from position −1,120 to +160 relative to the transcription start site). PCR, using primer 1 or 2, was performed under the following conditions: at 95°C for 10 min, 35 cycles of 95°C for 45 s, 62°C for 30 s and 72°C for 1 min, and 72°C for 7 min. PCR, using primer 3, was performed under the following conditions: at 95°C for 10 min, 45 cycles of 95°C for 45 s, 58°C for 30 s and 72°C for 1 min, and 72°C for 7 min. Individual PCR products were fully sequenced by a CEQ8000 autosequencer (Beckman Coulter, Fullerton, CA, USA) and compared with the original deposited sequence to identify variants.

Genotyping of the −1,007C/T SNP was performed using a PCR-based restriction-fragment-length polymorphism assay. Five hundred twenty seven base pair fragments were amplified using primers 5′-CATCACAAGGTCAGG AGATCGA-3′ and 5′-CAGTGGAATTTTGGGGCTGG AA-3′, and then the product was digested by *Taq*I. The amplified T allele variant was cleaved twice, whereas the C allele was cleaved once, which was visualized by 2% agarose gel electrophoresis and ethidium bromide staining.

Luciferase reporter assay

Two allelic forms of the SNP1 (−525G/A) were cloned into pGL3-Basic vector (Promega, Madison, WI, USA). The fragments containing the −525G/A SNP were amplified by PCR from genomic DNA using the primers 5′-AAAGGG CTGCTCCCTGGTGTG-3′ (5′ end at position −564, transcriptional start site as +1) and 5′-ATATCTGGGCCCGGG GATCG-3′ (5′ end at position +81). These primers were designed to incorporate *Nhe*I (forward) and *Hin*dIII (reverse) restriction sites, and the PCR product was inserted into the polylinker site upstream of the luciferase coding region in the pGL3-Basic vector. The inserted sequence was confirmed with the autosequencer CEQ8000 in both directions using primers supplied by the manufacturer (Promega; RV primer 3 and GL primer 2).

Human neuroblastoma IMR-32 cells and HeLa cells were cultured in Minimum Essential Medium (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (Invitogen, Carlsbad, CA, USA) and penicillin/streptomycin (Sigma). Human glioma Hs683 cells were cultured in Dulbecco's Modified Eagle Medium (Sigma) containing 10% fetal bovine serum and penicillin/streptomycin. Cells in 24-well plates were cotransfected in triplicate with 800 ng of pGL3-Basic firefly luciferase reporter vectors that included two allelic forms of the −525G/A SNP and 25 ng of Renilla luciferase expression vector (phRL-TK vector; Promega) as an internal control by using Lipofectamine 2000 Regent (Invitogen). As a negative control, an empty pGL3-Basic vector was transfected simultaneously in all the experiments. Cells were washed with phosphate-buffered saline and then harvested with luciferase lysis buffer (Promega) 24 h after transfection. A luciferase reporter assay was performed using a Dual-Luciferase Reporter Assay System (Promega) and a Lumat LB 9507 luminometer (Berthold, Bad Wildbad, Germany). Firefly and renilla luciferase activities were quantified sequentially as relative light units (RLU) by the addition of their respective substrates according to the protocol of the supplier. The ratio of firefly RLU to renilla RLU of each sample was automatically computed. The activity of each construct was expressed at the relative value compared to that of −525G allele, and these relative values were used for statistical analysis. Experiments were repeated three times in all cell lines.

Statistical analysis

Statistical analysis of association studies was performed using SNPAlyse software (DYNACOM, Yokohama, Japan). The presence of a Hardy–Weinberg equilibrium was examined using the χ^2 test for goodness of fit. Allele distributions between patients and comparison subjects were analyzed by the χ^2 test for independence. The measures of linkage disequilibrium (LD), denoted as D', was calculated from the haplotype frequency using an Expectation-Maximization algorithm. Case-control haplotype analysis was performed by the permutation method to obtain empirical significance [7]. Haplotype frequencies were determined by using the case-control haplotype analysis program of SNPAlyse software which was used in our previous study [15]. The global p values represent the overall significance using the χ^2 test when the observed vs expected frequencies of all the haplotypes are considered together. The individual haplotypes were tested for association by grouping all others together and applying the χ^2 test with 1 *df*. P values were calculated based on 10,000 replications. For luciferase reporter gene assay, Student's t test was used for comparison in relative luciferase expression between alleles inserted into vectors. All p values reported are two-tailed. Statistical significance was defined at $p<0.05$.

Results

The genotype distributions of all examined SNPs for the patients and controls were not significantly deviated from the Hardy–Weinberg equilibrium (data not shown). When we examined males and females separately, the genotype distributions of all examined SNPs for the male and female patients and controls were in the Hardy–Weinberg equilibrium (data not shown). Genotype distributions and allele

Table 1 Genotype and allele distributions for the SNPs in the GMIP gene among the patients with major depressive disorder and controls

SNP-ID	SNP	Controls				Major depressive disorder				OR (95% CI)
		Genotype frequency			Allele 2 frequency	Genotype frequency			Allele 2 frequency	
		1/1	1/2	2/2		1/1	1/2	2/2		
SNP1	G/A	134 (81.7%)	30 (18.3%)	0 (0%)	0.091	120 (73.2%)	41 (25.0%)	3 (1.8%)	0.143	1.66 (1.05–2.69)
SNP2	T/G	119 (72.6%)	44 (26.8%)	1 (0.6%)	0.140	106 (64.6%)	53 (32.3%)	5 (3.1%)	0.192	1.46 (0.93–2.26)
SNP3	G/C	120 (73.2%)	43 (26.2%)	1 (0.6%)	0.137	106 (64.6%)	53 (32.3%)	5 (3.1%)	0.192	1.50 (0.96–2.27)
SNP4	C/G	119 (72.6%)	44 (26.8%)	1 (0.6%)	0.140	106 (64.6%)	53 (32.3%)	5 (3.1%)	0.192	1.46 (0.96–2.23)

The major allele in each SNP represents allele 1

frequencies of the four SNPs among the patients and controls are shown in Table 1. The A allele of SNP1 was in excess in our MDD patients when compared to controls (χ^2=4.25, df=1, p=0.039), and there were trends toward increased frequencies of minor alleles of the other three SNPs (SNP2 p=0.075, SNP3 p=0.058, and SNP4 p=0.075). There was no difference in the allele frequency of SNP5 (8.7 kb away from the 5′ of the GMIP gene) or SNP6 (5.5 kb away from the 3′ of the GMIP gene) between patients and controls (p>0.2). As the association was strongest in the SNP1 in the putative promoter region, we have searched polymorphisms in the 5′-putative promoter region. We sequenced 1,280 bp of the 5′-upstream region of the GMIP gene using 24 unrelated Japanese subjects and detected one novel SNP (−1,007C/T). We genotyped this SNP by a PCR-based restriction-fragment-length polymorphism assay and found that the frequency of the minor allele (−1,007T) was rare (<1%), suggesting that this SNP has no major role in the pathogenesis of MDD.

As gender differences occur in MDD (female predominance), we examined males and females separately. Genotype distributions and allele frequencies of the four SNPs among the patients and controls in males (Table 2) and females (Table 3) are shown. There were significant differences in the allele frequencies for three SNPs between patients with MDD and controls in males (SNP2 p=0.0086, SNP3 p=0.0086, and SNP4 p=0.015), while there were no such differences for any SNPs between those with major depression and controls in females. Minor allele frequencies of SNP1 were overrepresented in both male and female patients with MDD, although the differences of the allele frequency did not reach statistical significance.

To further analyze the haplotype structure in our controls and MDD patients, we computed the LD between the SNPs using D' (Table 4). All D' values were more than 0.97, indicating tight LD across all the markers. Thus, we performed haplotype-based analysis consisting of the four SNPs. It was estimated that only two haplotypes represented more than 90% of the total haplotype diversity present in the population of total subjects. Estimated haplotype frequencies and individual p values corresponding to the haplotypes in patients with MDD and controls in males are shown in Table 5. The overall distribution of haplotypes was significantly different between MDD and controls in males (global p value=0.0087), while no significant difference was observed in females. The estimated frequency of the GTGC haplotype was significantly less in patients with MDD (86.4 vs 72.8%, p=0.0044) in males, while similar estimated frequencies of the GTGC haplotype were observed in females (MDD 84.8 vs control 85.2%). Our findings suggested that genetic variances in the genomic region of GMIP might be associated with MDD.

As −525G/A SNP in the putative promoter region had a significant association with MDD, the promoter analysis was carried out using luciferase reporter gene assay. To determine the effects of the −525G/A allele on transcription regulation, we cloned allele-specific promoter fragments into the pGL3-Basic vector (Promega). Promoter activity was assayed using a dual-luciferase system (Promega). Figure 2 shows observed relative luciferase expression

Table 2 Genotype and allele distributions for the SNPs in the GMIP gene among the patients with major depressive disorder and controls in male

SNP-ID	SNP	Controls				Major depressive disorder				OR (95% CI)
		Genotype frequency			Allele 2 frequency	Genotype frequency			Allele 2 frequency	
		1/1	1/2	2/2		1/1	1/2	2/2		
SNP1	G/A	45 (76.3%)	14 (23.7%)	0 (0%)	0.119	39 (66.1%)	17 (28.8%)	3 (5.1%)	0.195	180 (0.91–4.02)
SNP2	T/G	44 (74.6%)	15 (25.4%)	0 (0%)	0.127	32 (54.2%)	23 (39.0%)	4 (6.8%)	0.263	2.45 (1.23–5.23)
SNP3	G/C	44 (74.6%)	15 (25.4%)	0 (0%)	0.127	32 (54.2%)	23 (39.0%)	4 (6.8%)	0.263	2.45 (1.23–5.23)
SNP4	C/G	43 (72.9%)	16 (27.1%)	0 (0%)	0.136	32 (54.2%)	23 (39.0%)	4 (6.8%)	0.263	2.27 (1.20–4.72)

The major allele in each SNP represents allele 1

Table 3 Genotype and allele distributions for the SNPs in the GMIP gene among the patients with major depressive disorder and controls in females

SNP-ID	SNP	Controls				Major depressive disorder				OR (95% CI)
		Genotype frequency			Allele 2 frequency	Genotype frequency			Allele 2 frequency	
		1/1	1/2	2/2		1/1	1/2	2/2		
SNP1	G/A	89 (84.8%)	16 (15.2%)	0 (0%)	0.076	81 (77.1%)	24 (22.9%)	0 (5.1%)	0.114	1.56 (0.84–3.39)
SNP2	T/G	75 (71.4%)	29 (27.6%)	1 (1.0%)	0.148	74 (70.5%)	30 (28.6%)	1 (1.0%)	0.152	1.04 (0.61–1.80)
SNP3	G/C	76 (72.4%)	28 (26.7%)	1 (1.0%)	0.143	74 (70.5%)	30 (28.6%)	1 (1.0%)	0.152	1.08 (0.61–1.85)
SNP4	C/G	76 (72.4%)	28 (26.7%)	1 (1.0%)	0.143	74 (70.5%)	30 (28.6%)	1 (1.0%)	0.152	1.08 (0.61–1.85)

The major allele in each SNP represents allele 1

levels (RLEs) for the G or A alleles of the SNP1 of the GMIP gene compared to RLE without insertion of such alleles (empty pGL3-Basic vector). For all the cell lines examined (HeLa, IMR-32, and Hs683), RLE was robustly increased ($p<0.001$) due to insertion of the putative promoter region for both alleles compared to the empty pGL3-Basic vector. The RLE of the A allele, which was a higher allele frequency in patients, was significantly lower than that of the G allele in all cell lines (0.85±0.05 vs 1.00±0.03, $p<0.001$ in HeLa cells; 0.84±0.10 vs 1.00±0.07, $p=0.002$ in IMR-32 cells; 0.86±0.09 vs 1.00±0.07, $p=0.002$ in Hs683 cells, mean±SD). These results suggested that the putative promoter region, including −525G/A SNP, had a transcriptional activity, and that the difference in the transcription activity between the −525G and −525A alleles might influence the expression level of the GMIP gene.

Discussion

We found a significant association between genetic variations of the GMIP gene and MDD in a Japanese population. This significant association was observed among male subjects in particular. Men are about half as likely as women to experience a lifetime episode of major depression [5]. The exact etiology of this gender difference is unclear; however, psychological factors, changes in circulating gonadal hormones, and neurological factors are likely to contribute. Our results, stronger evidence for association in men, can lead to one of neurological factors, as the GMIP gene is a member of Rho GTPase-activating proteins which play a crucial role in neuronal development and synaptic functions [13]. Impairment of these regulations may alter the neural circuit in the central nervous system. Differences in brain development between the sexes arise from the differential actions of genes that are encoded on the sex chromosomes [2]. Actually, several genes of X-linked mental retardation, which occurs in males, are involved in Rho signaling pathways such as oligophrenin 1, PAK3, etc. [18]. Interestingly, a Rho GTPase-activating protein, the chimerin 2 gene, is reported to be associated with schizophrenia in men [8]. Taken together, Rho signaling genes can confer the susceptibility for mental disorders particularly in men.

Table 4 Marker-to-marker LD in the GMIP gene

	SNP1	SNP2	SNP3	SNP4
SNP1	–	0.97	0.97	0.97
SNP2	1.00	–	1.00	1.00
SNP3	1.00	1.00	–	1.00
SNP4	1.00	0.97	1.00	–

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

Table 5 Estimated haplotype frequencies for patients and controls in males

Haplotype	SNP1	SNP2	SNP3	SNP4	Haplotype frequency		P value
					Patients	Controls	
1	G	T	G	C	0.728	0.864	0.0044
2	A	G	C	G	0.186	0.119	0.11

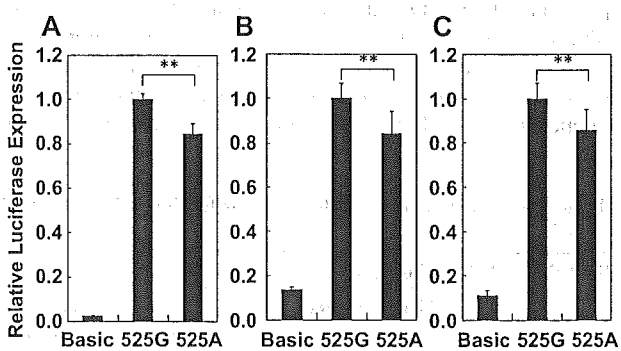

Fig. 2 Effects of human GMIP −525G/A SNP on promoter activity measured using HeLa cells (**a**), IMR-32 cells (**b**), and Hs683 cells (**c**). The figures show firefly luciferase activity relative to Renilla luciferase activity derived from the internal control vector phRL-TK. The construct containing the −525G allele was assigned a value of *1*. The pGL3-Basic vector (*Basic*), which does not contain promoter sequence, was used as a negative control. Results are means± SD of three independent transfection experiments performed in triplicate. ** indicates $p<0.005$

The four SNPs in the genomic region of GMIP were in strong LD in both MDD patients and controls. There were two common haplotypes (allele frequency in controls GTGC 0.86 and AGCG 0.12). When we computed LD between six SNPs, including two SNPs located outside of the GMIP gene, we also found strong LD (all D'>0.96). An unknown functional polymorphism, which is in LD with the associated SNPs and/or haplotypes in the GMIP gene and/or nearby genes, may be responsible for giving susceptibility for MDD.

Among all examined SNPs, the strongest evidence for association with MDD was observed for the −525G/A SNP. We calculated the power to detect a significant difference using our sample sizes for the −525G/A SNP in total samples (164), as well as males (59) or females (105), with a power of 90% when the critical p value was set at 0.05. The minor allele frequencies for the −525G/A SNP in controls were 0.091 (total), 0.119 (male), and 0.076 (female). The required odds ratios to detect a significant association between the risk allele and MDD were 1.7 (total), 3.0 (male), and 2.7 (female), while empirical odds ratios were 1.7 (total), 1.8 (male), and 1.6 (female). Thus, the reason why we did not detect the significant difference in males and females might be due to the power, which is not high enough to detect it. To detect the significant difference with a power of 90% when the critical p value was set at 0.05 in males (allele frequency 0.119, OR 1.8) and females (allele frequency 0.076, OR 1.6), sample sizes of 236 (males) and 561 (females) are required. The sample size we used did not have sufficient power to detect an interaction between the effect of the gene and gender on the disease. However, our results within the constraints of limited power indicate that the −525G/A SNP might be associated with MDD. Further studies to confirm the association between the SNP and MDD are warranted in such a larger sample size.

Since −525G/A SNP is located in the putative promoter region, it may play a role in transcriptional regulation. Luciferase reporter assay revealed the transcriptional activity of the DNA fragment of the 5′-upstream region of the GMIP gene, including −525G/A SNP. These findings were observed in three types of human cell lines (HeLa, IMR-32, and Hs683) where GMIP mRNA has been confirmed by reverse transcription–PCR (data not shown). Indeed, the −525A allele fragment, which was a higher allele frequency in MDD, showed lower promoter activity than the −525G allele fragment. The G to A nucleotide transition can alter the consensus sequence of binding sites of several transcription factors, including ZID (zinc finger protein with interaction domain), CP2, FTF (fetoprotein transcription factor), and MEF-3 [3, 10, 16, 17]. The G to A nucleotide transition might lose the binding activity on CP2 or ZID and might generate the binding activity on FTF or MEF-3. ZID binding activity was predicted to be most affected by motif sequences among the four transcription factors. Ribonuclease (Rnase) protection analysis indicated a wide expression of ZID, with highest levels in the brain, suggesting that ZID binding activity might be altered by the promoter SNP which was associated with MDD. Our polymorphism screening of the 5′-upstream region of the GMIP gene identified only one additional rare SNP (−1,007C/T). We searched a missense SNP in the databases (NCBI, JSNP, and Celera) and found only one validated missense SNP of the GMIP gene (rs12003; D641N). However, the minor allele of this SNP was not detected in our 328 Japanese samples, while the frequency of the minor allele in a Caucasian is 0.06. Further investigation is necessary to find a functional polymorphism which can be responsible for the susceptibility of MDD.

In conclusion, we have demonstrated for the first time the possible association between the genetic variation of the GMIP gene and MDD in a Japanese population. A false-positive association due to population stratification, and possible confounding factors such as stressful life events, adverse childhood experiences, and certain personality traits, socioeconomic status, etc., cannot be excluded in our case-control design, despite the precaution of ethnic matching of this study. Therefore, it is necessary to carry out further investigations to confirm our findings in other samples. If our results are replicated, potential −525G/A SNP function on transcriptional activity might contribute to understanding the molecular mechanisms of MDD.

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Relationship between XBP1 genotype and personality traits assessed by TCI and NEO-FFI

Ichiro Kusumi [a,*], Takuya Masui [a], Chihiro Kakiuchi [b], Katsuji Suzuki [a], Tatsuyuki Akimoto [a], Ryota Hashimoto [c], Hiroshi Kunugi [c], Tadafumi Kato [b], Tsukasa Koyama [a]

[a] *Department of Psychiatry, Hokkaido University Graduate School of Medicine, North 15, West 7, Kita-ku, Sapporo, Hokkaido 060-8638, Japan*
[b] *Laboratory for Molecular Dynamics of Mental Disorders, Brain Science Institute, RIKEN, Wako, Japan*
[c] *Department of Mental Disorder Research, National Institute of Neuroscience, NCNP, Kodaira, Japan*

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Abstract

There have been several researches on the role of personality in the pathophysiology of bipolar disorder. Recently, a polymorphism of XBP1, a pivotal gene in the endoplasmic reticulum (ER) stress response, was shown to contribute to the genetic risk factor for bipolar disorder. Therefore, in this study, we examined the relationship between the XBP1 gene polymorphism and the personality traits assessed by two self-rating scales, a shortened version of Temperament and Character Inventory (TCI) and NEO-Five Factor Inventory (NEO-FFI) in healthy subjects. The present results suggested that the XBP1 gene polymorphism was associated with the NEO-FFI score of neuroticism in female subjects. However, no significant differences in the other personality scale scores of both assessments were observed among normal subjects with −116C/C, C/G and G/G genotypes. Further investigations are necessary to examine the relationship in patients with bipolar disorder, or use full version of various self-rating personality assessments.

Keywords: XBP1; Polymorphism; Personality; TCI; NEO-FFI

Genetic factors significantly contribute to the determination of human personality traits although environmental influence is also important. Personality traits assessed by self-report questionnaires show moderate heritability [5]. Such inheritance is ultimately attributable to functional variants of genes programming brain development and function [4]. Some of these genes have also been implicated in the susceptibility to various psychiatric illnesses including mood disorders [5]. Several authors have paid attention to the premorbid personality traits of patients suffering from bipolar disorder. For example, Akiskal [1] showed that dysthymic, cyclothymic and hyperthymic temperaments represent putative development pathways to bipolarity in childhood and adolescence with clinically ascertained depressions. Bipolar patients might share a specific personality trait that represents the behavioral expression of some genetic neurochemical diathesis to the disease [2].

Recently, a polymorphism of XBP1 gene that plays a pivotal role in endoplasmic reticulum (ER) stress response was shown to contribute to the genetic risk factor for bipolar disorder [8], although negative findings were also reported [3,7]. Cell injury may develop under conditions where ER calcium homeostasis and, folding or processing of proteins is disturbed (referred to as ER stress), leading to the activation of unfolded protein response such as suppression of protein synthesis and expression of ER stress-related genes including *XBP1* [11]. The polymorphism (−116C → G) in the promoter region of the XBP1 gene was significantly more common in Japanese bipolar patients (odds ratio = 4.6). The XBP1-dependent transcription activity of −116G allele was lower than that of −116C allele, and induction of XBP1 expression after ER stress was markedly reduced in the cells with the G allele [8].

Therefore, it is possible that the XBP1 gene polymorphism may be involved in the development of personality specific for bipolar disorder. Recently, Kato et al. [9] reported a statistical trend for association between the XBP1 gene polymorphism and the Revised NEO Personality Inventory (NEO-PI-R) scores of

* Corresponding author. Tel.: +81 11 716 1161x5973; fax: +81 11 706 5081.
E-mail address: ikusumi@med.hokudai.ac.jp (I. Kusumi).

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Table 1
TCI scores in healthy subjects sorted by -116C/G polymorphism of XBP1 gene

Sample	TCI subscales	XBP1 polymorphism			ANCOVA
		C/C	C/G	G/G	
Total	(N=248)	(N=24)	(N=116)	(N=108)	
	Novelty seeking	50.7 ± 4.9	50.1 ± 6.0	50.2 ± 7.0	F=0.10, p=0.91
	Harm avoidance	54.1 ± 8.5	52.7 ± 7.7	53.4 ± 9.0	F=0.36, p=0.70
	Reward dependence	43.5 ± 4.9	43.2 ± 5.2	42.9 ± 4.9	F=0.34, p=0.71
	Persistence	12.5 ± 2.1	13.2 ± 2.9	12.8 ± 2.7	F=1.47, p=0.23
	Self-directedness	68.4 ± 10.0	71.3 ± 9.6	69.3 ± 10.0	F=1.67, p=0.19
	Cooperativeness	72.8 ± 5.8	71.8 ± 6.7	71.3 ± 6.7	F=0.29, p=0.75
	Self-transcedence	26.8 ± 4.9	28.0 ± 6.4	26.9 ± 5.6	F=1.30, p=0.28
Male	(N=141)	(N=7)	(N=71)	(N=63)	
	Novelty seeking	48.9 ± 3.4	49.9 ± 6.0	50.1 ± 6.0	F=0.30, p=0.74
	Harm avoidance	51.6 ± 4.5	52.5 ± 8.0	51.8 ± 8.3	F=0.43, p=0.65
	Reward dependence	42.4 ± 4.9	42.6 ± 5.4	42.1 ± 4.8	F=0.43, p=0.65
	Persistence	13.4 ± 1.7	13.5 ± 2.8	12.6 ± 2.8	F=2.02, p=0.14
	Self-directedness	67.4 ± 6.9	69.8 ± 10.5	69.0 ± 10.1	F=0.10, p=0.91
	Cooperativeness	71.9 ± 7.0	71.1 ± 7.3	70.6 ± 6.5	F=0.14, p=0.87
	Self-transcedence	25.7 ± 4.3	27.9 ± 6.6	26.8 ± 5.7	F=0.54, p=0.58
Female	(N=107)	(N=17)	(N=45)	(N=45)	
	Novelty seeking	51.4 ± 5.3	50.3 ± 6.1	50.3 ± 8.2	F=0.16, p=0.85
	Harm avoidance	55.2 ± 9.6	52.2 ± 7.4	55.7 ± 9.5	F=1.97, p=0.15
	Reward dependence	43.9 ± 3.6	44.2 ± 4.6	44.0 ± 4.8	F=0.03, p=0.97
	Persistence	12.1 ± 2.2	12.8 ± 3.1	13.2 ± 2.5	F=1.25, p=0.29
	Self-directedness	68.8 ± 10.3	73.7 ± 7.2	69.6 ± 10.4	F=3.03, p=0.05
	Cooperativeness	73.2 ± 5.4	72.8 ± 5.5	72.3 ± 7.0	F=0.17, p=0.84
	Self-transcedence	27.2 ± 5.2	28.2 ± 6.3	27.0 ± 5.6	F=0.49, p=0.61

TCI scores are expressed as the mean ± S.D.

agreeableness and neuroticism in healthy Japanese female volunteers. In this study we examined the relationship between the −116C/G polymorphism of the XBP1 gene and the personality traits measured by two representative self-report questionnaires, Temperament and Character Inventory (TCI) and NEO Five Factor Inventory (NEO-FFI), in the Japanese healthy male and female subjects.

Two hundred and forty-eight biologically unrelated healthy volunteers were all Japanese recruited from laboratory, office or hospital staff at Hokkaido University. They all underwent a direct interview to exclude clinical and family history of psychiatric disorders classified according to DSM-IV. There were 141 males and 107 females, and the average age was 31.6 ± 9.1 (mean ± S.D.) years. After complete description of the study, informed consent was obtained from all subjects. The research protocol was approved by the ethics committee of Hokkaido University Graduate School of Medicine.

DNA was extracted from 20 ml of whole blood by standard methods. Genotypes for XBP1 gene −116C/G polymorphism were determined using the TaqMan 5′-exonuclease allelic discrimination assay, described previously [6]. Briefly, primers and probes for detection of the SNP are: forward primer 5′-CTGTCACTCCGGATGGAAATAAGTC-3′, reverse primer 5′-ATCCCTGGCCAAAGGTACTTG-3′, probe 1 5′-VIC-CTCCCGCACGTAAC-MGB-3′, and probe 2 5′-FAM-TCCCGCAGGTAAC-MGB-3′. PCR cycling conditions were: at 95 °C for 10 min, 45 cycles of 92 °C for 15 s and 60 °C for 1 min.

After collecting the blood samples, all subjects filled out a shortened version of TCI, which consists of 125 questions with four possible answers [10]. Each score on the 4-point scale can range from 1 (strongly disagree) to 4 (strongly agree). A part of the participants (206 subjects, 119 males and 87 females, age: 32.5 ± 9.4 years) completed NEO-FFI, the shortened version of the NEO-PI-R, which consists of 60 questions with 5-point scales. The validity and reliability of the Japanese version of TCI and NEO-FFI have already been confirmed among different Japanese populations [8,12].

Age and sex are known to affect self-rating personality assessments. In order to examine the relationships between the XBP1 gene polymorphism and, TCI or NEO-FFI scores, one-way analysis of covariance (ANCOVA) was performed with XBP1 genotype as independent variables, and with age and sex as covariates. Statistical test was carried out using SPSS for Windows. P values less than .05 were considered statistically significant after Bonferroni's correction for multiple testing.

The TCI and NEO-FFI scores sorted by the −116C/G polymorphism of XBP1 gene are shown in Tables 1 and 2, respectively. Observed genotype distribution was consistent with Hardy–Weinberg equilibrium. The distribution of the XBP1 genotype in our sample was almost same as in the other Japanese samples [8,9]. Although there was no significant relationship between the XBP1-116C/G genotypes and seven personality dimension scores of TCI (Table 1), the NEO-FFI score of neuroticism showed a significant association with the XBP1 gene polymorphism in females (F=6.41, p=0.003),

Table 2
NEO-FFI scores in healthy subjects sorted by -116C/G polymorphism of XBP1 gene

Sample	NEO-FFI subscales	XBP1 polymorphism			ANCOVA
		C/C	C/G	G/G	
Total	(N=206)	(N=20)	(N=98)	(N=88)	
	Neuroticism	29.4 ± 8.1	25.0 ± 6.7	26.2 ± 7.7	F=1.97, p=0.14
	Extraversion	23.2 ± 5.5	24.2 ± 9.1	22.0 ± 6.3	F=2.28, p=0.11
	Openness	29.6 ± 6.5	29.2 ± 5.4	29.1 ± 5.0	F=0.05, p=0.95
	Agreeableness	30.0 ± 5.4	28.4 ± 5.6	28.6 ± 5.0	F=0.51, p=0.60
	Conscientiousness	24.7 ± 8.5	26.3 ± 7.0	24.5 ± 5.8	F=1.50, p=0.23
Male	(N=119)	(N=7)	(N=61)	(N=51)	
	Neuroticism	23.7 ± 8.6	25.1 ± 6.9	24.9 ± 7.8	F=0.47, p=0.62
	Extraversion	24.3 ± 5.5	24.2 ± 10.6	21.8 ± 5.7	F=1.25, p=0.29
	Openness	28.9 ± 7.3	29.2 ± 5.7	28.6 ± 5.5	F=0.08, p=0.92
	Agreeableness	31.6 ± 4.7	28.2 ± 5.9	27.8 ± 5.2	F=1.45, p=0.24
	Conscientiousness	29.7 ± 8.4	26.7 ± 6.8	24.2 ± 5.7	F=2.87, p=0.06
Female	(N=87)	(N=13)	(N=37)	(N=37)	
	Neuroticism	32.5 ± 6.2	25.0 ± 6.3	28.1 ± 7.4	F=6.41, p=0.003
	Extraversion	22.6 ± 5.7	24.2 ± 6.1	22.3 ± 7.2	F=0.69, p=0.51
	Openness	30.0 ± 6.3	29.3 ± 4.9	29.7 ± 4.2	F=0.15, p=0.86
	Agreeableness	29.1 ± 5.7	28.9 ± 5.1	29.6 ± 4.7	F=0.15, p=0.87
	Conscientiousness	22.0 ± 7.5	25.6 ± 7.3	24.8 ± 6.0	F=1.32, p=0.27

NEO-FFI scores are expressed as the mean ± S.D.

not in male or all subjects (Table 2). No significant associations were observed in the other four dimension scores of NEO-FFI.

If some personality trait might be involved in the vulnerability of bipolar disorder, it should be a continuous factor from normal control to bipolar disorder. On the other hand, the G allele of XBP1 gene, a risk for bipolar disorder, is also observed in normal controls, not only in bipolar disorder [8]. Accordingly, it is of significance to examine the relationship between the XBP1 genotype and the personality traits in normal subjects. The present study partially confirmed the finding of Kato et al. [9] reporting a trend for association between the XBP1 gene polymorphism and the NEO-PI-R scores of agreeableness and neuroticism in healthy volunteers. In contrast to the previous report [9], the present study examined the relationship between the XBP1 genotype and the personality traits assessed by not only NEO but also TCI in both male and female healthy subjects. Thus, it clearly demonstrated that a significant association between the XBP1 polymorphism and the NEO score of neuroticism was observed only in female subjects. This finding suggests that gender differences exist in contribution of genetic factors to behavioral phenotypes. The discrepancy for the finding of agreeableness is unknown, but it may arises from the methodological difference between the two reports that the questionnaire used is full or shortened version of NEO. Further studies are necessary to examine the relationship between the XBP1 genotype and the personality traits in patients with bipolar disorder. The limitation of this study is to use the shortened version of TCI and NEO-PI-R. Analyzing subscales in each dimension might enable us to assess more specific facets related to the XBP1 gene polymorphism.

In conclusion, the present study suggests that the XBP1 gene polymorphism is associated with the NEO-FFI score of neuroticism in healthy female subjects. Further investigations are needed to examine the relationship in patients with bipolar disorder, or use full version of various self-rating personality assessments.

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Effect of antipsychotic drugs on DISC1 and dysbindin expression in mouse frontal cortex and hippocampus

S. Chiba[1,2], R. Hashimoto[1], S. Hattori[1], M. Yohda[2], B. Lipska[3], D. R. Weinberger[3], and H. Kunugi[1]

[1] Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, and
[2] Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, Koganei, Japan
[3] Clinical Brain Disorders Branch, National Institute of Mental Health, National Institutes of Health, Bethesda, MD, USA

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Summary. Altered expression of Disrupted-In-Schizophrenia-1 (DISC1) and dysbindin (DTNBP1), susceptibility genes for schizophrenia, in schizophrenic brain has been reported; however, the possible effect of antipsychotics on the expression levels of these genes has not yet been studied. We measured the mRNA expression levels of these genes in frontal cortex and hippocampus of mice chronically treated with typical and atypical antipsychotics by a real-time quantitative RT-PCR method. We found that atypical antipsychotics, olanzapine and risperidone, in a clinically relevant dose increased DISC1 expression levels in frontal cortex, while a typical antipsychotic, haloperidol, did not. No significant effect on dysbindin expression levels was observed in either brain region. These data suggest that prior evidence of decreased expression of dysbindin in postmortem brain of schizophrenics is not likely to be a simple artifact of antemortem drug treatment. Our results also suggest a potential role of DISC1 in the therapeutic mechanisms of certain atypical antipsychotics.

Keywords: Antipsychotic, DISC1, dysbindin, schizophrenia, gene expression.

Introduction

Schizophrenia is a common neuropsychiatric disorder affecting 0.5–1% of the general population worldwide. The pathophysiology of schizophrenia is still unclear; however, this disease is highly heritable (Owen et al., 2004). Several genes, e.g. Disrupted-In-Schizophrenia 1 (DISC1), dysbindin, catechol-O-methyltransferase, neuregulin 1, the regulator of G-protein signaling-4, GRM3 and G72 have been proposed as susceptibility genes for schizophrenia (Harrison and Weinberger, 2005).

The DISC1 gene has initially been identified at the breakpoint of a balanced translocation (1;11) (q42.1;q14.3), which segregates with schizophrenia and related psychiatric

disorders in a large Scottish family (Millar et al., 2000). Genetic association and linkage studies have also suggested that the DISC1 gene may be implicated in schizophrenia in independent populations (Ekelund et al., 2001, 2004; Hennah et al., 2003; Hodgkinson et al., 2004; Callicott et al., 2005). The function of DISC1 is still unclear, however, increasing evidence suggests a role in cytoskeletal organization, as DISC1 interacting proteins are associated with the components of microtubule and actin (Millar et al., 2003; Miyoshi et al., 2003; Morris et al., 2003b; Ozeki et al., 2003). Expression analysis of DISC1 using lymphocytes from patients in a balanced translocation family revealed that patients with the breakpoint expressed lower expression of DISC1 compared with controls, suggesting that lower levels of DISC1 might be related to the pathogenesis of schizophrenia (James et al., 2004). Further recent evidence implicates DISC1 in transcription regulation (Sawamura et al., 2005).

A significant association between schizophrenia and genetic variation in dysbindin has been reported in various populations from Ireland, Wales, Germany/Hungary/Israel, Sweden, Bulgaria, United States, China, and Japan (Straub et al., 2002; Schwab et al., 2003; Tang et al., 2003; Van Den Bogaert et al., 2003; van den Oord et al., 2003; Funke et al., 2004; Kirov et al., 2004; Numakawa et al., 2004; Williams et al., 2004). One study, which failed to replicate a positive association based on single SNPs in an Irish population, was subsequently positive using a haplotype strategy (Morris et al., 2003a). Dysbindin is a binding partner of alpha- and beta-dystrobrevins, which are parts of the dystrophin-associated protein complex (Benson et al., 2001), and is a component of the biogenesis of lysosome-related organelles complex 1, which regulates trafficking to lysosome-related organelles (Li et al., 2003). Recently, dysbindin has been reported to play roles in glutamate release and in cell models of neuroprotection, which have also been hypothesized to be related to the pathophysiology of schizophrenia (Numakawa et al., 2004).

Abnormal expression of DISC1 and dysbindin in schizophrenic brain has been reported. The expression ratio of an isoform of DISC1 was increased within the nuclear fraction extracted from orbitofrontal cortex of brains from patients with schizophrenia and also major depression (Sawamura et al., 2005) and the mRNA levels of DISC1 tended to be increased in hippocampus in patients with schizophrenia (Lipska et al., 2004). The expression levels of dysbindin mRNA and protein were reduced in the prefrontal cortex and hippocampus in schizophrenic brain (McClintock et al., 2003; Talbot et al., 2004; Weickert et al., 2004). In studies of schizophrenic postmortem brain, patients have received antipsychotic medication at various times in their lives, including in most cases around the time of death, while control subjects do not. Thus, possible effects of antipsychotics on gene expression are an important potential confounder when interpreting results of postmortem tissue studies of schizophrenic cases. Here, we examined for a possible effect of chronic administration of typical and atypical antipsychotics on the mRNA expression levels of DISC1 and dysbindin in mouse frontal cortex and hippocampus.

Materials and methods

Drug preparation

Haloperidol, risperidone and clozapine were purchased from Sigma-Aldrich (Tokyo, Japan). Olanzapine was a gift from Eli Lilly and Company Lilly Corporate Center (Greenfield, IN). Haloperidol was dissolved in glacial acetic acid solution, diluted with saline up to 1 ml with adjustment to pH 5.5 with 1 N sodium hydroxide, and brought to a final concentration of 0.005 or 0.1 mg/ml. Clozapine was dissolved in glacial acetic acid solution, diluted with saline up to 1 ml with adjustment to pH 5.5 with 8 N sodium hydroxide, and brought to a final concentration of 0.05 or 1 mg/ml. Olanzapine and risperidone were dissolved in 1 N acetic acid solution, diluted

with saline up to 1 ml with adjustment to pH 5.5 with 1 N sodium hydroxide, and brought to a final concentration of 0.004 or 1 mg/ml (olanzapine) and 0.0025 or 0.075 mg/ml (risperidone), respectively.

Animals and drug treatment

Male C57BL/6J mice (CLEA, Japan) weighing 20–25 g received once-daily injections intraperitoneally (i.p.) for 21 days with haloperidol (clinical dose: 0.05 mg/kg; high dose: 1 mg/kg), olanzapine (clinical dose: 0.04 mg/kg; high dose: 10 mg/kg), risperidone (clinical dose: 0.025 mg/kg; high dose: 0.75 mg/kg), clozapine (clinical dose: 0.5 mg/kg; high dose: 10 mg/kg), or vehicle (0.1 N acetic acid in saline). This dose regimen was chosen to simulate the therapeutic range of doses given to patients (Kapur et al., 2000), and was shown to be effective in several behavioral and biochemical studies (Lipska et al., 2001; Parikh et al., 2004). Haloperidol is a typical (conventional) antipsychotic, whereas the others are termed atypical antipsychotics, which are associated with fewer motor side effects and possibly greater efficacy. Animals were sacrificed 20 hr after the final injection. Brain regions were removed, frozen in liquid nitrogen, and stored at −80°C. The experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of the National Institute of Neuroscience, Japan.

RNA extraction, DNAse treatment and reverse transcriptase reaction

Tissues from frontal cortex or hippocampus were homogenized in 4 mol/L guanidinium isothiocyanate (containing 25 nmol/L sodium citrate, pH 7.5, and 1% 2-mercaptoethanol), and total RNA was isolated by a standard phenol-chloroform extraction. The yield of total RNA determined by the absorbance at 260 nm and the quality of total RNA was also analyzed using agarose gel electrophoresis.

Total RNA was treated with DNase for removal of contaminating genomic DNA using DNase Treatment & Removal Reagents (Ambion, Austin, TX), according to the manufacturer's protocol. Total RNA (3.3 μg) treated with DNase was used in 50 μl of reverse transcriptase reaction to synthesize cDNA, by using a SuperScriptII First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. Briefly, total RNA (3.3 μg) was denatured with 1 mM of dNTP and 6 ng/μl of random primers at 65°C for 5 min. After addition of RT buffer, dithiothreitol (10 mM in final concentration), RNAsin Plus RNase Inhibitor (40 units) and SuperScriptII RT (200 units), the reaction mixture was incubated at 25°C for 10 min, at 42°C for 40 min, and at 70°C for 15 min. RNAse H (2 units) was added to the reaction mixture and then incubated at 37°C for 20 min.

Real-time quantitative PCR

The TaqMan® Endogenous Controls (Applied Biosystems, Foster City, CA) were used for measurements of house keeping genes, β-actin (Mm00607939_s1) and GAPDH (Mm99999915_q1). TaqMan® Gene Expression Assays (Applied Biosystems) were used for DISC1 (Mm00533313_m1) and dysbindin (Mm00458743_m1) genes. Both TaqMan assay kits included optimized concentrations of primers and probes to detect the target gene expression. The levels of mRNA expression of these genes were measured by a real-time quantitative RT-PCR using an ABI Prism 7900 sequence detection system with 384-well format (Applied Biosystems), described previously (Hashimoto et al., 2004). Briefly, each 20 μl PCR reaction mixture contained 6 μl of cDNA, 0.5 μl of TaqMan assay kit and 10 μl of TaqMan Universal PCR Mastermix (Applied Biosystems). PCR cycling conditions were: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. PCR data were obtained with the Sequence Detector Software (SDS version 2.1, Applied Biosystems) and quantified by a standard curve method. Standard curves were prepared using serial dilutions (1:4) of pooled cDNA from total RNA derived from whole brain of three mice.

Statistical analysis

An analysis of variance (ANOVA) was used to compare gene expression levels between drug treatment groups with SPSS 11.0J for Windows (SPSS Japan Inc, Tokyo, Japan). Bonferroni post hoc comparisons were performed when applicable. Statistical significance was defined at $p < 0.05$.

Results

The expression levels of the two standard "housekeeping" genes, β-actin and GAPDH in frontal cortex and hippocampus of control mice and mice treated with typical or atypical antipsychotics for three weeks in clinical or high dose are shown in Table 1. The expression levels of both genes in frontal cortex and hippocampus were not significantly influenced by drug treatments at clinical dosing (all p values >0.4, ANOVA), however, there was a significant drug treatment effect on expression of the two house keeping genes

Table 1. Expression analysis of house keeping genes in frontal cortex and hippocampus in clinical and high dose

Drugs		Clinical dose		High dose			
		Frontal cortex (n)	Hippocampus (n)	Frontal cortex (n)	*p* value	Hippocampus (n)	*p* value
VEH	β-actin	100.0 ± 36.4 (19)	100.0 ± 33.1 (19)	100.0 ± 36.4 (19)		100.0 ± 33.1 (19)	
	GAPDH	100.0 ± 22.8 (19)	100.0 ± 26.6 (19)	100.0 ± 22.8 (19)		100.0 ± 26.6 (19)	
HPD	β-actin	105.4 ± 33.0 (10)	91.5 ± 16.7 (10)	72.2 ± 22.6 (12)	NS	102.8 ± 39.3 (12)	NS
	GAPDH	95.8 ± 15.9 (10)	96.7 ± 24.5 (10)	86.1 ± 15.5 (12)	NS	112.8 ± 29.7 (12)	NS
OZP	β-actin	139.4 ± 34.8 (10)	90.3 ± 40.4 (10)	67.4 ± 19.4 (12)	0.023	65.8 ± 22.1 (12)	0.041
	GAPDH	118.3 ± 22.8 (10)	89.4 ± 26.3 (10)	73.5 ± 11.3 (12)	0.002	77.5 ± 19.9 (12)	NS
RPD	β-actin	99.2 ± 32.7 (10)	83.4 ± 16.8 (10)	75.6 ± 24.8 (11)	NS	75.0 ± 33.1 (11)	NS
	GAPDH	92.3 ± 24.9 (10)	93.0 ± 30.4 (10)	88.0 ± 20.9 (11)	NS	94.7 ± 34.9 (11)	NS
CZP	β-actin	105.7 ± 40.9 (9)	88.1 ± 27.3 (9)	67.2 ± 25.1 (11)	0.027	84.5 ± 22.8 (12)	NS
	GAPDH	93.1 ± 36.3 (9)	85.6 ± 20.3 (9)	72.9 ± 14.0 (11)	0.002	90.3 ± 22.7 (12)	NS

VEH vehicle, *HPD* haloperidol, *OZP* olanzapine, *RPD* risperidone, *CZP* clozapine, *NS* not significant, *n* number of animals used. Data are the means ± SD. Post hoc *p* values compared with VEH are shown

at high dosing (frontal cortex: β-actin, $F_{4, 60} = 3.97$, $p = 0.006$, GAPDH, $F_{4, 60} = 5.73$, $p = 0.001$; hippocampus: β-actin, $F_{4, 61} = 3.42$, $p = 0.014$, GAPDH, $F_{4, 61} = 2.79$, $p = 0.034$). Post hoc analysis revealed that the expression levels of β-actin and/or GAPDH were significantly decreased in mice received clozapine or olanzapine in high dose. Body weight loss or lower level of body weight gain after three weeks of drug administration was also observed in clozapine or olanzapine treated mice in high dose compared with control mice (body weights change ± standard deviation for clozapine: -0.73 ± 0.51 g, $p = 0.00005$; olanzapine: 0.67 ± 0.81 g, $p = 0.083$, control: 1.57 ± 1.62 g), while no significant difference was observed at the clinical dose (clozapine: 2.5 ± 1.02 g, $p = 0.13$; olanzapine: 2.31 ± 0.88 g, $p = 0.19$; control: 1.57 ± 1.62 g). These results suggest that olanzapine and clozapine treatment in high dose might affect the general health of mice, which could result in the altered expression levels of house keeping genes. Thus, we focused on possible effects on the gene expression levels of DISC1 and dysbindin at the clinical dose only.

The expression levels of DISC1 mRNA normalized by β-actin and GAPDH (to reduce effects of possible mRNA degradation not detectable by electrophoresis and possible variations in RT efficiency) in frontal cortex of mice administrated with a typical antipsychotic (haloperidol) or atypical antipsychotics (olanzapine, risperidone, clozapine) at the clinical dose are shown in Fig. 1. Analysis of the DISC1 expression demonstrated significant effects of drug treatments (normalized by β-actin, $F_{4, 53} = 6.41$, $p < 0.001$, or GAPDH, $F_{4, 53} = 5.25$, $p = 0.001$). Post hoc analysis revealed that DISC1 expression levels were increased by treatments with atypical antipsychotics, olanzapine (normalized by β-actin: 36%, $p = 0.0029$; or GAPDH: 64%, $p = 0.016$) and risperidone (normalized by β-actin: 39%, $p = 0.0077$; or GAPDH: 55%, $p = 0.0031$)

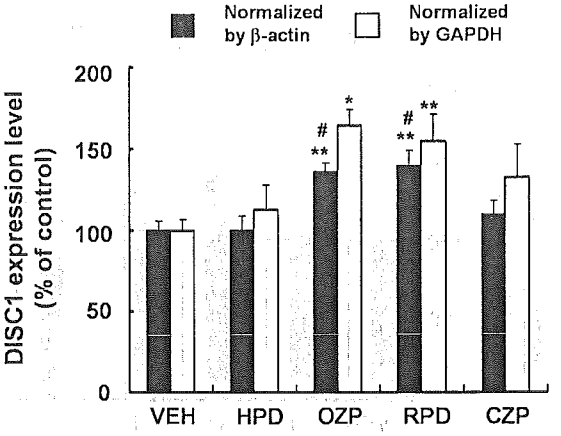

Fig. 1. Relative expression levels of DISC1 in frontal cortex in clinical dose. DISC1 mRNA expression levels normalized by β-actin or GAPDH in control mice (treated with vehicle: VEH) and mice treated with haloperidol (HPD), olanzapine (OZP) risperidone (RPD), or clozapine (CZP) are shown. Expression levels were calculated by comparison to percentage of average of those of control mice. Data are the means ± SEM from 19 control mice or mice treated with HPD (n = 10), OZP (n = 10), RPD (n = 10) or CZP (n = 9). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the control group. $\#p < 0.05$, compared with the haloperidol treated group

compared with the control group. No significant difference of DISC1 expression levels was observed after treatment with the typical antipsychotic (haloperidol). Elevated expression levels of the DISC1 gene normalized by β-actin were also found in olanzapine (36%, $p = 0.013$) and risperidone (39%, $p = 0.028$) treatment groups compared with haloperidol. Similar trends were obtained after normalization with GAPDH (olanzapine: 45%, $p = 0.095$; risperidone: 37%, $p = 0.30$). Treatment with clozapine tended to increase the expression levels of the DISC1 gene compared with control group, although they did not reach statistical significance.

The expression levels of DISC1 mRNA normalized by β-actin and GAPDH in hippocampus of mice administrated with a typical antipsychotic or atypical antipsychotics at the clinical dose are shown in Fig. 2. Analysis of the DISC1 expression in hippocampus

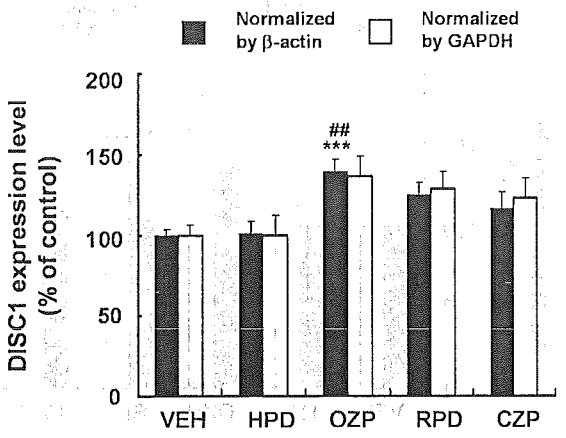

Fig. 2. Relative expression levels of DISC1 in hippocampus in clinical dose. DISC1 mRNA expression levels normalized by β-actin or GAPDH in control mice (treated with vehicle: VEH) and mice treated with haloperidol (HPD), olanzapine (OZP) risperidone (RPD), or clozapine (CZP) are shown. Expression levels were calculated by comparison to percentage of average of those of control mice. Data are the means ± SEM from 19 control mice or mice treated with HPD (n = 10), OZP (n = 10), RPD (n = 10) or CZP (n = 9). ***$p < 0.001$, compared with the control group. ##$p < 0.01$, compared with the haloperidol treated group

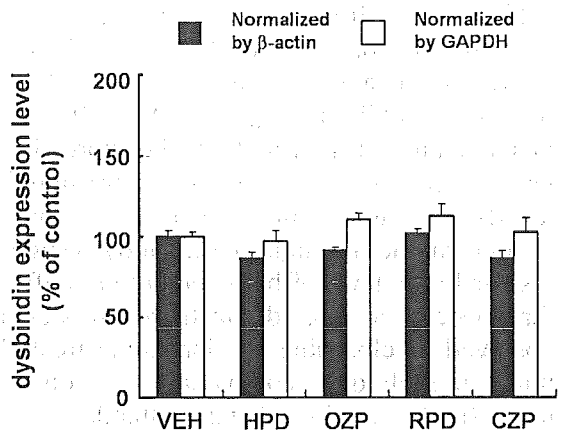

Fig. 3. Relative expression levels of dysbindin in frontal cortex in clinical dose. Dysbindin mRNA expression levels normalized by β-actin or GAPDH in control mice (treated with vehicle: VEH) and mice treated with haloperidol (HPD), olanzapine (OZP) risperidone (RPD), or clozapine (CZP) are shown. Expression levels were calculated by comparison to percentage of average of those of control mice. Data are the means ± SEM from 19 control mice or mice treated with HPD (n = 10), OZP (n = 10), RPD (n = 10) or CZP (n = 9)

demonstrated significant effects of drug treatments (normalized by β-actin, $F_{4,53} = 6.09$, $p < 0.001$, or GAPDH, $F_{4,53} = 2.82$, $p = 0.034$). In post hoc analysis, DISC1 expression levels normalized by β-actin were significantly increased by the atypical antipsychotic, olanzapine, compared with control (39%, $p = 0.0006$) or haloperidol (29%, $p = 0.0054$) and similar trend was observed in risperidone compared with control (25%, $p = 0.079$). On the other hand, a slight increase of DISC1 expression was also found when normalizing by GAPDH (olanzapine vs control: 37%, $p = 0.094$; olanzapine vs haloperidol: 29%, $p = 0.23$; risperidone vs control: 29%, $p = 0.39$), which did not reach statistical significance. No effect of haloperidol or clozapine treatment was found in either normalization. These findings suggest that the mRNA expression levels of the DISC1 gene are increased by the chronic administration of some atypical antipsychotics in frontal cortex and possibly in hippocampus.

The expression levels of dysbindin mRNA normalized by β-actin and GAPDH in frontal cortex and hippocampus of mice administered treatment with a typical antipsychotic or atypical antipsychotics at the clinical dose are shown in Figs. 3 and 4. Dysbindin gene expression normalized by either β-actin or GAPDH in frontal cortex or hippocampus did not significantly differ between the treatment groups (frontal cortex: GAPDH, $F_{4,53} = 1.45$, $p = 0.23$; hippocampus: β-actin, $F_{4,53} = 0.64$, $p = 0.64$, GAPDH, $F_{4,53} = 0.46$, $p = 0.77$), except for that in frontal cortex normalized by β-actin ($F_{4,53} = 3.68$, $p = 0.01$). However, post hoc analysis demonstrated no significant difference in dysbindin expression in frontal cortex normalized by β-actin in any of the drug treatments, although there were trends towards slightly decreased expression of dysbindin in mice

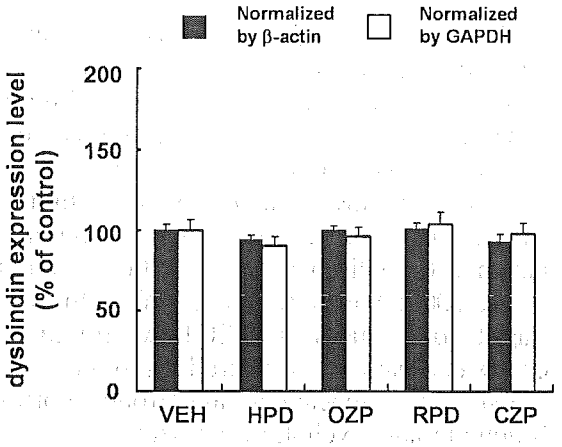

Fig. 4. Relative expression levels of dysbindin in hippocampus in clinical dose. Dysbindin mRNA expression levels normalized by β-actin or GAPDH in control mice (treated with vehicle: VEH) and mice treated with haloperidol (HPD), olanzapine (OZP) risperidone (RPD), or clozapine (CZP) are shown. Expression levels were calculated by comparison to percentage of average of those of control mice. Data are the means ± SEM from 19 control mice or mice treated with HPD (n = 10), OZP (n = 10), RPD (n = 10) or CZP (n = 9)

treated with haloperidol, compared with control (14%, $p = 0.074$) and in mice treated with risperidone (16%, $p = 0.094$). These data suggest that administration of typical and atypical antipsychotics do not have a consistent influence on mRNA expression levels of the dysbindin gene in frontal cortex or in hippocampus.

Discussion

In this study, we have measured mRNA expression levels of two susceptibility genes for schizophrenia, DISC1 and dysbindin, in frontal cortex and hippocampus using a real-time quantitative RT-PCR in mice treated chronically with typical or atypical antipsychotics. We found preliminary evidence that the expression levels of DISC1 may be altered by treatment with the atypical agents in frontal cortex and possibly in hippocampus and that the expression levels of dysbindin may not be changed under these conditions. Upregulation of DISC1 mRNA in frontal cortex by olanzapine and risperidone was observed in both normalizations by β-actin and GAPDH, however, that in hippocampus by olanzapine was found only in normalization by β-actin. As DISC1 has been shown to interact with actin (Miyoshi et al., 2003), it is possible that the DISC1 mRNA expression level normalized by β-actin in hippocampus may be somehow affected by the interaction. Upregulation of DISC1 mRNA in hippocampus by atypical antipsychotics appears to be marginal while that in frontal cortex is more apparent. As DISC1 expression is dominant in hippocampus compared with frontal cortex (Miyoshi et al., 2003), there is a possibility that this differential expression of DISC1 might affect the degree of the upregulation of DISC1 mRNA by the atypical antipsychotics.

Specifically, there was an increase of DISC1 expression levels after treatment with olanzapine and risperidone and possibly with clozapine in a simulated clinical dose in frontal cortex. As consistent results were obtained from normalization of the DISC1 expression by two house keeping genes, our findings would seem to be robust at least in comparison to results that might have been based on using only one control gene. However, it should be noted that there were some effects of antipsychotics on housekeeping gene expression, though largely nonsignificant. It is conceivable that some of the effect on our measures of DISC1 expression could be exaggerated by these effects on our control genes, as significant effects of drug treatments on the raw expression levels of DISC1 (non-normalized) were not observed in either frontal cortex or hippocampus (data not shown). Our data raise the possibility that DISC1 may be involved in the treatment of schizophrenia. However, as our study did not include the measurement of DISC1 proteins, or expression in other brain regions, or of treatment with other psychotropic drugs, further work is necessary to clarify whether

changes in DISC1 mRNA impact on protein expression and are specific for brain regions and psychotropic drugs. It also should be noted that we measured expression only of the common transcript for both of these genes. It is not currently known whether schizophrenia involves alternate processing of these genes into disease related transcripts or isoforms and we cannot rule out that treatment may impact on variable splicing or processing of these genes.

A balanced translocation in the DISC1 gene segregates with schizophrenia and other major psychiatric illnesses in a Scottish family (Millar et al., 2000). However, little is known about how the translocation affects the expression and/or function of the DISC1 gene. DISC1 protein expression in lymphoblasts derived from the family member with the translocation was observed to be decreased but the mutant truncated form of DISC1, which should be produced by the translocation, was not found (James et al., 2004). It is unknown whether the expression of DISC1 in brains of the family members is altered or not, however, this observation in peripheral cells suggested that the translocation might decrease the expression of DISC1. Alternatively, mutant truncated form of DISC1, which has been shown to play a role in inhibiting neurite outgrowth (Ozeki et al., 2003), might down-regulate the DISC1 protein expression and/or function. These findings suggest that reduced expression of DISC1 in brain might be expected in schizophrenic brain if DISC1 is involved in the pathogenesis of schizophrenia. On the other hand, gross expression levels of DISC1 protein have not been found to be changed in frontal cortex in patients with schizophrenia (Sawamura et al., 2005) and expression levels of DISC1 mRNA tended to be increased in hippocampus of schizophrenia patients (Lipska et al., 2004). Our data suggest that increased expression of DISC1 mRNA may be, at least in part, related to treatment with some atypical antipsychotics.

Evidence that dysbindin is associated with schizophrenia is now quite strong, although no functional mutation in dysbindin gene has yet been identified. Recent postmortem studies have found decreased expression of dysbindin mRNA and protein in hippocampus and frontal cortex in schizophrenic patients (McClintock et al., 2003; Talbot et al., 2004; Weickert et al., 2004). In contrast to our data with DISC1, we found no consistent pattern of altered dysbindin expression in hippocampus and frontal cortex following antipsychotic treatment.

Knowledge about protein functions of DISC1 and dysbindin is insufficient, however, we discuss a possibility how these genes affect the mechanisms of schizophrenia. As DISC1 has a prominent role in the neurite extension and its expression is developmentally regulated (Ozeki et al., 2003), upregulation of DISC1 could support the maturation of dendritic spine, which is believed to be affected in schizophrenia. As dysbindin promotes glutamate release in neuronal culture (Numakawa et al., 2004), reduced expression of dysbindin in schizophrenic brain could be relevant to glutamatergic dysfunction, which has been implicated in the pathophysiology of schizophrenia.

In summary, our findings offer preliminary evidence that altered expression of DISC1 may be caused by certain antipsychotic drugs, suggesting a role for DISC1 in therapeutic actions of these drugs. Additional studies are warranted to examine DISC1 and dysbindin expression, including western blotting analysis, in situ hybridization, immunohistochemistry, and the effect of other psychotropic drugs.

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