INTRODUCTION

The disrupted-in-schizophrenia 1 (DISC1) gene was initially identified at the breakpoint of a balanced translocation (1;11)(q42.1;q14.3), which segregated with major mental disorders in a large Scottish family (1). In this family, patients with schizophrenia, bipolar disorder and recurrent major depressive disorder (MDD) had been identified as carriers of the translocation (1,2). Subsequent genetic studies in several independent populations, including association and linkage studies, have also suggested that the DISC1 gene may be implicated in both schizophrenia and bipolar disorder (3–10). Previous studies suggest that genetic variations within the DISC1 gene, which increase risk for schizophrenia, are associated with the cerebral cortical gray matter and hippocampal volumes and function (9,11,12). However, no association study between the DISC1 gene and MDD has not yet been reported, despite there are more patients with MDD than those with schizophrenia in the original Scottish family members with translocation.

DISC1 is a multi-functional protein. Several research groups have identified DISC1 interacting proteins, which are associated with the components of the cytoskeleton and centrosome, such as dynein, Nudel, elongation protein zeta-1, etc. (13–18). DISC1 plays critical roles on the cerebral cortex development via microtubular dynamics and the DISC1–dynein complex (13). Another function of DISC1 may be modulation of cAMP signaling via an interaction with phosphodiesterase 4B, which also has been found to be disrupted by a balanced translocation in a patient with schizophrenia (19). Other functions of DISC1, including the mitochondrial and nuclear related functions, have also been suggested (15,20,21).

Here, we report an association of the genetic variations of DISC1, including Ser704Cys (rs821616) single-nucleotide polymorphism (SNP) and MDD. The risk allele (Cys704) for MDD is associated with the reduced gray matter volume and fractional anisotropy (FA) in prefrontal white matter in healthy subjects. Furthermore, the risk allele is linked to lower ERK activity (extracellular signal-regulated kinase), which has been suggested to have a role in the pathophysiology in MDD.

RESULTS

Association between the genetic variants of the DISC1 gene and major depression

We examined possible association between genetic variants in the DISC1 gene, including Ser704Cys SNP and MDD or schizophrenia. We genotyped 13 SNPs to cover the DISC1 locus at an average density of 31 kb. We found a significant association between genetic variations in DISC1 and MDD (Table 1). The Cys allele frequency of SNP12 (Ser704Cys) was greater in patients with MDD when compared with controls ($\chi^2 = 7.88$, df = 1, $P = 0.005$, odds ratio = 1.46, 95% CI 1.12–1.92). There was a weak evidence for an association with SNP1 ($P = 0.048$). As previous association studies of the DISC1 gene applied three-marker haplotype analysis (4,8,9), we performed this analysis. Consistent with the individual marker results, the three marker haplotypes containing SNPs 11–13 were associated with MDD: SNP11 (G allele)–SNP12(Cys)–SNP13(C allele) ($P = 0.002$; Table 2). When we examined the association between genetic variations of DISC1 and schizophrenia, a weak evidence for association was observed with SNP7 ($P = 0.0496$), whereas SNP12 (Ser704Cys) was not significantly associated with schizophrenia (Table 1). Because a prior study reported sex-dependent effects on association with schizophrenia (4), we examined males and females separately. This analysis revealed stronger evidence for association with SNP9 in female patients with schizophrenia ($P = 0.0088$), but no association in male subjects, whereas a prominent gender effect was not found in MDD (Table 3). Three marker haplotypes were not associated with schizophrenia (data not shown). SNP12 showed a strong linkage disequilibrium (LD) with SNP10 and SNP11, moderate LD with SNP1 and SNP13 and weak LD with SNP6 in controls, and similar LD results were obtained in MDD and schizophrenia (Supplementary Material, Tables S1–S3). LD pattern of our data was similar to that of HapMap database in the Japanese population. Our results suggest that DISC1 is associated with MDD and with schizophrenia and that Ser704Cys SNP, in particular, is associated with MDD in our sample.

Effects of the DISC1 Ser704Cys polymorphism on *in vivo* brain structure

As the abnormalities in the brain gray matter volume and white matter microstructure have been implicated in the biology of mood disorders and schizophrenia (22,23), we examined the possible effects of the Ser704Cys SNPs and other associated SNPs with schizophrenia on brain structure in healthy subjects (demographic information for Ser704Cys, Table 4). We found a bilateral (left dominant) reduction in the gray matter volume in the anterior cingulate cortex (ACC) [Brodmann area (BA) 24, Talairach coordinates x, y, $z = -6$, 27, 17, respectively, $t = 3.58$], cingulate gyrus (BA24, Talairach coordinates x, y, $z = 0$, 3, 34, respectively, $t = 3.7$) and the posterior cingulate gyrus (BA31, Talairach coordinates x, y, $z = -9$, -39, 36, respectively, $t = 3.39$) in cys-DISC1 carriers (cys/cys and cys/ser) compared with ser/ser-DISC1 individuals by tensor-based morphometry (TBM) analysis (Fig. 1, upper panel). When we analyzed the effect of this SNP on brain morphology in the other direction (i.e. cys > ser), we found a significant reduction in the volumes of the lateral ventricle, interhemispheric fissure and the bilateral Sylvian fissure in ser/ser-DISC1 individuals compared with cys-DISC1 carriers (Fig. 1, lower panel). This finding suggests that cys-DISC1 carriers have expanded cerebrospinal fluid (CSF) space compared with ser/ser-DISC1 individuals, possibly consistent with our finding that cys carriers in our analysis have reductions in tissue volume measures. We did not detect significant differences in hippocampal volume related to this DISC1 polymorphism with even at a lenient threshold at $P < 0.05$. In comparison with ser/ser-DISC1 individuals, cys-DISC1 carriers demonstrated a significantly decreased FA value in the frontal white matter ($P < 0.001$) (Fig. 2). The reversed contrast, i.e. increased FA in cys-DISC1 carriers, was not observed even at the level of

Table 1. Allele distributions for 13 SNPs in the DISC1 gene in patients with MDD, those with schizophrenia and controls

SNP	dbSNP	Distance from SNP1	Major/minor polymorphism	Amino acid substitution	Controls $n = 717$	MDD $n = 373$	*P*-value	OR	SZ $n = 658$	*P*-value	OR
1	rs6541281		C/T		0.122	0.094	*0.048*	*0.75*	0.105	0.16	—
2	rs3738401	17161	G/A	Gln264Arg	0.260	0.263	0.89	—	0.264	0.83	—
3	rs1340982	48898	C/T		0.461	0.456	0.82	—	0.459	0.92	—
4	rs1322784	115801	T/C		0.351	0.371	0.36	—	0.375	0.19	—
5	rs1535529	141475	C/T		0.425	0.441	0.48	—	0.410	0.42	—
6	rs7551537	159083	C/T		0.363	0.363	0.98	—	0.382	0.29	—
7	rs999710	197809	C/T		0.404	0.424	0.39	—	0.441	*0.05*	1.16
8	rs967433	218681	T/C		0.409	0.416	0.76	—	0.387	0.24	—
9	rs821577	253923	G/T		0.201	0.166	0.05	—	0.172	0.06	—
10	rs821597	289130	T/C		0.414	0.438	0.28	—	0.404	0.60	—
11	rs843979	317474	C/G		0.335	0.358	0.29	—	0.334	0.92	—
12	rs821616	331464	A/T	Ser704Cys	0.104	0.145	*0.005*	*1.46*	0.122	0.14	—
13	rs2806465	353160	G/C		0.376	0.378	0.92	—	0.370	0.75	—

Minor allele frequencies in controls are shown. SZ, schizophrenia, OR, odds ratio. Significant results ($P < 0.05$) indicated by italics.

Table 2. Three-marker haplotype analysis among patients with major depression and controls

SNP	Haplotype										
1	2										
2	1	1									
3	1	1	2								
4		2	1	1							
5			2	1	1						
6				2	2	2					
7					1	1	1				
8						2	2	1			
9							2	2	2		
10								1	2	2	
11									1	2	2
12										2	2
13											2
Global *P*-value	*0.030*	0.19	*0.043*	0.81	0.74	0.55	0.69	0.20	*0.012*	0.17	0.057
Individual *P*-value	*0.017*	*0.026*	*0.018*	0.24	0.25	0.25	0.19	*0.023*	*0.019*	*0.017*	*0.002*
Haplotype frequency in control	0.050	0.201	0.180	0.123	0.136	0.108	0.081	0.064	0.018	0.096	0.074
Haplotype fequency in MDD	0.026	0.250	0.233	0.104	0.116	0.089	0.060	0.035	0.000	0.130	0.116

Major allele = 1, minor allele = 2. Individual *P*-value indicates the best *P*-value among the haplotypes. Significant results ($P < 0.05$) indicated by italics.

$P < 0.05$. Our results suggest that the Ser704Cys SNP of the DISC1 gene might affect human gray matter volume and white matter microstructure, particularly in peri-cingulate area. On the other hand, genotype effects of other SNPs, i.e. SNP1, SNP7 and SNP9 on the brain morphology or white matter microstructure, were not detected.

DISC1 and ERK signaling

ERK signaling and Akt signaling have been implicated in MDD (24,25). Thus, we investigated the possible involvement of DISC1 on ERK and Akt signaling pathways. The endogenous DISC1 function in cortical cultures was examined using small interfering RNA (siRNA) for DISC1, and robust decrease (70%) of endogenous DISC1 protein was confirmed (Fig. 3Aa and d). We found decreased levels of phosphorylation of ERK1/2 (pERK1/2: an activated form of ERK) and phosphorylation of Akt (pAkt: an activated form of Akt) after DISC1-siRNA transfection, suggesting that endogenous DISC1 protein is involved in ERK and Akt activations (Fig. 3Aa–c). In contrast, the levels of total ERK1/2, Akt and TUJ1 (class III β-tubulin, a neuronal marker) proteins were not altered (Fig. 3Aa, quantified data (mean ± SD): ERK1: 1.15 ± 0.22, 0.95 ± 0.27; ERK2: 1.01 ± 0.17, 0.99 ± 0.22; Akt: 1.05 ± 0.06, 1.09 ± 0.04; TUJ1: 1.03 ± 0.07, 1.01 ± 0.07; relative to none; scramble, si-DISC1, respectively).

To examine the effect of Ser704Cys SNP on ERK and Akt signaling, we overexpressed each type of DISC1 protein (sDISC1: DISC1 protein with Ser704; cDISC1: DISC1 protein with Cys704) using a sindbis virus-mediated gene delivery system. Neuronal cultures infected with the control viral construct [green fluorescence protein (GFP) only] were doubly stained with GFP signal and immunostaining signal

Table 3. Gender difference of allele distributions for 13 SNPs in the DISC1 gene among patients with schizophrenia, those with major depression and controls

SNP	female							Male						
	Control	SZ			MDD			Control	SZ			MDD		
	$n = 366$	$n = 318$	*P*-value	OR	$n = 226$	*P*-value	OR	$n = 351$	$n = 340$	*P*-value	OR	$n = 147$	*P*-value	OR
1	0.135	0.102	0.061	—	0.091	*0.021*	*0.64*	0.108	0.107	0.96	—	0.099	0.65	—
2	0.258	0.274	0.52	—	0.279	0.44	—	0.262	0.254	0.74	—	0.238	0.43	—
3	0.466	0.459	0.80	—	0.465	0.97	—	0.456	0.459	0.91	—	0.442	0.69	—
4	0.344	0.385	0.12	—	0.356	0.68	—	0.359	0.366	0.78	—	0.395	0.29	—
5	0.414	0.399	0.58	—	0.469	0.063	—	0.437	0.421	0.53	—	0.398	0.25	—
6	0.373	0.381	0.77	—	0.354	0.51	—	0.352	0.384	0.22	—	0.378	0.44	—
7	0.404	0.447	0.12	—	0.427	0.44	—	0.405	0.437	0.23	—	0.418	0.69	—
8	0.411	0.390	0.42	—	0.425	0.65	—	0.406	0.384	0.40	—	0.401	0.89	—
9	0.209	0.154	*0.0088*	*1.45*	0.164	0.054	—	0.192	0.190	0.90	—	0.170	0.41	—
10	0.399	0.426	0.31	—	0.442	0.14	—	0.430	0.384	0.08	—	0.432	0.96	—
11	0.328	0.343	0.56	—	0.369	0.14	—	0.343	0.325	0.47	—	0.340	0.92	—
12	0.111	0.137	0.14	—	0.146	0.073	—	0.097	0.107	0.52	—	0.143	*0.035*	*0.64*
13	0.383	0.363	0.46	—	0.363	0.50	—	0.369	0.376	0.77	—	0.401	0.34	—

Minor allele frequencies in controls are shown. SZ, schizophrenia; OR, odds ratio. Significant results ($P < 0.05$) indicated by italics.

Table 4. Demographic information for the brain MRI study

Variables	ser/ser-DISC1 ($n = 86$)	cys-DISC1 carriers ($n = 22$)	*P*-value
Age	35.1 (11.5)	40.1 (13.8)	0.08
Gender (M/F)	33/53	7/15	0.81
Education years	16.7 (2.8)	16.8 (3.4)	0.89
Full-scale IQ	111.8 (11.5)	109.2 (13.7)	0.39

Cys-DISC1 carriers ($n = 22$): cys/ser-DISC1 ($n = 21$) and cys/cys-DISC1($n = 1$). Mean values (SD) are presented.

by anti-microtubule-associated protein 2 (MAP2: a neuronal dendritic marker) antibody, as well as viral constructs of two types of DISC1 (sDISC1 and cDISC1) (Fig. 3Ba). About 85% of MAP2-positive cells in control-, sDISC1- or cDISC1-infected cortical cultures were GFP positive, indicating that the majority of neurons were infected, respectively [control (mean $\pm$ SD): 84.4 $\pm$ 6.4%; sDISC1: 86.6 $\pm$ 5.1%; cDISC1: 86.3 $\pm$ 9.8%, n=6 fields, selected randomly]. When cortical cultures infected by sDISC1 or cDISC1 were doubly stained with GFP signal and immunostaining signal by the DISC1 antibody, the sDISC1 and cDISC1 proteins were located in both cell body and neurites in punctuate manner, especially perinuclear region and neurite branch (Fig. 3Bb). As expression levels of GFP and TUJ1 were not altered by viral infections and both sDISC1- and cDISC1-infected cultures showed similar levels of DISC1 expression (Fig. 3Ca, quantified data (mean $\pm$ SD): GFP: 1.00 $\pm$ 0.10, 0.92 $\pm$ 0.15; TUJ1: 0.98 $\pm$ 0.07, 0.98 $\pm$ 0.09; DISC1: 5.43 $\pm$ 0.52, 5.43 $\pm$ 0.33, relative to control, sDISC1, cDISC1, respectively), we examined the phosphorylation of ERK and Akt in this system. Two-way ANOVA indicated significant main effects of viral infection ($F = 202$, df = 2, $P < 0.001$) and measurement of protein levels ($F = 161$, df = 8, $P < 0.001$) and interaction of these two factors ($F = 44.4$, df = 16, $P < 0.001$). There were significant effects of viral infection in the measurements of pERK1/2 and pAkt; however, neither total ERK1/2 nor Akt was altered after virus infection (pERK1: $F = 104$, $P < 0.001$; pERK2: $F = 29.2$, $P < 0.001$; pAkt: $F = 9.0$, $P < 0.01$; ERK1: $F = 1.8$, $P > 0.2$; ERK2: $F = 3.0$, $P > 0.1$; Akt: $F = 0.2$, $P > 0.8$) (Fig. 3C). *Post hoc* comparison revealed significant increases in pERK1/2 in sDISC1- or cDISC1-infected cultures compared with control (pERK1: sDISC1 $P < 0.01$; cDISC1 $P < 0.01$; pERK2: sDISC1 $P < 0.05$; cDISC1 $P < 0.05$) (Fig. 3Ca and b). The levels of ERK1 activation in sDISC1-overexpressing cultures were more intensive than those in cDISC1-overexpressing cultures ($P < 0.01$) (Fig. 3Ca and b). A significant elevation of pAkt was observed in the sDISC1-overexpressing cultures compared with control ($P < 0.05$), although an increase in the pAkt level by cDISC1 overexpression was not significant ($P > 0.1$) (Fig. 3Ca and c). However, there were no significant difference of pAkt levels between sDISC1 and cDISC1 ($P > 0.1$).

We next examined rescue experiments, transfection with sDISC1 or cDISC1 into primary culture knocked down to DISC1. In this experiment, siRNA for DISC1 decreased phosphorylation of ERK by ~50% and there were significant effects of viral infection in the measurements of pERK1/2 (pERK1: $F = 7.3$, $P < 0.01$; pERK2: $F = 9.4$, $P < 0.01$) (Fig. 3D). *Post hoc* comparison revealed a significant elevation of pERK1 levels in the sDISC1-overexpressing cultures compared with si-DISC1 treatment ($P < 0.01$), although an increase in the pERK1 level by cDISC1 overexpression was not statistically significant ($P > 0.1$) (Fig. 3D). A significant elevation of pERK2 was observed in sDISC1- and cDISC1-infected cultures compared with si-DISC1 treatment (sDISC1: $P < 0.05$; sDISC1: $P < 0.05$) (Fig. 3D). Western blots showed that total ERK, Akt and TUJ1 protein levels were not changed and that pAkt levels were also rescued by DISC1 overexpression (Fig. 3Da). These results suggest the recovery of the activation of ERK1/2 and Akt after sDISC1- and cDISC-overexpression in DISC1 knockdown cultures. The effect of rescue on phosphorylation of ERK was larger in sDISC1 compared with cDISC1, although the difference did not reach the statistical significance (Fig. 3D). This

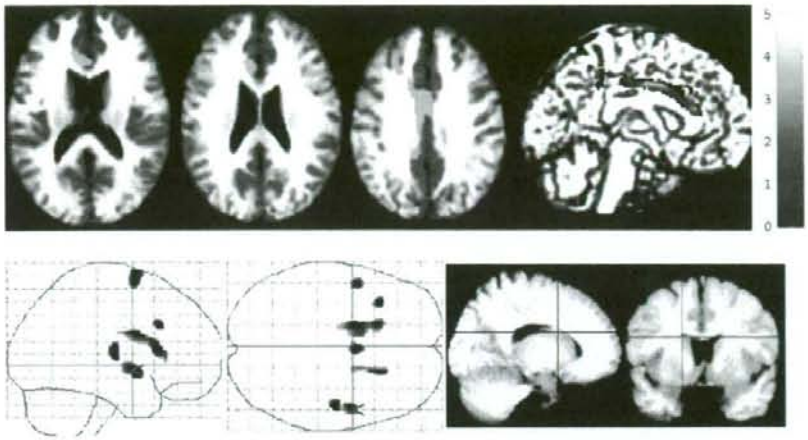

Figure 1. Impact on the brain morphology of the Ser704Cys SNP in healthy subjects. (Upper panel) The SPM {t} is displayed onto T1-weighted MR images. Cys-DISC1 carriers ($n = 22$) had reduced volumes in the bilateral ACC, cingulate gyrus and the posterior cingulate gyrus compared with ser/ser-DISC1 individuals ($n = 86$). (Lower panel) The SPM{t} is displayed on a standard maximum intensity projection images and T1-weighted MR images. Ser/Ser-DISC1 individuals demonstrated decreased volumes of the lateral ventricle, interhemispheric fissure and bilateral Sylvian fissure, indicating an expansion of the CSF space in cys-DISC1 carriers.

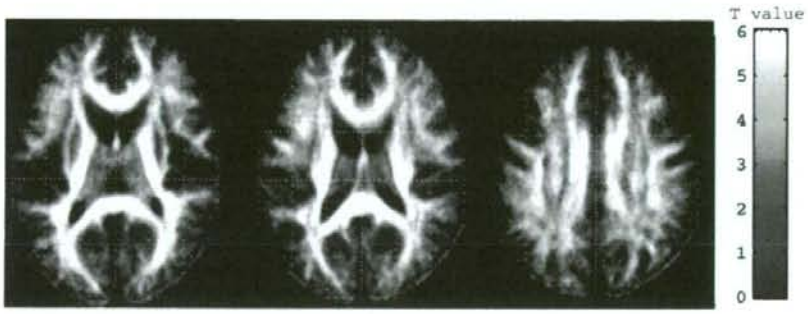

Figure 2. Disruption of white matter integrity revealed by DTI. The SPM{t} is displayed onto a FA map. A significant reduction in FA in the prefrontal white matter was found in the cys-DISC1 carriers ($n = 22$) when compared with individuals with ser/ser-DISC1 ($n = 86$).

might be due to the complexity of this experiment (siRNA plus overexpression) compared with the overexpression only experiments, as the standard deviations of these experiments were larger than those of overexpression experiments. Our results suggest a possible role of DISC1 in the ERK and Akt signaling and an impact of Ser704Cys on ERK activation.

DISCUSSION

Here, we report the evidence for association between MDD and the Ser704Cys SNP as well as several haplotypes including the Ser704Cys SNP. We also replicated earlier evidence for a weak association with schizophrenia which is stronger in female patients (4). Previous association studies with schizophrenia suggested several regions in the DISC1 gene, such as intron2–intron3, intron4–intron9 and intron9–exon13 (4, 5, 8, 9). The region of intron8–intron9 is common across the studies. Consistent with the previous studies, two SNPs in intron9 were associated with schizophrenia in our study. Both false-positive and false-negative associations due to population stratification cannot be excluded in our case–control study, despite the precaution of ethnic matching of this study. Differences in gender ratio and ages between groups could be potential confounding factors. Therefore, it is necessary to carry out further investigations to confirm our findings in other samples. It has also suggested that allelic heterogeneity exists for association between the DISC1 gene and psychiatric illness (26), and this may explain different alleles being associated with illness in our sample compared with others.

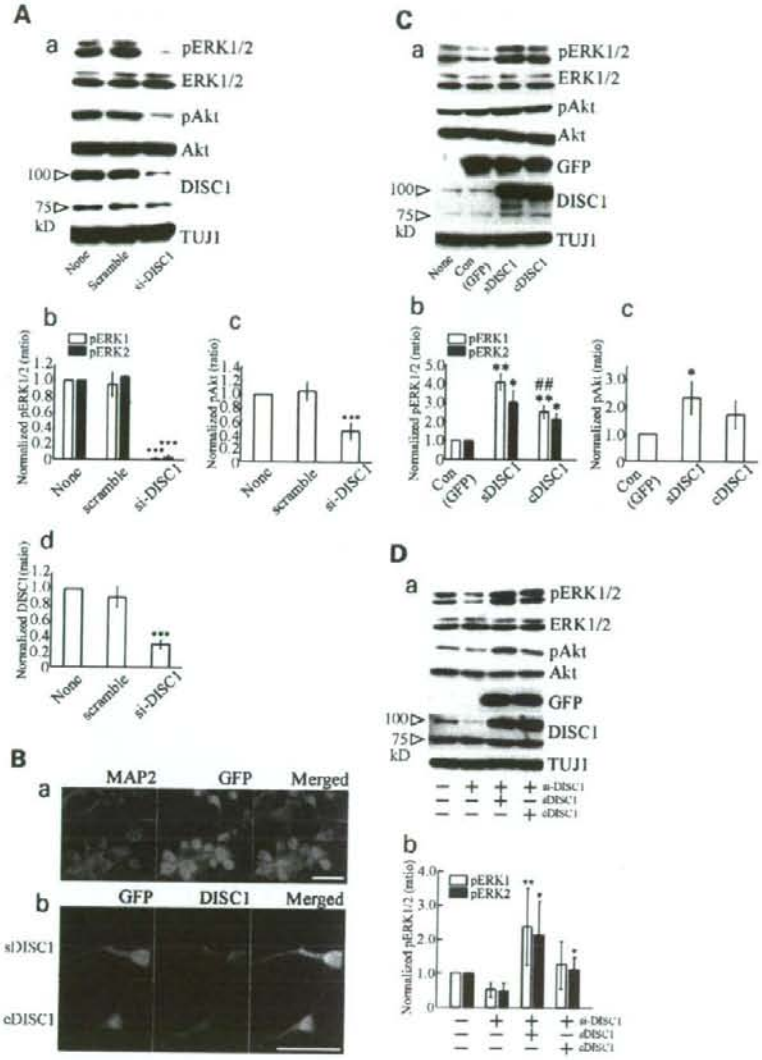

Figure 3. Effects of the DISC1 protein on the ERK and Akt signaling in cortical neurons. (**A**) Suppression of phosphorylation of ERK and Akt in DISC1-siRNA-transfected cultures. Cortical cultures after DIV4 were treated with siRNA for DISC1 (si-DISC1; 100 nM) or control (scramble; 100 nM) for 72 h. Cortical cultures were harvested at DIV7 for western blotting for pERK1/2, ERK1/2, pAkt, Akt, DISC1 or TUJ1. The immunoblots shown are representative of four independent experiments (a). Quantification of the immunoreactivity of pERK1/2 (b), pAkt (c) or DISC1 (d). Quantitative data represent the mean ± SD ($n = 4$). $^{***}P < 0.001$ versus scramble. (**B**) (a) Double staining with GFP (green) signal and immunostaining signal by anti-MAP2 (red, a neuronal marker) antibody after sindbis virus-mediated gene transfer. Representative control (GFP only)-infected cortical cultures were shown. (b) GFP and DISC1 signal after sDISC1 (*upper*) or cDISC1 (*lower*) gene transfer, respectively. DISC1 localization was detected as a red signal. Virus infection was performed at DIV4 and infected cultures were fixed at DIV6 for immunostaining. Bar = 50 μm. (**C**) Differential activation of ERK and Akt between sDISC1 and cDSIC1. Samples for blotting pERK1/2, ERK1/2, pAkt, Akt, GFP, DISC1 or TUJ1 were prepared 24 h (DIV5) after viral infection at DIV 4 (a). The quantification of pERK1/2 (b) or pAkt (c) levels after overexpression of sDISC1 or cDISC1 was shown. The immunoblots shown are representative of four independent experiments. Quantitative data represent the mean ± SD ($n = 4$). $^{**}P < 0.01$, $^{*}P < 0.05$ versus control. $^{\#\#}P < 0.001$ versus sDISC1. (**D**) (a) Recovery of the activation of ERK1/2 and Akt after sDISC1 and cDISC overexpressing in DISC1 knockdown cultures. To downregulate endogenous DISC1, si-DISC1 was applied at DIV4 or DIV5 cultures. Sindbis virus-infection for sDISC1 or cDISC1 overexpression was performed 48 h after the si-DISC1 treatment. Samples for blotting for pERK1/2, ERK1/2, pAkt, Akt, GFP, DISC1 or TUJ1 were prepared 48 h after viral infection. The immunoblot images are representative of five independent experiments. (b) The quantification of pERK1/2 for each experimental condition was shown. Quantitative data represent the mean ± SD ($n = 5$). $^{**}P < 0.01$, $^{*}P < 0.05$ versus si-DISC1.

We demonstrated that healthy subjects with the risk allele carriers for MDD (cys-DISC1) had relatively reduced the gray matter volumes in cingulate cortex, relatively expanded CSF space and reduced the FA values in the prefrontal white matter. This pattern of changes on magnetic resonance imaging (MRI) scanning, specifically the gray matter volume deficits in the ACC, expanded the CSF and reduced the FA values in prefrontal cortex, has been repeatedly reported in the studies of patients with schizophrenia and MDD (22,27–29). Several studies demonstrated a decreased volume in the ACC in patients with MDD in remission, MDD with a family history or in early onset depression (30–33) and abnormalities of cortical neuronal organization in postmortem brain of MDD have been reported in the ACC (34). It has been reported that relatively higher FA is associated with remission of MDD, following treatment with drugs or electroconvulsive therapy; however, reduced prefrontal FA has not been reported consistently in MDD (29,35–37). These various findings suggest that decreased gray matter volume and FA in the frontal area might be associated with the increased risk for MDD. Previous studies found that the risk haplotype of the DISC1 gene affected cortical gray matter and that Ser704Cys SNP had an impact on the hippocampal structure and function (9,11); however, we did not observe either effects of SNPs associated with schizophrenia in our sample on cortical gray matter or effects of Ser704Cys SNP on hippocampal volume. Moreover, in our study of the effect of Ser/Cys genotype on brain imaging derived phenotypes and clinical association, it is the cys allele that is relatively deleterious, whereas in an earlier study, it was the ser allele (9). These inconsistencies may relate to sample differences, methodological differences, and also to possible genetic and allelic heterogeneity.

We found robust effects of DISC1 on ERK and Akt signaling and evidence that the cDISC1 (the risk allele for MDD) might exert a weaker effect on the ERK activation than sDISC1. The involvement of ERK in the therapeutic mechanisms of mood disorder has been proposed (38,39). It has been shown that ERK can phosphorylate PDE4 and alter its activity (40,41) and that PDE inhibitors might have antidepressant efficacy (24). Taken together, the regulation of ERK signaling by DISC1 may contribute, at least in part, to the mechanisms of the risk for MDD. Structural imaging studies have demonstrated reduced gray matter volumes and white matter abnormality in several brain areas of patients with mood disorders relative to healthy controls, and postmortem morphometric brain studies also demonstrated cellular atrophy and/or loss (24). As the ERK kinase signaling is implicated in cytoskeletal remodeling, neurite outgrowth and cell survival (24) and decreased expression of ERK was observed in the postmortem brain of depressive patients (42), impaired ERK signaling could be related to the structural abnormality in major depression.

In conclusion, we have found evidence for association between genetic variation of DISC1 and MDD, brain morphology and ERK signaling pathway. Our data suggest that Ser704Cys might be a functional variant that impacts on neural mechanisms implicated in the biology of major depression.

MATERIALS AND METHODS

Subjects

Subjects for the clinical association study were recruited at Fujita Health University School of Medicine, Showa University School of Medicine and National Center of Neurology and Psychiatry, Japan. They were 373 patients with MDD [147 males and 226 females with mean age of 54.0 years (SD 16.0); mean age of onset of 46.5 years (SD 15.3)], 658 patients with schizophrenia [340 males and 318 females with mean age of 43.6 years (SD 14.6); mean age of onset of 24.2 years (SD 8.6)] and 717 healthy comparison subjects [351 males and 366 females with mean age of 41.3 years (SD 16.9)]. All the subjects were Japanese. 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). Control subjects were healthy volunteers who had no current or past contact to psychiatric services.

One hundred and eight healthy Japanese (biologically unrelated) for MR experiments were recruited at the National Center of Neurology and Psychiatry and screened by a questionnaire on medical history and excluded if they had neurological, psychiatric or medical conditions that could potentially affect the central nervous system, such as substance abuse or dependence, atypical headache, head trauma with loss of consciousness, asymptomatic or symptomatic cerebral infarction detected by the T2-weighted MRI, hypertension, chronic lung disease, kidney disease, chronic hepatic disease, cancer or diabetes mellitus. Detail demographics of subjects in genotypes of SNP1, SNP7, SNP9 and SNP12 (Ser704Cys) were noted in Supplementary Material. After description of the study, written informed consent was obtained from every subject. The study protocol was approved by institutional Ethics Committees.

Genetic analysis

Venous blood was drawn from subjects and genomic DNA was extracted from the whole blood according to the standard procedures. Thirteen SNPs were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay as described previously (43,44). Primers and probes for detection of the SNPs are available upon request. Statistical analysis of association studies was performed using SNPAlyse (DYNACOM, Yokohama, Japan). Allele distributions between patients and controls were analyzed by the χ^2 test for independence. The measure of LD, denoted as D′ and r^2, was calculated from the haplotype frequency using the expectation–maximization algorithm. Case–control haplotype analysis was performed by the permutation method to obtain the empirical significance (45). The global P-values represent the overall significance using the χ^2 test when the observed versus 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 on the basis of 10 000 replications. All P-values reported are two tailed. Statistical significance was defined at $P < 0.05$.

Neuroimaging analysis

Brain MR procedure is described in Supplementary Material. The basic principle of TBM is to analyze the local deformations of an image and to infer local differences in the brain structure. The method was described in detail previously (46) (Supplementary Material). Diffusion tensor imaging (DTI) analysis was performed using FA maps by a voxel-by-voxel analysis (Supplementary Material). The statistical parametric maps of Jacobian determinants and FA values were analyzed using statistical parametric mapping (SPM) 2, which implements a 'general linear model'. To test hypotheses about regional population effects, data were analyzed by a two-sample *t*-test without global normalization. We used $P < 0.001$ without a correction for multiple comparisons to avoid type-II error to explore whole brain and then applied small-volume correction ($P < 0.01$) to each cluster. Since there has been no a priori hypothesis for FA changes associated with DISC1 polymorphism, we applied conservative statistical threshold ($P < 0.001$) for the analysis of FA values. The resulting sets of *t*-values constituted the statistical parametric maps {SPM (t)}.

Molecular biology

Primary cultures were prepared from the cerebral cortex of postnatal 2-day-old rats (Wister ST; SLC, Shizuoka, Japan) as reported previously (47,48).

The siRNA transfection was performed as reported previously (49). We used 21 nt siRNA duplexes with two nucleotides of the rat DISC1 mRNA coding region (113–131, GACCAGGCTACATGAGAAG, NM_175596). Sense (GAC CAGGCUACAUGAGAAGtt) and antisense (CUUCUCAU GUAGCCUGGUCtc) strands were chemically synthesized by Ambion Ltd (Cambridge, UK). The siRNA (GCGCGC UUUGUAGGAUUCG) named ScrambleII from Dharmacon Research Inc. was used as a scramble control. The plasmid for viral construction of the DISC1 gene was derived from pSinRep5 (Invitrogen, USA) and had two subgenomic promoters followed by a multiple cloning site for an arbitrary gene insertion and an enhanced GFP open-reading frame, thus the virus can produce both arbitrary protein and enhanced GFP independently in the infected cell (50). The control virus produces GFP only, whereas DISC1 virus produces both DISC1 and GFP independently. Detail procedure for viral construction is in Supplementary Material.

Immunocytochemistry was performed, as described previously (51). We used anti-MAP2 (1:1000; Sigma) or anti-DISC1 (1:100) (17) antibodies as a primary antibody, respectively. Alexa Fluor (1:1000, Molecular Probes) was applied as a secondary antibody. Fluorescent images were observed by an inverted microscope (Axiovert 200, ZEISS) with a CCD (cool SNAPfx, ZEISS).

Immunoblotting was carried out as described previously (47). Primary antibodies for immunoblotting were used at the following dilutions: anti-Akt (1:1000, Cell Signaling), anti-phospho-Akt (1:1000, Cell Signaling), anti-ERK (1:1000, Cell Signaling), anti-phospho-ERK (1:1000, Cell Signaling), anti-TUJ1 (1:5000, Berkeley antibody company), anti-GFP (1:1000, Medical & Biological Laboratories) and anti-DISC1 antibodies (1:1000) (17). To quantify the amount of proteins after immunoblotting, we measured the density of immunoblots with an image-analysis software (Science Lab 98 Image Gauge; Fuji Photo Film Co. Ltd, Tokyo, Japan). The level of protein expression was indicated as a ratio that was normalized to control the condition (none, sole GFP-infected, or scramble-transfection, respectively) in each experiment. Statistical analysis was performed with unpaired *t*-test or ANOVA, followed by the Tukey *post hoc* comparisons when applicable.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. The authors declare that they have no conflict of interests.

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Association study of the vesicular monoamine transporter 1 (VMAT1) gene with schizophrenia in a Japanese population

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Abstract

Background: Vesicular monoamine transporters (VMATs) mediate accumulation of monoamines such as serotonin, dopamine, adrenaline, and noradrenaline from the cytoplasm into storage organelles. The VMAT1 (alternatively solute carrier family 18: SLC18A1) regulates such biogenic amines in neuroendocrine systems. The VMAT1 gene maps to chromosome 8p21.3, a locus with strong evidence of linkage with schizophrenia. A recent study reported that a non-synonymous single nucleotide polymorphism (SNP) of the gene (Pro4Thr) was associated with schizophrenia.

Methods: We attempted to replicate this finding in a Japanese sample of 354 schizophrenics and 365 controls. In addition, we examined 3 other non-synonymous SNPs (Thr98Ser, Thr136Ile, and Val392Leu). Genotyping was performed by the TaqMan allelic discrimination assay.

Results: There was no significant difference in genotype or allele distribution of the three SNPs of Pro4Thr, Thr136Ile, or Val392Leu between patients and controls. There was, however, a significant difference in genotype and allele distributions for the Thr98Ser polymorphism between the two groups ($P = 0.01$ for genotype and allele). When sexes were examined separately, significant differences were observed in females ($P = 0.006$ for genotype, $P = 0.003$ for allele), but not in males. The Thr98 allele was more common in female patients than in female controls (odds ratio 1.69, 95% CI 1.19–2.40, $P = 0.003$). Haplotype-based analyses also provided evidence for a significant association in females.

Conclusion: We failed to replicate the previously reported association of Pro4Thr of the VMAT1 gene with schizophrenia. However, we obtained evidence for a possible role of the Thr98Ser in giving susceptibility to schizophrenia in women.

Background

Vesicular monoamine transporters (VMATs) mediate accumulation of monoamines such as serotonin, dopamine, adrenaline, noradrenaline, and histamine from the cytoplasm into storage organelles with an absolute dependence on a vacuolar ATPase-generated proton gradient to transport the cationic amine substrates into the storage organelle in exchange for protons [1-3]. There are two isoforms of VMATs identified in rats and humans [4-8]: VMAT1 (previously known as chromaffin granule amine transporter; CGAT) and VMAT2 (alternatively designated as synaptic vesicle monoamine transporter; SVMT). They are also the first and second members of the solute carrier family 18 (SLC18A1 and SLC18A2, respectively). These proteins share 60% sequence identity; however, they demonstrate a range of differences in their physiologic and pharmacologic properties. VMAT1 is expressed primarily in neuroendocrine cells such as the adrenal medulla and pineal gland, while VMAT2 is expressed in all aminergic neurons in the mammalian CNS [6,9,10]. The expression of the two isoforms in a given cell type is usually, but not always, mutually exclusive [2,11]. Furthermore, the two isoforms differ in recognition of substrates (e.g., histamine) and sensitivity to inhibitors such as tetrabenazine and methamphetamine [12]. Since biogenic amines play critical roles in consciousness, mood, thought, motivation, cognition, perception, and autonomic responses, alterations in genes encoding VMATs might play an important role in the pathogenesis of neuropsychiatric diseases including schizophrenia.

With respect to the human VMAT2 gene, we previously reported exon/intron boundaries, novel polymorphisms, and association analysis with schizophrenia; however, we did not find any polymorphism that resulted in an amino acid change [13]. In addition, we failed to obtain evidence for a significant association of the detected polymorphisms with schizophrenia [13]. The other VMAT, VMAT1, is also an attractive candidate gene for schizophrenia not only because it plays a critical role in the maintenance of monoaminergic endocrine systems but also it maps to chromosome 8p21.3 [14], a locus with strong evidence for linkage with schizophrenia [15-21]. In accordance with the possible role of the VMAT1 gene in schizophrenia, a recent study reported that an SNP in exon 3 of the gene that results in an amino acid change (277C > A resulting in Pro4Thr) was significantly associated with schizophrenia [22]. The C/C genotype (homozygosity for proline residue at codon 4) occurred in 21.4% of the schizophrenic group and only 2.6% of the control group. The A/A genotype (homozygosity for threonine), on the other hand, occurred in 28.6% of the schizophrenic group and 73.6% of the control group. Such a dramatic difference in one polymorphism of the VMAT1 gene in a Caucasian population prompted us to attempt replication of this finding in a Japanese population. In addition, we examined other non-synonymous polymorphisms in the VMAT1 gene for association with schizophrenia.

Methods

Subjects

Subjects were 354 patients with schizophrenia (212 males, mean age of 44.0 years [SD 13.7]) and 365 healthy controls (113 males, mean age of 39.7 years [SD 14.1]). All subjects were biologically unrelated Japanese and recruited from the same geographical area (Western part of Tokyo Metropolitan). Consensus diagnosis by at least two psychiatrists was made for each patient according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) criteria [23] on the basis of unstructured interviews and information from medical records. The majority of the patients (318 patients, 90%) had a history of admission to a psychiatric hospital. Mean age of onset was 24.4 years [SD 8.6]. Twenty-nine percent of the patients (102 patients) had a family history of schizophrenia spectrum disorders within the second degree relatives. The controls were healthy volunteers recruited from hospital staffs and their associates. Control individuals were interviewed and those who had current or past history of psychiatric treatment were not enrolled in the study. The study protocol was approved by the ethics committee at the National Center of Neurology and Psychiatry, Japan. After description of the study, written informed consent was obtained from every subject.

Genotyping

Since genetic variations that result in an amino acid change are most likely to alter function, we searched for non-synonymous polymorphisms of the VMAT1 gene *in silico* based on the NCBI dbSNP database and found 4 well-validated SNPs with a heterozygosity value of > 0.10. They were rs2270641 (SNP1, 277C > A, Pro4Thr), rs2270637 (SNP2, 560C > G, Thr98Ser), rs1390938 (SNP3, 674C > T, Thr136Ile), and rs17092104 (SNP4, 1441G > C, Val392Leu). The numbers of base and amino acid positions were according to NM_003053 and NP_003044, respectively. Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to the standard procedures. The SNPs were genotyped using the TaqMan 5'-exonuclease allelic discrimination assay; the assay ID (Applied Biosystems) for each SNP was C_22271506_10 for SNP1, C_2716008_1 for SNP2, C_8804621_1 for SNP3, and C_2715953_10 for SNP4. Thermal cycling conditions for polymerase chain reaction (PCR) were 1 cycle at 95°C for 10 minutes followed by 50 cycles of 92°C for 15 seconds and 60°C for 1 minute. Genotype data were read blind to

the case-control status. Ambiguous genotype data were not included in the analysis.

Statistical analysis

Deviations of genotype distributions from the Hardy-Weinberg equilibrium were assessed with the χ^2 test for goodness of fit. Genotype and allele distributions were compared between patients and controls by using the χ^2 test for independence. These tests were performed with the SPSS software ver 11 (SPSS Japan, Tokyo, Japan). Haplotype-based association analyses were examined with the COCAPHASE software ver 2.4 [24]. The expectation-maximization (EM) and "droprare" options were used. Haplotypes with frequencies less than 3 % were considered to be rare. We examined associations by permutation procedure (10,000 replications) to determine the empirical significance.

Results

Genotype and allele distributions of the examined SNPs in patients and controls are shown in Table 1. The genotype distributions did not significantly deviate from the Hardy-Weinberg equilibrium in patients and controls for any SNPs. With respect to SNP1, there was no significant difference in genotype or allele distributions between patients and controls. Both genotype and allele distributions were approximately the same in the two groups; therefore, we failed to replicate the finding of Bly [22]. For the remaining SNPs, however, we found a significant difference in genotype and allele distributions of SNP2, but not SNP3 or SNP4, between patients and controls. For SNP2, the Thr98 (560C) allele was significantly more common in patients than in controls ($P = 0.01$, odds ratio = 1.39, 95% CI 1.09–1.77). When men and women were examined separately, genotype and allele distributions of SNP2 significantly differ in females, but not in males, between the two groups (Table 2). The excess of the Thr98 allele in patients was highly significant in females ($\chi^2 = 8.54$, df = 1, $P = 0.003$, odds ratio = 1.69, 95% CI 1.19–2.40), whereas genotype and allele distributions were quite similar in male patients and controls.

Pair-wise linkage disequilibrium values between neighbouring SNPs are shown in Table 3. Fairly tight linkage disequilibrium was observed in any pair of the SNPs. We obtained no significant difference in haplotype frequencies for two-, three-, or four-marker analyses between patients and controls in males (data not shown). In females, however, we obtained significant differences in estimated haplotype distributions for any comparisons when SNP2 was included in the analysis (Table 4). The most significant result was obtained by the two-marker haplotype (C-C) consisting of SNP2 and SNP3 (permutation $P = 0.007$).

Discussion

We failed to replicate the finding of Bly [22] who reported a significant association between the Pro4Thr polymorphism (SNP1) of the VMAT1 gene and schizophrenia. This discrepancy may be attributable to ethnic differences in the effects of SNP1 between Caucasians and Asians. The possibility of a type-II error is unlikely because our sample size had a power of approximately 100% to detect the difference in the frequency of C/C genotype reported in Bly's study (21.4% in patients and 2.6% in controls). Moreover, both the genotype and allele distributions of SNP1 were almost the same in our patients and controls. An alternative possibility might be that the finding of Bly [22] had arisen by chance due to the small sample size (28 schizophrenics and 38 controls) and thus obtained evidence of statistical significance was not strong ($P = 0.036$) in spite of the marked difference in the frequency of C/C genotype between patients and controls in his sample.

When additional SNPs were genotyped, we found that the 98Thr (560C) allele of SNP2 was significantly increased in schizophrenics compared to controls, although no significant results were obtained for SNP3 or SNP4. This significant excess of the 98Thr allele in patients was observed in females, but not in males, suggesting that the Thr98 allele has a sexually dimorphic effect of giving susceptibility to schizophrenia. Considering that the frequency of the 98Thr allele was greater than the 98Ser allele, it might be more appropriate to infer that the 98Ser allele has a protective effect against the development of schizophrenia. Haplotype-based analyses also yielded several significant differences in haplotype frequencies between female patients and controls only when SNP2 was included in the analysis, providing further support for the possible role of SNP2 in female schizophrenia. However, since we examined only non-synonymous SNPs that had been deposited in the public database (dbSNP) and did not perform polymorphism screening, we may have missed unknown functional polymorphisms. It is possible that such unknown polymorphisms nearby which are in linkage disequilibrium to the SNP2 might be "truly" responsible in giving susceptibility to schizophrenia.

The Thr98Ser polymorphism may affect the processing and overall function of VMAT1 through altering cell signaling and protein trafficking pathways. The human VMAT1 gene is composed of 18 exons which encode 525 amino acids [5]. There are 12 predicted transmembrane domains in the VMAT1 secondary structure and a large luminal loop between transmembrane domains 1 and 2. The Thr98Ser polymorphism is located on this luminal loop, in which there are three potential sites for N-linked glycosylation (asparagines residues at codons 58, 87 and 104) [6]. This loop is the main site of N-glycosylation on the VMAT1 protein, which is believed to regulate targeting

Table 1: Genotype and allelic distributions of the VMAT1 SNPs in patients with schizophrenia and controls

dbSNP ID	Position[a]	Inter-SNP distance (bp)	Group	N	Genotype frequency (GF)			Allele frequency (AF)		Odds ratio (95% CI)	Chi-square test HWE (df = 1)	GF (df = 2)	AF (df = 1)
	7883394				C/C	A/C	A/A	C	A				
SNP1 rs2270641	Exon 2	____	Patients	351	45 (0.13)	153 (0.44)	153 (0.44)	243 (0.35)	459 (0.65)	0.83 – 1.29	$\chi^2 = 0.48$, $P = 0.49$	$P = 0.95$	$P = 0.75$
	Pro4Thr		Controls	360	49 (0.14)	157 (0.44)	154 (0.43)	255 (0.35)	465 (0.65)	1.04	$\chi^2 = 0.78$, $P = 0.37$	$\chi^2 = 0.11$	$\chi^2 = 0.10$
	7881755				C/C	G/C	G/G	C	G				
SNP2 rs2270637	Exon 3	1639	Patients	352	11 (0.03)	130 (0.37)	211 (0.60)	152 (0.22)	552 (0.78)	1.09 – 1.77	$\chi^2 = 2.9$, $P = 0.09$	<u>$P = 0.01$</u>	<u>$P = 0.01$</u>
	Thr98Ser		Controls	362	28 (0.08)	144 (0.40)	190 (0.52)	200 (0.28)	524 (0.72)	1.39	$\chi^2 = 0.0$, $P = 0.92$	$\chi^2 = 9.09$	$\chi^2 = 7.00$
	7881641				C/C	T/C	T/T	C	T				
SNP3 rs1390938	Exon 3	114	Patients	352	188 (0.53)	135 (0.38)	29 (0.08)	511 (0.73)	193 (0.27)	0.70 – 1.13	$\chi^2 = 0.46$, $P = 0.50$	$P = 0.44$	$P = 0.33$
	Thr136Ile		Controls	360	200 (0.56)	139 (0.39)	21 (0.06)	539 (0.75)	181 (0.25)	0.89	$\chi^2 = 0.24$, $P = 0.62$	$\chi^2 = 1.62$	$\chi^2 = 0.95$
	7850482				G/G	G/C	C/C	G	C				
SNP4 rs17092104	Exon 13	31159	Patients	352	0 (0.00)	23 (0.07)	329 (0.93)	23 (0.03)	681 (0.97)	0.38 – 1.34	$\chi^2 = 0.40$, $P = 0.53$	$P = 0.28$	$P = 0.29$
	Val392Leu		Controls	363	0 (0.00)	17 (0.05)	346 (0.95)	17 (0.02)	709 (0.98)	0.71	$\chi^2 = 0.21$, $P = 0.65$	$\chi^2 = 1.16$	$\chi^2 = 1.13$

[a]Chromosome position was according to the dbSNP database.
HWE: Hardy-Weinberg equilibrium
P values of < 0.05 are underlined.

Table 2: Genotype and allele distributions of SNP2 (Thr98Ser) in patients with schizophrenia and controls for each sex

		N	Genotype distribution (frequency)							Allele distribution (frequency)						HWE	
			CC		GC		GG	χ^2	P	C		G		χ^2	P	χ^2	P
Total																	
	Patients	352	11	(0.03)	130	(0.37)	211	(0.60)	9.09	<u>0.011</u>	152	(0.22)	552	(0.78)	7.00	<u>0.008</u>	2.90	0.089
	Controls	362	28	(0.08)	144	(0.40)	190	(0.52)			200	(0.28)	524	(0.72)			0.01	0.921
Male																		
	Patients	211	9	(0.04)	79	(0.37)	123	(0.58)	2.27	0.322	97	(0.23)	325	(0.77)	0.20	0.655	0.70	0.404
	Controls	112	9	(0.08)	37	(0.33)	66	(0.59)			55	(0.25)	169	(0.75)			1.31	0.252
Female																		
	Patients	141	2	(0.01)	51	(0.36)	88	(0.62)	10.12	<u>0.006</u>	55	(0.20)	227	(0.80)	8.54	<u>0.003</u>	3.26	0.071
	Controls	250	19	(0.08)	107	(0.43)	124	(0.50)			145	(0.29)	355	(0.71)			0.39	0.534

HWE: Hardy-Weinberg equilibrium
Significant P values are underlined.

Table 3: Pair-wise linkage disequilibrium between neighbouring SNPs in the VMAT1 gene

	SNP1 rs2270641	SNP2 rs2270637	SNP3 rs1390938	SNP4 rs17092104
SNP1		0.70	0.99	1.00
SNP2	0.29		1.00	1.00
SNP3	0.19	0.12		1.00
SNP4	0.02	0.01	0.01	

Upper diagonal figures are D' and lower diagonal figures are r^2.
Pairs in LD (D' > 0.8 or r^2 > 0.8) are underlined.

of the protein. Since the Thr98Ser is closely located to N-linked glycosylation sites, it is possible that the Thr98Ser polymorphism may affect glycosylation status. Another possibility is that the Thr98Ser polymorphism may lead to altered phosphorylation in the VMAT1 protein, since serine and threonine residues play a central role in phosphorylation (activation/inactivation) of proteins. Indeed, some serine residues have been shown to undergo phosphorylation in the isoform protein VMAT2 [25]. However, conclusions remain purely speculative and additional research on protein structure, cell signaling, and protein trafficking pathways within VMAT1 are required.

We detected a significant association between the VMAT1 gene and schizophrenia only in females. This observation is not surprising, because there is substantial evidence for sex differences in the pathogenesis and pathophysiology of schizophrenia, which may have arisen from interplay between sex hormones and other developmental factors [26]. Indeed, there are several other genes (e.g., ZDHHC8 [27] and chimerin 2 [28]) that have been suggested to have a sexually dimorphic effect on the development of schizophrenia. Furthermore, there is evidence for crucial regulation by ovarian steroids on the expression of the VMAT2 gene [29]. Although there is little information on such regulation for the VMAT1 gene, it is possible that similar regulation exists, which may be related to our observation of the differential effect of the VMAT1 gene between males and females.

Recently, Lohoff et al [30] reported a significant association between the VMAT1 gene and bipolar I disorder. They genotyped three non-synonymous SNPs (Thr4Pro, Thr98Ser, and Thr136Ile) and 4 non-coding SNPs, and found that allele frequencies in the Thr136Ile, and polymorphisms in the promoter region and intron 8 differed significantly between patients and controls of European descent. Although the associated SNP was again different with our results, the results of Lohoff et al [30] and ours might support the view that schizophrenia and bipolar has several similarities and share susceptibility genes [31].

Conclusion

In conclusion, although we failed to replicate the finding of Bly [22], our results suggest that another amino acid substitution (Thr98Ser) of the VMAT1 gene may have a sexually dimorphic effect of giving susceptibility to schizophrenia in the Japanese population. If our results are replicated, further investigations on VMAT1 function may elucidate molecular mechanisms of schizophrenia, permitting the development of novel therapeutic agents.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

MR, YI, HitK, and TS performed genotyping and statistical analyses. MR helped to draft the manuscript. HH, KA, and

Table 4: Estimated haplotype frequencies and significance of differences between patients and controls in females

Haplotype				Haplotype frequency (%)		*P*-values		
SNP1	SNP2	SNP3	SNP4	Patients	Controls	Individual	Global	Permutation global
C	G	/	/	0.21	0.14	0.015	0.004	0.008
C	C	/	/	0.13	0.23	0.002		
/	C	C	/	0.20	0.29	0.003	0.012	0.007
C	G	C	/	0.21	0.14	0.017	0.010	0.011
C	C	C	/	0.14	0.23	0.002		
/	C	C	C	0.20	0.29	0.004	0.021	0.012
C	G	C	C	0.21	0.14	0.025	0.012	0.021
C	C	C	C	0.14	0.23	0.003		

Haplotype individual p-values of < 0.05 are listed.

OS recruited and assessed the subjects and helped to draft the manuscript. HirK designed the study, recruited the subjects, and drafted the manuscript. All authors read and approved the final manuscript.

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

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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%, $p = 0.0031$)

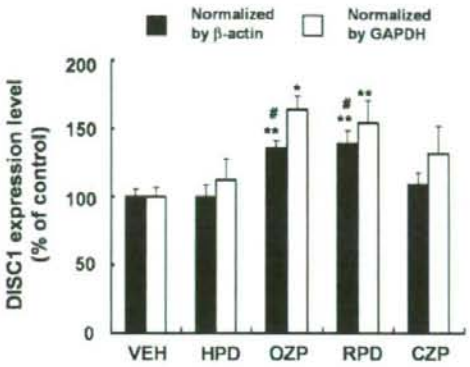

Fig. 1. Relative expression levels of DISC1 in frontal cortex in clinical dose. DISC1 mRNA expression levels normalized by β-actin or GAPDH in control mice (treated with vehicle: VEH) and mice treated with haloperidol (HPD), olanzapine (OZP) risperidone (RPD), or clozapine (CZP) are shown. Expression 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