Table 2 Results of image analyses

Anatomical regions	Brodmann area	Cluster size	Corrected P FDR	*T*-value (voxel level)	Talairach coordinates		
					x	*y*	*z*
Main effects							
Diagnosis effects (control > schizophrenia) (Fig. 3)							
Limbic system							
R insula	BA13	4682	0.000	6.41	33	11	−2
L insula	BA13	4017	0.000	8.81	−33	11	4
R parahippocampal gyrus, amygdala-uncus	BA36	4682	0.000	7.32	30	1	−17
R parahippocampal gyrus	BA36	186	0.000	5.04	30	−41	−8
L parahippocampal gyrus, hippocampus-amygdala	BA34/36	637	0.000	5.46	−20	−41	−8
R anterior cingulate cortex	BA32	147	0.000	4.9	9	33	20
L anterior cingulate cortex	BA32	200	0.000	4.63	−11	32	20
L cingulate gyrus	BA32	275	0.001	4.2	−12	−16	39
Prefrontal cortex							
R inferior frontal gyrus	BA47,11	145	0.000	4.99	27	28	−11
R superior frontal gyrus	BA8/9	1889	0.000	6.08	12	43	39
L medial frontal gyrus	BA9	1333	0.000	5.13	−8	47	19
L inferior frontal gyrus	BA45	141	0.000	4.55	−44	23	15
L middle frontal gyrus	BA8	482	0.000	4.44	−30	24	43
L superior frontal gyrus	BA8	482	0.000	4.39	−35	17	51
Premotor area							
R dorsal premotor area	BA6	429	0.000	4.37	41	13	45
Temporal cortex							
R superior temporal gyrus	BA22	806	0.000	5.04	47	−23	−1
R middle temporal gyrus	BA21	806	0.000	4.87	56	−15	−3
L superior temporal gyrus	BA38	4017	0.000	7	−36	1	−17
Central grey matter							
L thalamus		4017	0.000	7.26	−15	−17	2
Diagnosis effects (control < schizophrenia) (Fig. 4)							
L sylvian fissure		621	0.000	6.7	−45	17	−3
R sylvian fissure		774	0.000	6.59	44	17	−8
Lateral ventricle (anterior horn)		279	0.000	5.27	−5	21	4
Lateral ventricle (L inferior horn)		248	0.000	6.18	−41	−30	−10
Lateral ventricle (R inferior horn)		137	0.000	5.02	36	−40	−1
Interhemisphrenic fissure		154	0.000	5.28	3	55	−12
Genotype effects (Val/Val-COMT < Met-COMT carriers) (Fig. 5)							
Limbic system							
L anterior cingulate cortex	BA24/25	334	0.033	4.29	−8	17	−13
Temporal cortex							
R middle temporal gyrus	BA21	285	0.016	5.10	59	−3	−14
Genotype-diagnosis interaction effects (Fig. 6)							
Limbic system							
L anterior cingulate gyrus	BA24/25/32	264	0.044	3.77	−6	25	−6
L parahippocampal gyrus, amygdala-uncus	BA34	219	0.048	3.74	−24	−6	−14
The effects of polymorphism in control group (no significant difference)							
The effects of polymorphism in schizophrenia							
Val/Val-COMT < Val/Met, Met/Met-COMT (Fig. 7)							
Limbic system							
L parahippocampal gyrus, amygdala-uncus	BA28	81	0.010	4.17	−26	2	−22
L anterior cingulate cortex	BA24/25/32	263	0.007	4.38	−7	20	−8
Central grey matter							
L thalamus		91	0.014	3.94	−21	−28	6

and IQ, however, a significant genotype-by-diagnosis interaction effect was found in a visual memory measure (F = 4.605, df = 1, P = 0.03) (Table 1). However, a *post hoc t*-test (Bonferroni test) demonstrated no genotype effect in each diagnostic category (control: P = 0.15, schizophrenia: P = 0.11).

Morphological changes in schizophrenia (diagnosis effects)

In comparison with controls, patients with schizophrenia demonstrated a significant reduction of volumes in multiple brain areas, such as the limbic and paralimbic systems, neocortical areas and the subcortical regions (Table 2 and Fig. 3).

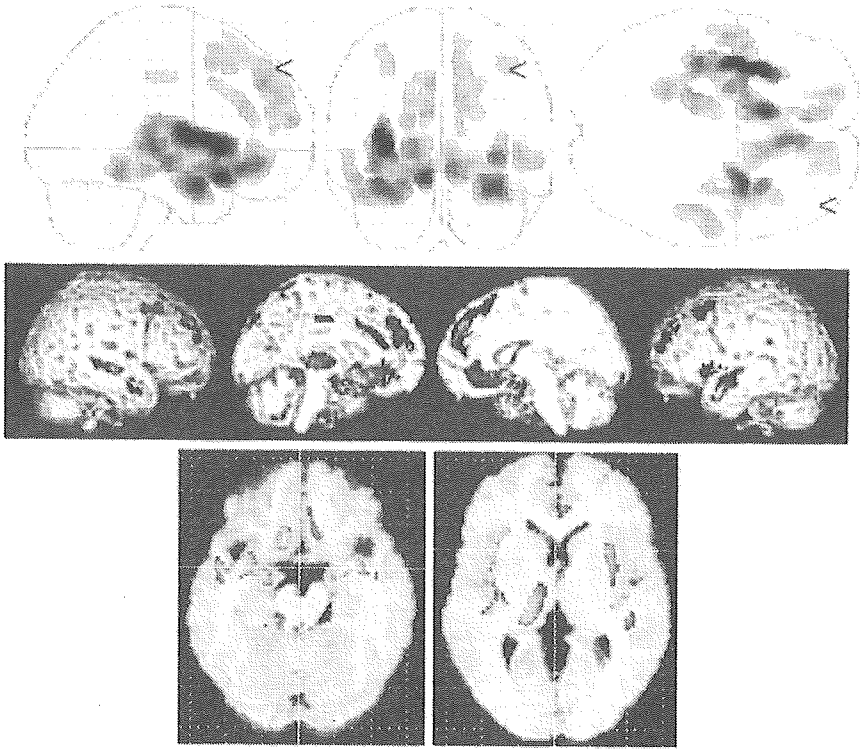

Fig. 3 Decreased volumes in schizophrenics ($n = 47$) as compared to controls ($n = 76$). *Top*: The SPM {t} is displayed in a standard format as a maximum-intensity projection (MIP) viewed from the right, the back and the top of the brain. The anatomical space corresponds to the atlas of Talairach and Tournoux. Representation in stereotaxic space of regions with significant reduction of volume in schizophrenia was demonstrated. Schizophrenics demonstrated a significant reduction of volumes in the multiple brain areas, such as the limbic and paralimbic systems, neocortical areas and the subcortical regions. *Middle*: The SPM {t} is rendered onto T_1-weighted MR images. *Bottom*: The SPM {t} is displayed onto axial T_1-weighted MR images. A significantly decreased volume of the amygdala-uncus, bilateral insular cortices, ACC, temporal cortex and the left thalamus in schizophrenics was noted.

In the limbic and paralimbic systems, patients with schizophrenia showed reduction of volumes in the parahippocampal gyri, amygdala-uncus, insular cortices and the anterior cingulate cortices (ACC). They also demonstrated reduced volumes in the frontal and temporal association areas, dorsal premotor areas and the left thalamus. In comparison with controls, patients with schizophrenia showed significantly increased volume in the CSF space such as lateral ventricle, sylvian and the interhemispheric fissures but not in the grey matter (Table 2 and Fig. 4).

Morphological changes associated with the Val158Met polymorphism (genotype effects)

In comparison with Met-COMT carriers, individuals homozygous for the Val-COMT allele demonstrated a significant reduction of volumes in the left ACC and the right middle temporal gyrus (MTG) (Table 2 and Fig. 5). The hypothesis-driven analysis demonstrated a genotype effect on volumes in the bilateral DLPFC (right BA9, left BA8) at a lenient threshold (uncorrected $P = 0.05$) (data are not shown), however, no voxels could survive after the correction for multiple comparisons (FDR < 0.05) within the ROI. There were no areas that individuals homozygous for the Val-COMT allele demonstrated a significant increment of volume compared to Met-COMT carriers.

Genotype—diagnosis interaction effects

We found significant genotype-diagnosis interaction effects on brain morphology. The stronger effects of Val158Met polymorphism on brain morphology in schizophrenia than those in controls were noted in the left ACC and the left amygdala-uncus (Table 2 and Fig. 6). The hypothesis-driven analysis demonstrated a genotype-diagnosis interaction effect on the volume of the right DLPFC (BA9/46) at a lenient threshold (uncorrected $P = 0.05$) (data not shown), however, no voxels could survive after the correction of multiple comparisons (FDR < 0.05) within the ROI.

Effects of the Val58Met polymorphism on brain morphology

Since genotype–disease interaction effects were found, we estimated the effects of genotypes on brain morphology in the control groups and the schizophrenic groups separately.

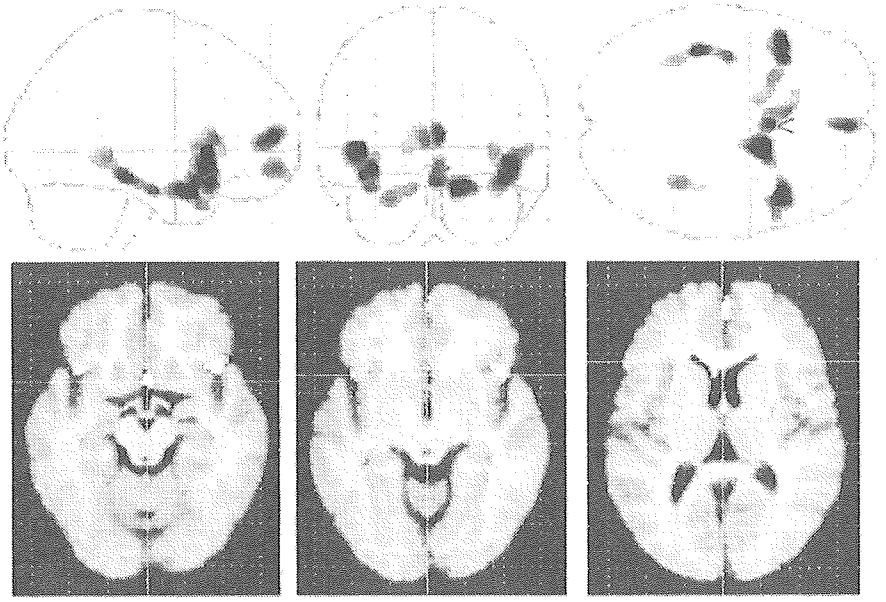

Fig. 4 Increased volumes in schizophrenics as compared to controls. *Top*: The SPM {*t*} is displayed in a standard format as a MIP. Patients with schizophrenia showed a significantly increased volume of the CSF space. *Bottom*: The SPM {*t*} is displayed onto axial T_1-weighted MR images. A significantly increased volume of the CSF space such as the lateral ventricle, sylvian fissures and the interhemisphrenic fissure was noted.

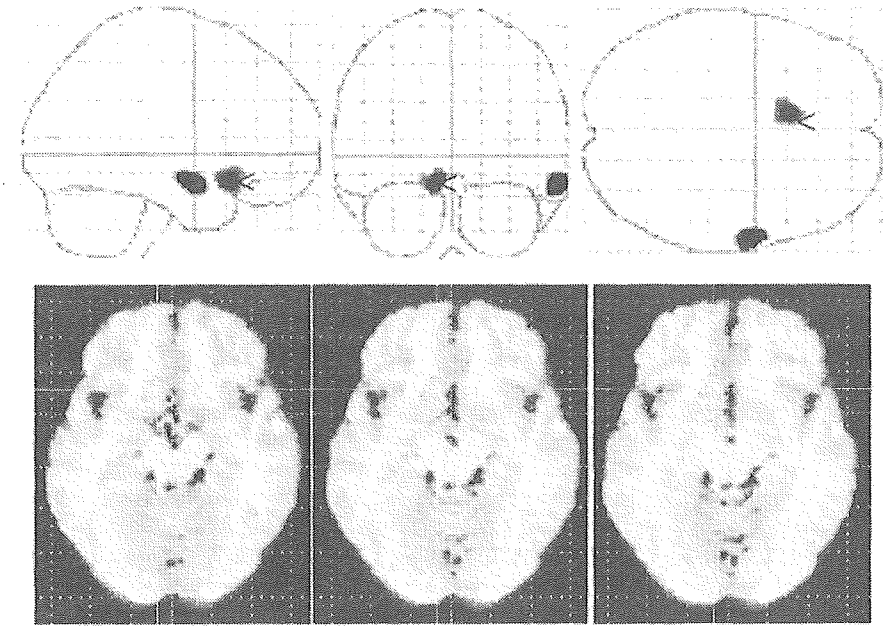

Fig. 5 The result of comparison between individuals homozygous for the Val-COMT allele (n = 57) and Met-COMT carriers (n = 66) (genotype effects). *Top*: Representation in stereotaxic space of regions with significant reduction of volume in individuals homozygous for the Val-COMT allele demonstrated. *Bottom*: The SPM {*t*} is displayed onto axial T_1-weighted MR images. Individuals homozygous for the Val-COMT allele demonstrated a significant reduction of volumes in the left ACC and right MTG as compared to Met-COMT carriers.

In the control group, we found no significant morphological differences between individuals homozygous for the Val-COMT allele and Met-COMT carriers. Even the hypothesis driven analysis with a lenient statistical threshold ($P < 0.05$) could not detect any significant morphological changes in the DLPFC between the two groups. Contrary to the control group, schizophrenics homozygous for the Val-COMT allele showed a significant reduction of volumes in the left amygdala-uncus, bilateral ACC, right MTG and the left thalamus when compared to the patients carrying the Met-COMT

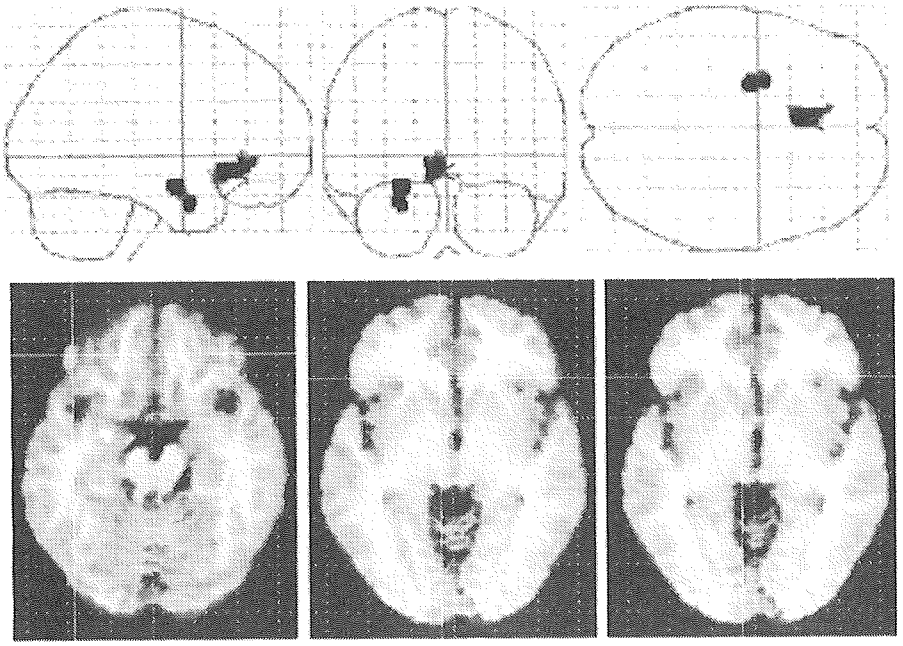

Fig. 6 Results of genotype-diagnosis interaction effects on brain morphology. *Top*: The SPM {*t*} is displayed in a standard format as a MIP. The stronger effects of Val158Met polymorphism on brain morphology in schizophrenia than those in controls were noted in the left ACC, left parahippocampal gyrus and the amygdala-uncus. *Bottom*: The SPM {*t*} is displayed onto axial T_1-weighted MR images.

allele (Table 2, Fig. 7). The hypothesis-driven analysis demonstrated a significantly decreased volume of the bilateral DLPFC in schizophrenics homozygous for the Val-COMT allele when compared to the Met-COMT schizophrenics at a lenient threshold (uncorrected $P = 0.05$) (data not shown). However, no voxels could survive after the correction for multiple comparisons (FDR < 0.05) within the ROI. There are no significantly increased volumes in the schizophrenics homozygous for the Val-COMT allele. All the results were essentially unchanged even if all the left-handed subjects were excluded in all analyses (data not shown).

Discussion

In this study, we found reduction of volumes in the limbic and paralimbic systems, neocortical areas (prefrontal and temporal cortices) and thalamus in patients with schizophrenia when compared to control subjects. The schizophrenia patients demonstrated a significant enlargement of CSF spaces including the lateral and sylvian fissure, which could be interpreted as a result of impaired neurodevelopment and/or global brain atrophy. These findings are concordant with previous studies of MR morphometry of schizophrenia. According to a recent review and meta-analyses of the morphometry of schizophrenia, the consistent abnormalities in schizophrenia are as follows; (i) ventricular enlargement (lateral and third ventricles); (ii) medial temporal lobe involvement; (iii) superior temporal gyrus involvement (iv) parietal lobe involvement; and (v) subcortical brain region involvement including the thalamus (Okubo *et al.*, 2001; Shenton *et al.*, 2001; Davidson and Heinrichs, 2003). The other regions observed in this study, such as the insula, DLPFC and the ACC have also often been demonstrated as abnormal areas in schizophrenia (Shenton *et al.*, 2001; Takahashi *et al.*, 2004; Yamasue *et al.*, 2004). Using the TBM technique, we replicated the morphological abnormalities observed in previous MR studies on schizophrenia, suggesting that TBM was able to detect morphological changes associated with this disease. As well as neuroimaging studies, post-mortem studies have also reported morphological abnormalities in schizophrenia, but not necessarily as common neuropathological features. Regions including the hippocampus, ACC, thalamus and the DLPFC are regularly associated with abnormalities of cell size, cell number and neuronal organization (Bogerts, 1993; Arnold and Trojanowski, 1996; Selemon, 2001; Selemon and Lynn, 2002, 2003). Selemon *et al.* reported that schizophrenics demonstrated abnormalities in overall and laminar neuronal density in the DLPFC (Brodmann area 9) and suggested that the DLPFC should be a particularly vulnerable target in the disease process (Selemon 2001; Selemon and Lynn, 2002, 2003).

Importantly, our results suggest that some of the morphological changes in schizophrenia mentioned above are associated with the Val158Met polymorphism of the COMT gene. In the schizophrenic group, the polymorphism was associated with the volumes in the limbic and paralimbic systems, temporal cortices and the left thalamus, whereas no morphological changes related to the polymorphism were found in

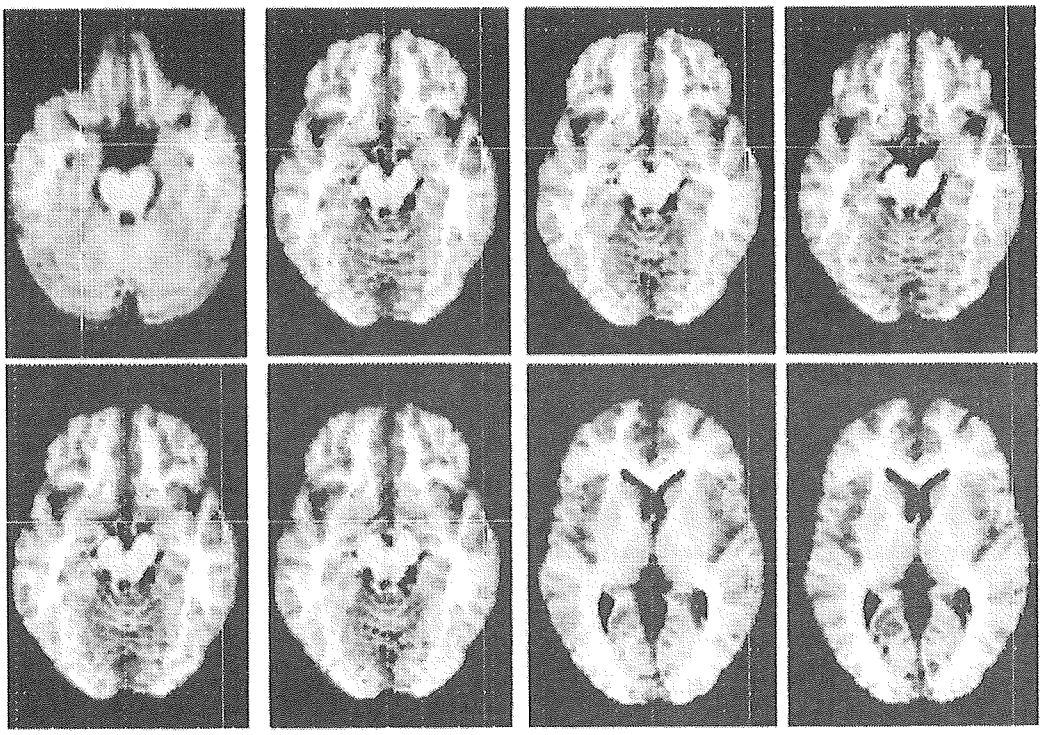

Fig. 7 The effects of the Val158Met polymorphism of the COMT gene on brain morphology in schizophrenics. The SPM {*t*} is displayed onto axial T_1-weighted MR images. The schizophrenics homozygous for the Val-COMT allele (n = 19) showed a significant reduction of volumes in the left parahippocampal gyrus, amygdala-uncus, ACC, left thalamus and the right MTG when compared to patients who carried the Met-COMT allele (n = 28).

normal individuals. As a consequence, significant genotype-diagnosis interaction effects were found in the left ACC and the amygdala-uncus. These results indicate that the Val158-Met polymorphism of the COMT gene is strongly associated with morphological changes in schizophrenia, particularly those in the limbic and paralimbic systems. Longitudinal MRI studies of schizophrenia strongly suggest that progressive changes should occur after onset of the illness (Okubo *et al.*, 2001; Ho *et al.*, 2003). Recent studies have demonstrated that antipsychotic drugs, particularly haloperidol, have considerable effects on brain morphology (Arango *et al.*, 2003; Lieberman, 2005; Dorph *et al.*, 2005). Because of the long duration of illness and medication taken by our subjects, the effects of antipsychotics may be a possible confounding factor for our findings. However, the duration of medication and the dose of antipsychotics taken by the Val/Val-COMT schizophrenics did not differ from those of the Met-COMT schizophrenics. Although the effects of antipsychotics on brain morphology may contribute to the observed morphological changes in patients with schizophrenia in this study, it is unlikely that the effects of antipsychotics contributed to morphological differences between the two schizophrenic groups.

When we were preparing this manuscript, another study demonstrated no genotype and genotype-diagnosis interaction effects of the Val158Met polymorphism on morphology of the frontal lobe in controls and schizophrenia (Ho *et al.*, 2005). Although there are differences between the two studies, such as mean ages of subjects, duration of illness, methods for image analysis and a racial factor (Caucasians versus Japanese), that study also demonstrated no genotype and genotype-diagnosis interaction effects on morphology of the DLPFC. However, we found these effects on DLPFC morphology at a very lenient statistical threshold. Further studies with a larger sample will clarify whether Val158Met polymorphism does affect DLPFC morphology. As well as prefrontal morphology, we found no significant genotype or genotype-diagnosis interaction effects on working memory, however, schizophrenics homozygous for the Val-COMT allele tended to have poorer performances on working memory measures, compared to Met-COMT carriers with schizophrenia. Although there were no significant effects of Val158Met polymorphism on working memory and other neuropsychological measures, a significant effect of the polymorphism was noted in brain morphology. The brain morphology has been considered to be useful as an intermediate phenotype in genetic research in neuropsychiatric disorders (Baare *et al.*, 2001; Durston *et al.*, 2005). Therefore, morphological changes might be more sensitive to the effects of genotype than behavioural measures such as the performance of working memory measures. In a previous study (Ho *et al.*, 2005) a similar phenomenon—no significant effect of Val158Met polymorphism on working memory performance but significant

effects on brain activities during a working memory task—was found. Further studies with a larger sample size are needed to clarify whether morphological changes are a more sensitive marker of genotype effects than behavioural measures.

Unexpectedly, we found effects of the polymorphism on the ACC volume rather than the DLPFC which is crucial for working memory. Since the ACC is associated with a variety of cognitive tasks involving mental efforts, and also plays important roles in working memory (Paus *et al.*, 2001; Kondo *et al.*, 2004), it is feasible that the Val158Met polymorphism may be associated with the ACC morphology. In fact, a previous study demonstrated that the Val-COMT allele was associated with abnormal ACC function as well as abnormal prefrontal cortical function, relative to the Met-COMT allele, as measured by cognitive tests and fMRI activation in normal subjects (Egan *et al.*, 2001).

One would argue that the effects of one polymorphism of the gene could not explain the morphological changes in schizophrenia. As well as the effects of the Val158Met polymorphism, we agree that other polymorphisms of schizophrenia susceptibility genes and genotype–genotype interaction may relate to individual brain morphology. Such interactions might contribute to the different effects of the Val158Met polymorphism on brain morphology observed in this study. Further studies of each effect and interaction of several schizophrenia susceptibility genes on brain morphology, brain functions and performances of neuropsychological tests should be conducted to clarify how polymorphisms of these genes affect intermediate phenotypes of schizophrenia.

In conclusion, we found an association between the Val158Met polymorphism and morphological abnormalities in schizophrenia. Although the underlying mechanisms of our observation remain to be clarified, our data indicate that brain morphology as an intermediate phenotype should be useful for investigating how genotypes affect endophenotypes of schizophrenia.

Acknowledgements

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Susceptibility genes for schizophrenia

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Key Words: schizophrenia, susceptibility gene, dysbindin, neuregulin-1, DISC1, COMT, G72, RGS4, Akt

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The Val66Met polymorphism of the brain-derived neurotrophic factor gene affects age-related brain morphology

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Abstract

We investigated the effects of the brain-derived neurotrophic factor (BDNF) Val66Met polymorphism on age-associated changes of brain morphology in 109 Japanese healthy subjects using MRI with optimized voxel-based morphometry technique. A significant age-related volume reduction was found in the dorsolateral prefrontal cortices (DLPFC), anterior cingulate cortices, and temporal and parietal cortices in all subjects. Further analysis revealed a significantly negative correlation between age and the volume of the bilateral DLPFC only in the Met-BDNF carriers, and a significant interaction between the polymorphism and age-associated volume changes in the bilateral DLPFC. Furthermore, Met-carriers showed a significant interaction ($p < 0.0001$) between the gender and the genotype on the gray matter volume in the DLPFC, and female Met-carriers showed more widespread age-associated volume reduction in DLPFC than male Met-carriers. Our data suggest that the Val66Met polymorphism may impact on age-related changes of the brain, which might be associated with the functional variance of neuroprotective effects of the BDNF. Furthermore, we suggest that genotype effects of the BDNF gene on brain morphology might differ in female from in male.

Keywords: Brain-derived neurotrophic factor; Val66Met polymorphism; Magnetic resonance imaging; Voxel-based morphometry; Dorsolateral prefrontal cortex; Aging

Brain-derived neurotrophic factor (BDNF), a member of neurotrophin family, has important roles in hippocampal plasticity and hippocampal-related learning and memory through long-term potentiation [15]. It also plays an important role in preventing death of neurons during development and protecting cholinergic neurons of the basal forebrain and the hippocampus from induced death in the adult brain [21].

A common missense polymorphism of the BDNF gene producing a valine to methionine amino acid substitution (Val66Met) affects the activity dependent secretion of BDNF in neurons and affects memory function [6,8]. Neuroimaging studies revealed that this polymorphism affected memory-related neuronal activities measured by functional magnetic resonance imaging (MRI) and macroscopic morphology of the hippocampus [8,12,23,28]. Regarding the brain morphology in normal individuals, Pezawas et al. [23] reported that Met-BDNF carriers had smaller volumes of the hippocampi and the prefrontal cortices as compared to individuals with homozygous Val-BDNF. This result was recently replicated in another mixed study of healthy and schizophrenic subjects [28]. Although several neuroimaging studies have indicated that environmental factors considerably impact on human brain structures even in normal adult brains [18], these data suggest that genetic factors such as polymorphism of BDNF might also strongly affect human brain morphology, and contribute to individual differences of brain morphology.

Aging is another factor which strongly affects brain morphology in human. There are several studies that demon-

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strated morphological changes associated with normal aging in vivo [10,24]. A general trend in the in vivo volumetric studies of healthy volunteers points to the prefrontal cortex as the cortical region in which the largest age-related volume reduction is observed. Considering the previous findings that BDNF is expressed abundantly in the prefrontal cortex [25] and that BDNF has a neuroprotective effect, Val66Met polymorphism might have some impacts on age-related morphological changes. However, there is no datum whether this polymorphism is associated with age-related morphological changes.

To clarify whether the BDNF polymorphism impacts on morphological changes associated with aging, we analyzed structural MR images in 109 normal individuals using optimized voxel-based morphometry (VBM) technique.

One hundred and thirty healthy subjects participated in the study. Written informed consent was obtained from all subjects in accord with ethical guidelines in place at local ethical committee. All of the subjects were recruited from local advertisements and underwent a Japanese version of National Adult Reading Test (JART) that is essentially the same as National Adult Reading Test [22] and MRI scanning. We employed JART as a convenient tool to measure IQ for each participant because previous study reported that it showed high correlation with IQ in healthy subjects [20]. All subjects were screened by a questionnaire regarding 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 T2 weighted MRI, hypertension, chronic lung disease, kidney disease, chronic hepatic disease, cancer, or diabetes mellitus. Template creation for the optimized VBM was based on a sample of the 120 subjects, aged 36.2 ± 12.1 years (range 20–72). All subjects were Japanese. Since single nucleotide polymorphism (SNP) genotyping, described in the next section, was done successfully in 109 subjects, the MR images of these 109 subjects were used for subsequent analyses. According to the polymorphism, subjects were categorized into the following three groups: a homozygous Val-BDNF group ($n = 41$), a Val/Met-BDNF group ($n = 51$), or a homozygous Met-BDNF group ($n = 17$). The genotype distribution of this SNP was not deviated with Hardy–Weinberg equilibrium (chi-square = 0.03, $p = 0.86$). Because of the small number of subjects with homozygous Met-BDNF, the Val/Met-BDNF group and homozygous Met-BDNF group were treated as one group, the Met-BDNF carriers ($n = 68$). The demographic data of these groups are the following; the homozygous Val-BDNF comprised 26 females and 15 males, two were left-handed, aged 36.9 ± 13.0 years (range 21–68), and the mean education period and JART score were 16.2 ± 2.8 years (range 12–24) and 75.5 ± 13.3 (equivalent to 108.8 ± 9.55 for full scale IQ (range 50–96; equivalent to 90.5–123.6 for full scale IQ), respectively. The Met-BDNF carriers comprised 45 females and 23 males, three were left-handed, aged 35.8 ± 11.6 years (range 20–72), and their mean education period and JART score were 16.9 ± 3.0 years (range 12–28) and 78.0 ± 11.6 (equivalent to 110.7 ± 8.3) for full scale IQ (range 45–99; equivalent to 86.9–125.8 for full scale IQ), respectively. The mean age, gender ratio, handedness, education period, or JART score did not differ between the two groups (two sample t-test, data not shown).

The detail process of genotyping of BDNF Val66Met SNP (dbSNP accession: rs6265) was described previously [13]. Primers and probes for detection of the SNP (TaqMan SNP Genotyping assays on demand) were purchased from Applied Biosystems (ABI, Foster City, CA, USA). PCR cycling conditions were: at 95 °C for 10 min, 50 cycles of 92 °C for 15 s and 60 °C for 1 min.

All MR studies were performed on a 1.5 T Siemens Magnetom Vision plus system. A three dimensional volumetric acquisition of a T1-weighted gradient echo sequence produced a gapless series of 144 sagittal sections using an MPRage sequence (TE/TR, 4.4/11.4 ms; flip angle, 15°; acquisition matrix, 256 × 256; 1NEX, field of view, 31.5 cm; slice thickness, 1.23 mm).

Data were analyzed with Statistical Parametric Mapping 2 (SPM2) (http://www.fil.ion.ucl.ac.uk/spm/; Wellcome Department of Imaging Neuroscience, London, UK) running on MATLAB 6.5 R1 (MathWorks, Natick, MA). Before analyses, each image was confirmed by a neuroradiologist to eliminate images with artifacts, and then anterior commissure–posterior commissure line was adjusted. First, we made a customized anatomical T1 template and prior probability images from the sample of 120 brains [10]. Then, images were processed using an optimized VBM script (dbm.neuro.uni-jena.de/vbm.html). The detail of this process is described elsewhere [2,10]. The normalized segmented images were modulated by multiplication with Jacobian determinants of the spatial normalization function to encode the deformation field for each subject as tissue density changes in the normal space. Finally, images were smoothed using a 12 mm full width half maximum of isotropic Gaussian kernel. Statistical analyses were performed with SPM2, which implemented a General Linear Model. Proportional scaling was used to achieve global normalization of voxel values between images. First, we used a two-sample t-test to test regional population effect on gray matter volume. For this analysis, we set $p < 0.005$ without a correction for multiple comparisons, followed by applying small volume correction to each cluster with a false discovery rate (FDR) < 0.05. For the small volume correction, spheres with radius 10 mm around the peak were set as regions of interest (ROIs). The resulting sets of t-values constituted the statistical parametric maps {SPM (t)}. Anatomic localization was according to both MNI coordinates and Talairach coordinates, obtained from M. Brett's transformations (http://www.mrc-cbu.cam.ac.uk/Imaging/Common/mnispace.shtml) and presented as Talairach coordinates. Since a previous study with Caucasians demonstrated a significant reduction of volumes in the hippocampi and the frontal cortices in Met-BDNF carriers, we applied an additional hypothesis-driven ROI method to test regional population effects in these regions by using the Wake Forest University PickAtlas [19].

The genotype effects on age-related morphological changes were tested using a single subject condition and covariate model. Since several studies reported gender different age-related mor-

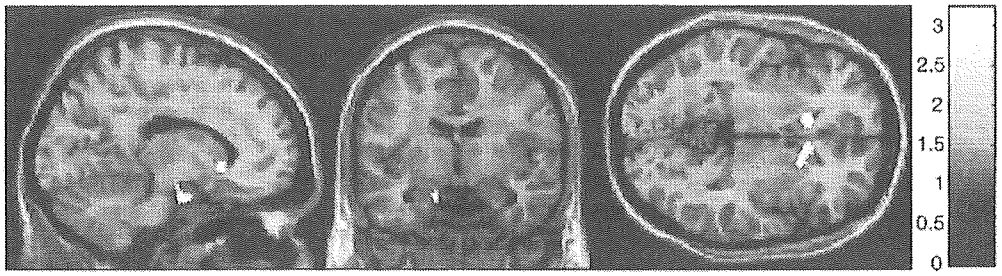

Fig. 1. The volume reduction of Met-BDNF carriers compared to that of individuals with homozygous Val-BDNF ($p < 0.05$, small volume correction with FDR). A significant reduction of volumes of the left parahippocampal gyrus (t-value: 2.92, Talairach coordinates (TAL): −12, −3, −19) and the bilateral heads of caudate nucleus (left: t-value; 3.23, TAL: −9, 22, −3, right: t-value; 3.02, TAL: 10, 21, −4) in the Met-BDNF carriers was noted.

phological changes in the brain [7], we additionally examined genotype effects on age-related morphological changes in each gender, separately. Orthogonalized first order polynomial expansion of age was treated as a covariate of interest to determine the linear effects of age [5]. Since second- and third-order polynomial expansions did not contribute to the age effect model of our sample, we removed them from a design matrix. Considering the possible association between IQ and brain morphology, we treated JART score as a nuisance variable. For this analysis, we applied $p < 0.001$, corrected for multiple comparisons with FDR < 0.05 as a statistical threshold [9]. MarsBar program (marsbar.sourceforge.net/) was also used to extract data from the regions of interest.

Fig. 1 shows a significant reduction of gray matter volumes of the left parahippocampal gyrus (Brodmann area (BA) 34), and bilateral heads of the caudate nucleus in Met-BDNF carriers when compared to homozygous Val-BDNF individuals. Even in hypothesis-driven ROI approach with a lenient statistical threshold (uncorrected $p = 0.05$), we could not find any significant differences of hippocampal nor prefrontal cortical volumes between the two groups. The results were essentially unchanged even when the restricted samples of subjects (female group, male group, or young group aged under 40 years old) were analyzed (data not shown).

Fig. 2 shows morphological changes related to normal aging. A significant negative correlation between age and the gray matter volumes was noted in the bilateral dorsolateral prefrontal cortices (DLPFC; BA9, 46), right superior temporal gyrus (STG; BA22), bilateral insulae (BA13), bilateral caudate nuclei, left anterior cingulate gyrus (BA24), bilateral inferior parietal lobules (BA40), bilateral precunei (BA7), and bilateral fusiform gyri (BA37) in all subjects. In homozygous Val-BDNF individuals, a significant age-related volume reduction was found in the bilateral insulae (BA13) and right STG (BA22). On the other hand, Met-BDNF carrier showed an additional negative correlation of the gray matter volumes in the bilateral DLPFC (BA9, 46) and right dorsal premotor area (BA6) with age. Additional analyses in each gender revealed a significant interaction ($p < 0.0001$) in Met-carriers between the gender and the genotype on the gray matter volume in the DLPFC, and female Met-carriers showed more widespread age-associated volume reduction in DLPFC than male Met-carriers. Male Met-carrier also showed volume

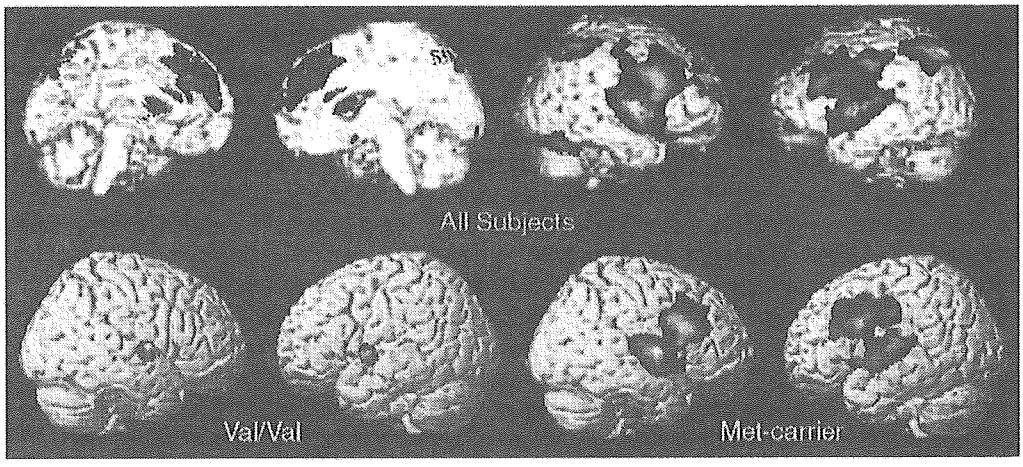

Fig. 2. (Top) The volume reduction associated with normal aging in all subjects ($p < 0.05$, FDR corrected). All subjects showed negative correlation with age in the bilateral DLPFC, right STG, bilateral insulae, bilateral caudate nuclei, left anterior cingulate gyrus, bilateral inferior parietal lobules, bilateral precunei, and bilateral fusiform gyri. (Bottom) The volume reduction associated with normal aging in each genotypic group ($p < 0.05$, FDR corrected). (Left) Results of individuals with homozygous Val-BDNF. Individuals with homozygous Val-BDNF showed negative correlation with age in the bilateral insulae (right: t-value: 4.36, TAL: 42, −2, 4; left: t-value: 4.52, TAL: −43, −2, 4) and the right superior temporal gyrus (t-value: 4.57, TAL: 47, 9, −4). (Right) Results of Met-BDNF carriers. The Met-BDNF carriers showed negative correlation with age in the bilateral dorsolateral prefrontal cortices (right: t-value: 6.5, TAL: 52, 21, 26; left: t-value: 6.12, TAL: −48, 19, 32) as well as the bilateral insulae and the superior temporal gyri.

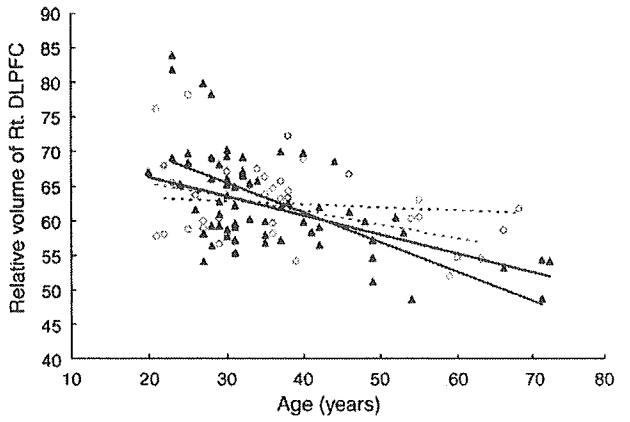

Fig. 3. Scatter plot of relative gray matter volume of the right DLPFC against age in each genomic group. The Met-BDNF carriers showed more significant volume reduction with normal aging compared to homozygous Val-BDNF subjects in the bilateral DLPFC in each gender (right: male Met-BDNF carriers: $y=-0.27x+71.8$, $r=-0.71$, $p<0.0001$, male homozygous Val-BDNF subjects: $y=-0.046x+64.2$, $r=-0.12$, $p=0.67$, female Met-BDNF carriers: $y=-0.43x+78.4$, $r=-0.56$, $p<0.001$, female homozygous Val-BDNF subjects: $y=-0.20x+69.5$, $r=-0.41$, $p=0.03$; left: male Met-BDNF carriers: $y=-0.20x+67.2$, $r=-0.53$, $p=0.01$, male homozygous Val-BDNF: $y=-0.11x+65.3$, $r=-0.25$, $p=0.367$, female Met-BDNF carriers: $y=-0.48x+77.0$, $r=-0.71$, $p<0.0001$, female homozygous Val-BDNF: $y=-0.14x+65.3$, $r=-0.27$, $p=0.18$). Due to limitations of space, only the plot at the right DLPFC in each gender is shown. Blue stands for male subjects and red stands for female subjects. Open circle: homozygous Val-BDNF; closed triangle: Met-BDNF carrier. Dotted lines are the regression line of homozygous Val-BDNF, whereas solid lines are those of Met-BDNF carrier.

reduction in the right inferior parietal lobules (BA40, t-value: 3.86, Talairach coordinates: 40, −43, 53). We found a significant interaction effect (male: $p=0.003$, female: $p<0.0001$) between the aging effect and the genotype on the gray matter volume in the DLPFC in each gender. (right: male Met-BDNF carriers: $r=-0.71$, $p<0.001$, male homozygous Val-BDNF subjects: $r=-0.12$, $p=0.67$; female Met-BDNF carriers: $r=-0.56$, $p<0.001$, female homozygous Val-BDNF subjects: $r=-0.41$, $p=0.03$; left: male Met-BDNF carriers: $r=-0.53$, $p=0.01$, male homozygous Val-BDNF subjects: $r=-0.25$, $p=0.367$, female Met-BDNF carriers: $r=-0.71$, $p<0.0001$, female homozygous Val-BDNF subjects: $r=-0.27$, $p=0.18$) (Fig. 3).

This is the first study which investigated the impacts of BDNF Val66Met polymorphism on age-associated brain morphological changes in normal individuals. We found an exaggerated age-related volume reduction of the DLPFC in the Met-BDNF carriers.

Several studies demonstrated morphological changes associated with normal aging in the STG, insula, inferior parietal lobules, motor cortex, ACC, and DLPFC [10,24]. In consistent with previous studies, our data also showed age-related volume reduction in similar regions in all subjects' analysis of each gender. Further analysis revealed that the Met-BDNF carriers showed a stronger negative correlation between age and gray matter volume in the DLPFC and right precentral gyrus when compared to individuals with homozygous Val-BDNF. Though the mechanisms underlying the predilection of the prefrontal cortex for age-related volume reduction are still unclear, the prefrontal cortex exhibits the greatest age-related alteration of GABA and glutamate [11], and glucose metabolism and age-related declines in regional cerebral blood flow [4]. Though there has been no study investigating the relationship between Val66Met SNP and vulnerability to age-related changes, BDNF protein itself is reported to be associated with aging. Amounts of BDNF protein in hippocampal pyramidal neurons and dentate granule cells are decreased during aging in monkeys [14]. Further, several studies demonstrated neuroprotective effects of BDNF [3,29]. Our data suggest that the Met-BDNF carriers, particularly females carrying Met-BDNF allele, may be more vulnerable to aging than individuals with homozygous Val-BDNF. Considering the fact that prefrontal cortex is one of the regions in which BDNF is expressed abundantly [25], we suggest that the Val66Met polymorphism may be associated with functional variances of neuroprotective and stress resistant effects of BDNF, which results in different effects on age-related morphological changes. Furthermore, we found a reduction of the striatal volumes in met-BDNF carriers as compared to individuals with homozygous Val-BDNF. It has been postulated that enhancement of BDNF in the cortex may be involved in protection of striatal neurons against damage via anterograde transport because BDNF exerts neuroprotective effects against excitotoxicity in the striatum [1,16]. The result, reduced volumes in the striatum in met-BDNF carriers, may again suggest the reduced neuroprotective effects of met-BDNF. Since there has been no direct evidence of differential regulation of vulnerability to neurodegenerative process by BDNF Val66Met polymorphism, further study such as investigating how Val66Met SNP affects cell survival in a cellular model is required to clarify our speculation.

Although we could not replicate results of the previous studies, the smaller hippocampus in the Met-BDNF carriers [23,28], our data also suggest that BDNF polymorphism should have impacts on brain morphology associated with episodic memory. The discrepancy between our results and those of the previous studies could be partially explained by the racial difference. Binding its receptor TrkB, BDNF activates several pathways including the PI3-kinase/Akt, the mitogen-activated protein kinase, and PLC-gamma1 pathway [15]. These signals are known to be critical for survival of neuron, suggesting that not only Val66Met polymorphism of BDNF, but also interaction of polymorphism of each signal or molecule has effects on brain morphology. Racial differences might be related to such interactions, resulting in the different findings. This may partially contribute to the discrepancy in associations between BDNF polymorphism and the prevalence of neuropsychiatric diseases in Asian and Caucasian populations [17,27].

Finally, we mention a limitation of this study. To explore the association between aging effects on the brain morphology and the Val66Met polymorphism, we performed a cross-sectional study. There is a secular bias, which can be resolved by a longitudinal study. In this context, our data may be considered preliminary rather than conclusive. However, a recent longitudinal MR study of normal aging demonstrated that cross-sectional and longitudinal estimates of atrophy rates were similar [26].

In conclusion, we found that Val66Met polymorphism of BDNF had impacts on age-associated morphological changes in Japanese subjects. Our data suggest that Val66Met polymorphism of BDNF may play important roles for vulnerability to age-related morphological changes as well as the efficiency of plasticity, especially in DLPFC. Furthermore, we suggest that genotype effects of the BDNF gene on brain morphology might differ in female from in male.

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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%, $p = 0.0031$)

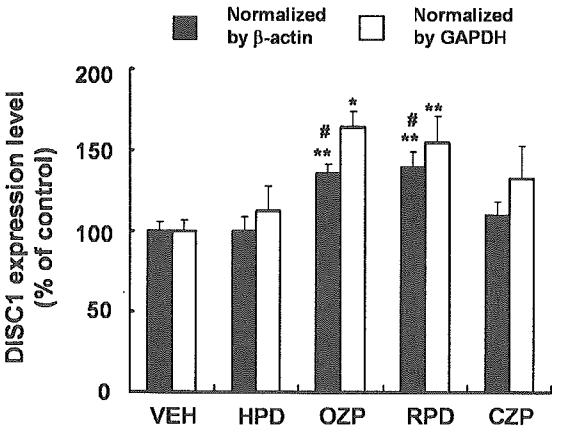

Fig. 1. Relative expression levels of DISC1 in frontal cortex in clinical dose. DISC1 mRNA expression levels normalized by β-actin or GAPDH in control mice (treated with vehicle: VEH) and mice treated with haloperidol (HPD), olanzapine (OZP) risperidone (RPD), or clozapine (CZP) are shown. Expression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

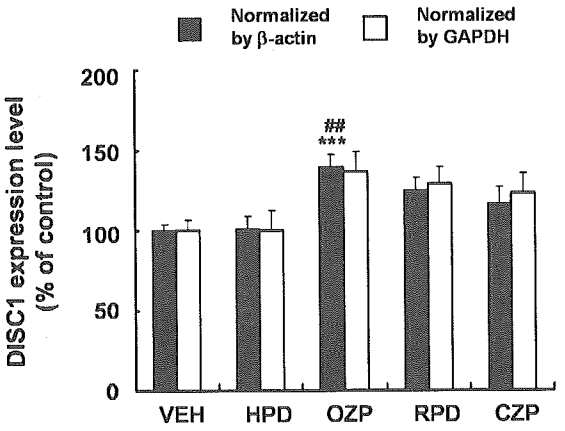

Fig. 2. Relative expression levels of DISC1 in hippocampus in clinical dose. DISC1 mRNA expression levels normalized by β-actin or GAPDH in control mice (treated with vehicle: VEH) and mice treated with haloperidol (HPD), olanzapine (OZP) risperidone (RPD), or clozapine (CZP) are shown. Expression levels 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

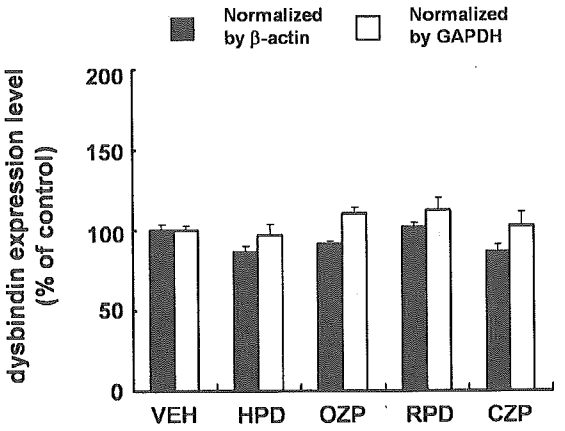

Fig. 3. Relative expression levels of dysbindin in frontal cortex in clinical dose. Dysbindin mRNA expression levels normalized by β-actin or GAPDH in control mice (treated with vehicle: VEH) and mice treated with haloperidol (HPD), olanzapine (OZP) risperidone (RPD), or clozapine (CZP) are shown. Expression 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

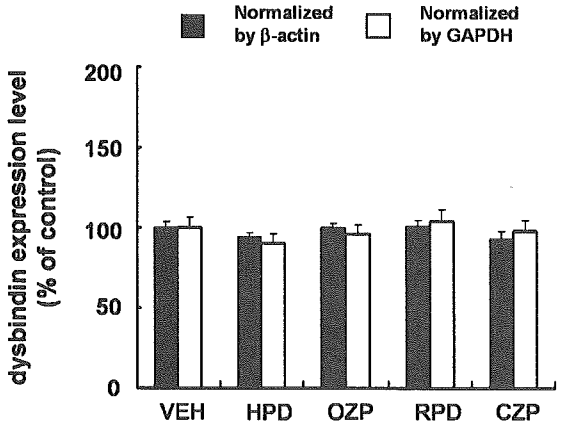

Fig. 4. Relative expression levels of dysbindin in hippocampus in clinical dose. Dysbindin mRNA expression levels normalized by β-actin or GAPDH in control mice (treated with vehicle: VEH) and mice treated with haloperidol (HPD), olanzapine (OZP) risperidone (RPD), or clozapine (CZP) are shown. Expression 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