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Genetic analysis of the gene coding for DARPP-32 (*PPP1R1B*) in Japanese patients with schizophrenia or bipolar disorder

Akira Yoshimi [a], Nagahide Takahashi [b], Shinichi Saito [b,*], Yoshihito Ito [b], Branko Aleksic [b], Hinako Usui [b], Yukiko Kawamura [a], Yukari Waki [a], Takeo Yoshikawa [c], Tadafumi Kato [d], Nakao Iwata [e], Toshiya Inada [f], Yukihiro Noda [a], Norio Ozaki [b]

[a] *Division of Clinical Science and Neuropsychopharmacology, Graduate School of Pharmacy, Meijo University, Aichi, 468-8503, Japan*
[b] *Department of Psychiatry, Nagoya University Graduate School of Medicine, Aichi, 466-8550, Japan*
[c] *Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute, Saitama, 351-0198, Japan*
[d] *Laboratory for Molecular Dynamics of Mental Disorders, RIKEN Brain Science Institute, Saitama, 351-0198, Japan*
[e] *Department of Psychiatry, Fujita Health University School of Medicine, Aichi, 470-1192, Japan*
[f] *Department of Psychiatry, Teikyo University School of Medicine, Chiba Medical Center, Chiba, 299-0111, Japan*

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Abstract

Several lines of evidence, including genome-wide linkage scans and postmortem brain studies of patients with schizophrenia or bipolar disorder, have suggested that DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, 32 kDa), a key regulatory molecule in the dopaminergic signaling pathway, is involved in these disorders. After evaluating the linkage disequilibrium pattern of the gene encoding DARPP-32 (*PPP1R1B*; located on 17q12), we conducted association analyses of this gene with schizophrenia and bipolar disorder. Single-marker and *haplotypic* analyses of four single nucleotide polymorphisms (SNPs; rs879606, rs12601930, rs907094, and rs3764352) in a sample set (subjects with schizophrenia=384, subjects with bipolar disorder=318, control subjects=384) showed that *PPP1R1B* polymorphisms were not significantly associated with schizophrenia, whereas, even after Bonferroni corrections, significant associations with bipolar disorder were observed for rs12601930 (corrected genotypic $p=0.00059$) and rs907094 (corrected allelic $p=0.040$). We, however, could not confirm these results in a second independent sample set (subjects with bipolar disorder=366, control subjects=370). We now believe that the significant association observed with the first sample set was a result of copy number aberrations in the region surrounding these SNPs. Our findings suggest that *PPP1R1B* SNPs are unlikely to be related to the development of schizophrenia and bipolar disorder in the Japanese population.
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Keywords: Schizophrenia; Bipolar disorder; Dopamine- and cAMP-regulated phosphoprotein; 32 kDa; Japanese population

1. Introduction

A number of studies have proposed that disruption of monoaminergic pathways, and in particular the dopaminergic pathway, contributes to both schizophrenia and bipolar disorder (Catapano and Manji, 2007; Murray

* Corresponding author. Department of Psychiatry, Nagoya University, Graduate School of Medicine, 65 Tsuruma-cho, Showa-Ku, Nagoya 466-8550, Japan. Tel.: +81 52 744 2282; fax: +81 52 744 2293.
E-mail address: s-saito@med.nagoya-u.ac.jp (S. Saito).

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et al., 2004). DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, 32 kDa), a critical molecule in *the* striatal neurons, regulates the dopaminergic signaling pathway through phosphorylation of protein phosphatase-1 and protein kinase A (Fienberg et al., 1998). Recently, it has been revealed that DARPP-32 also plays an important role in the regulation of *glutamatergic* signaling pathway (Nishi et al., 2005), which is also thought to contribute to the development of these disorders (Beneyto et al., 2007; Svenningsson et al., 2003).

DARPP-32 knockout mice have been shown to have abnormal responses to psychoactive drugs, such as the decrease of cage climbing behavior induced by dopamine agonists (Fienberg et al., 1998) and the decrease of attenuating effect of antidepressants on immobility (Svenningsson et al., 2002).

Moreover, reduced expression of DARPP-32 has been observed in the postmortem brain of schizophrenic patients (Albert et al., 2002). This is suggested to be related to neostriatal volume, activation, and functional connectivity in the prefrontal cortex, all of which are thought to be abnormal in patients with schizophrenia (Meyer-Lindenberg et al., 2007).

Additionally, several lines of evidence have demonstrated that genetic factors contribute to the development of schizophrenia and bipolar disorder, and genome-wide linkage scans have shown that several chromosomal regions are simultaneously linked to the development of these disorders. Namely, a chromosomal region within 17q, which includes the gene encoding DARPP-32 (*PPP1R1B*; located on 17q12), has been demonstrated to have high logarithm of the odds scores for schizophrenia (Cardno et al., 2001) and bipolar disorder (Dick et al., 2003), i.e. 2.54 and 3.63, respectively.

Therefore, *PPP1R1B* is considered to be one of the candidate genes that contribute to these disorders. In the present study, we performed linkage disequilibrium analysis of *PPP1R1B*, and investigated the association of polymorphisms in this gene with schizophrenia and bipolar disorder in Japanese patients. We employed a two-stage analysis using two independent sets of samples as a previous report (Ikeda et al., 2005). Additionally, copy number variations (CNVs), which have been observed for many genes (Lee and Lupski, 2006; Redon et al., 2006) can affect the accuracy of genotyping with single nucleotide polymorphisms (SNPs). Therefore, we also explored copy number differences of this gene to test the accuracy of genotyping with the SNPs, which deviated from the Hardy–Weinberg equilibrium (HWE).

2. Materials and methods

2.1. Subjects

The subjects for the case-control analysis consisted of 384 patients with schizophrenia (226 males and 158 females; 52.1 ± 15.3 years old), 318 patients with bipolar disorder (162 males and 156 females; 44.0 ± 20.7 years old), and 384 control subjects (159 males and 225 females; 43.9 ± 15.9 years old). To confirm a significant association with bipolar disorder, a second sample set was used, which consisted of 366 patients with bipolar disorder (181 males and 185 females; 50.1 ± 13.4 years old), and 370 control subjects (185 males and 185 females; 50.6 ± 12.6 years old).

For the analysis of copy number differences, we selected 12 male and 12 female subjects (schizophrenia patients: 54.9 ± 12.5 years old; bipolar disorder patients: 46.3 ± 17.4 years old; control subjects: 43.0 ± 12.6 years old) for screening, and another independent sample set of 36 male and 36 female subjects (schizophrenia patients: 41.1 ± 12.2 years old; bipolar disorder patients: 47.1 ± 15.6 years old; control subjects: 43.5 ± 12.7 years old) was used to confirm the results. The subjects used for the copy number analysis were also included in the first sample set used for the association study.

All subjects were unrelated and ethnically Japanese. The patients were diagnosed by at least two experienced psychiatrists according to the DSM-IV-TR (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision) criteria for schizophrenia and bipolar disorder on the basis of unstructured interviews and reviews of their medical records. All healthy control subjects were also psychiatrically screened on the basis of unstructured interviews.

This study was approved by the Ethics Committees of the Nagoya University Graduate School of Medicine, Fujita Health University, and the RIKEN Brain Science Institute. Written informed consent was obtained from each subject.

2.2. Linkage disequilibrium (LD) analysis and tagging SNP selection

For LD analysis, we consulted the HapMap database (release #21a; population: Japanese in Tokyo; minor allele frequency: more than 0.05) in order to obtain SNPs throughout the entire coding region of *PPP1R1B* (GenBank accession No. NM_032192) as well as in flanking regions 500 base pairs (bp) upstream and 500 bp downstream of the coding regions. For the gene-spanning analysis, we selected four SNPs (rs2271309,

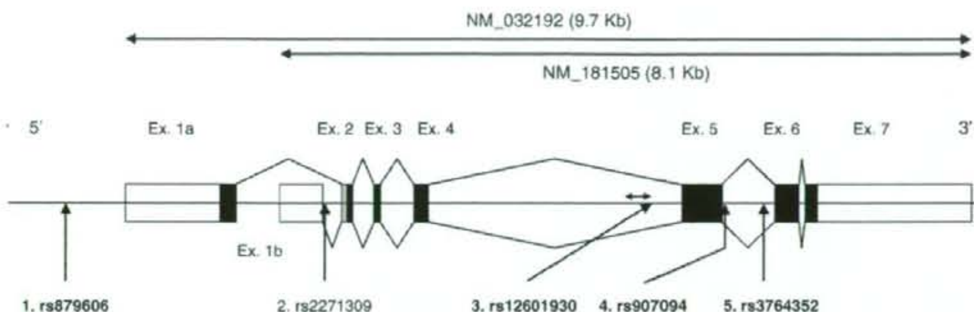

Fig. 1. Genomic structure of *PPP1R1B*. Black boxes indicate protein-coding regions, whereas open boxes denote untranslated regions (UTR). Each box represents the *PPP1R1B* exons. Numbers under the arrows represent the SNP IDs. Bold numbers represent tagging SNPs (pairwise tagger; $r^2>0.8$; Haploview 3.32). All SNPs in the coding region are listed as well as those within the 500-bp upstream 5′-flanking region and the 500-bp downstream 3′ UTR of *PPP1R1B*. Arrows ~200 bp upstream of rs12601930 shows the sites that were PCR amplified for copy number analysis.

rs12601930, rs907094, and rs3764352) in addition to a dbSNP (rs879606) located in the promoter region (Fig. 1). After evaluating the LD pattern with 48 control subjects using Haploview version 3.32, rs2271309 was excluded according to the criterion for pairwise tagging, $r^2>0.8$ (de Bakker et al., 2005) (see Fig. 2).

2.3. SNP genotyping

Genotyping of tagging SNPs was carried out primarily using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. TaqMan assays were used when available (Applied Biosystems, California, USA). TaqMan probes and Universal PCR Master Mix were obtained from Applied Biosystems. Allelic-specific fluorescence was measured using an ABI PRISM 7900 Sequence Detector System (Applied Biosystems). Information about each primer pair and enzyme is available upon request.

2.4. Quantitative real-time PCR

The *PPP1R1B* copy number was analyzed using real-time PCRs with a specific primer set and FAM-labeled fluorescent probe or TaqMan expression assays (Applied Biosystems). The test region for *PPP1R1B* was ~200 bp upstream of the SNP that produced results that deviated from the HWE (Fig. 1) and the prostaglandin transporter gene (*SLCO2A1*) was used as a single-copy control gene (Wilson et al., 2006). We did not use glucose-6-phosphate dehydrogenase (G6PD), which is located on the X chromosome and was used as single-copy control gene in the study from Wilson et al., because it showed an unstable copy number value (not an integral value) in their report.

To determine the relative copy number, 10 ng of genomic DNA was assayed in triplicate in 20 μL of reaction solution containing 1 × final concentration TaqMan Universal Master Mix (Applied Biosystems) and 1 × final concentration TaqMan probe (Applied Biosystems) specific for *PPP1R1B* or 700 nM each primer and 200 nM probe specific for *SLCO2A1*.

Each experiment was performed using an ABI PRISM 7900 Sequence Detector System (Applied Biosystems). To evaluate the relative copy number of *PPP1R1B*, we calculated the relative quantity of the dose of *PPP1R1B* using a comparative C_T method (*PPP1R1B* vs. *SLCO2A1*). The TaqMan specific primers and FAM-labeled fluorescent probe used for the PCR amplifications were as follows: *PPP1R1B*-FAM probe (5′-FAM-CCCCTTGCTCCTTTCC-MGBNFQ-3′), *PPP1R1B*-for (5′-GCCTTGGCCCCTTTCTCTAA-3′), *PPP1R1B*-rev (5′-GCAGCTGGAGACAAGTTTCC-3′), *SLCO2A1*-FAM (5′-FAM-CCATCCATGTCCTCATCTC-

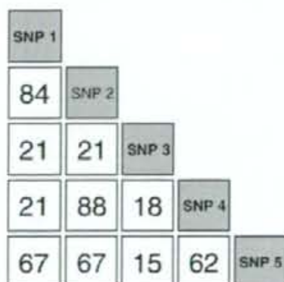

Fig. 2. LD analysis of *PPP1R1B*. Numbers in the top gray boxes correspond to the SNP ID numbers in Fig. 1. Numbers in the white boxes represent the r^2 values after the decimal point.

Table 1
Genotype and allele frequency of *PPP1R1B* in schizophrenia and controls

Gene symbol	SNP ID (M/m)	Method of genotyping	Genotype[a]						p value	Armitage's	Allele				p value	Global
			SCZ[b]			CON[b]				p value	SCZ		CON			p value[c]
PPP1R1B	rs879606 (G/A)	RFLP	G/G 62 (34%)	G/A 81 (45%)	A/A 39 (21%)	G/G 58 (32%)	G/A 91 (49%)	A/A 35 (19%)	0.631	0.985	G 205 (56%)	A 159 (44%)	G 207 (56%)	A 161 (44%)	0.985	0.697
	Rs12601930 (C/T)	real time PCR	C/C 256 (68%)	C/T 110 (29%)	T/T 11 (3%)	C/C 240 (63%)	C/T 134 (35%)	T/T 8 (2%)	0.190	0.271	C 622 (83%)	T 132 (18%)	C 614 (80%)	T 150 (20%)	0.287	
	rs907094 (T/C)	real time PCR	T/T 99 (27%)	T/C 202 (54%)	C/C 71 (19%)	T/T 114 (30%)	T/C 193 (50%)	C/C 77 (20%)	0.518	0.673	T 400 (54%)	C 344 (46%)	T 421 (55%)	C 347 (45%)	0.681	
	rs3764352 (A/G)	RFLP	A/A 86 (24%)	A/G 193 (54%)	G/G 77 (22%)	A/A 104 (28%)	A/G 195 (52%)	G/G 79 (21%)	0.582	0.419	A 365 (51%)	G 347 (49%)	A 403 (53%)	G 353 (47%)	0.434	

[a]M: major allele, m: minor allele [b]SCZ: schizophrenia, CON: control [c]global p value: haplotypic analysis.

MGBNFQ-3′), *SLCO2A1*-for (5′-ATCCCCAAAG-CACCTGGTTT-3′), and *SLCO2A1*-rev (5′-AGAGGC-CAAGATAGTCCTGGTAA-3′).

2.5. Statistical analysis

Genotype deviations from the HWE and single-marker association were analyzed using Haploview software. We evaluated the allelic and genotypic associations by the χ^2-test. Genotypic association of SNPs that deviated from the HWE was analyzed using Cochran-Armitage trend tests for multiplicative model of inheritance (Balding, 2006). Haplotypic analyses were performed with Unphased version 2.403 (Dudbridge, 2003). The significance level for all statistical tests was 0.05. Bonferroni corrections were used for multiple comparisons. Power calculations were performed using the genetic statistical package on a genetic power calculator (Purcell et al., 2003).

3. Results

The genotype and allele frequency of each SNP in schizophrenic patients, bipolar disorder patients, and control subjects are summarized in Table 1 and Table 2-1, respectively. The observed genotype frequencies of the tagging SNPs were within the distribution expected from the HWE except for rs12601930.

Neither the genotype nor the allele frequency of any of the examined *PPP1R1B* SNPs in the schizophrenic patients differed significantly from those observed for the control subjects (Table 1). Moreover, the distribution of haplotypic frequencies in the schizophrenia patients did not differ significantly from that in control subjects. Power analysis showed that more than 80% power in detecting an association with schizophrenia was obtained when the genotype relative risk (GRR) was set from 1.35 to 1.51 in a multiplicative model of inheritance.

A significant association was observed with both the genotype and the allele containing rs907094 ($p=0.036$ and $p=0.010$, respectively), whereas a significant association was only found with the genotype for rs12601930 ($p=0.000147$). Haplotypic analysis supported this association (global $p=0.030$; Table 2-1). After Bonferroni corrections, the observed positive associations were no longer significant for the rs907094 genotype (corrected $p=0.144$) and haplotype (corrected global $p=0.120$), whereas, even after the corrections, the associations remained significant for the rs907094 allele (corrected $p=0.040$) and the rs12601930 genotype (corrected $p=0.000588$). We, however, could not confirm these

Table 2-1
Genotype and allele frequency of *PPP1R1B* in bipolar disorder and controls (first sample set)

Gene symbol	SNP ID (M/m)	Method of genotyping	Genotype[a] BP[b]			Genotype[a] CON[b]			p value	Armitage's p value	Allele BP		Allele CON		p value	Global p value[c]
PPP1R1B	rs879606 (G/A)	RFLP	G/G 87 (30%)	G/A 146 (50%)	A/A 61 (21%)	G/G 58 (32%)	G/A 91 (49%)	A/A 35 (19%)	0.857	0.580	G 320 (54%)	A 268 (46%)	G 207 (56%)	A 161 (44%)	0.306	0.030
	rs12601930 (C/T)	real time PCR	C/C 169 (69%)	C/T 57 (23%)	T/T 18 (7%)	C/C 240 (63%)	C/T 134 (35%)	T/T 8 (2%)	0.000147	0.804	C 395 (81%)	T 3 (19%)	C 614 (80%)	T 150 (20%)	0.802	
	rs907094 (T/C)	real time PCR	T/T 63 (23%)	T/C 202 (54%)	C/C 71 (19%)	T/T 114 (30%)	T/C 193 (50%)	C/C 77 (20%)	0.036	0.010	T 267 (48%)	C 293 (52%)	T 421 (55%)	C 347 (45%)	0.010	
	rs3764352 (A/G)	RFLP	A/A 64 (22%)	A/G 170 (58%)	G/G 58 (20%)	A/A 104 (28%)	A/G 195 (52%)	G/G 79 (21%)	0.176	0.385	A 298 (51%)	G 286 (49%)	A 403 (53%)	G 353 (47%)	0.407	

[a]M: major allele, m: minor allele [b]BP: bipolar disorder, CON: control [c]global p value: haplotypic analysis.

Table 2-2
Genotype and allele frequency of *PPP1R1B* in bipolar disorder and controls (second sample set)

Gene symbol	SNP ID (M/m)	Method of genotyping	Genotype[a] BP[b]			Genotype[a] CON[b]			p value	Armitage's p value	Allele BP		Allele CON		p value	Global p value[c]
PPP1R1B	Rs12601930 (C/T)	real time PCR	C/C 232 (63%)	C/T 119 (33%)	T/T 15 (4%)	C/C 248 (68%)	C/T 105 (29%)	T/T 12 (3%)	0.419	0.192	C 583 (80%)	T 149 (20%)	C 601 (82%)	T 129 (18%)	0.191	0.307
	Rs907094 (T/C)	real time PCR	T/T 102 (28%)	T/C 190 (53%)	C/C 70 (19%)	T/T 107 (29%)	T/C 180 (49%)	C/C 81 (22%)	0.565	0.731	T 394 (54%)	C 330 (46%)	T 394 (54%)	C 342 (46%)	0.734	

[a]M: major allele, m: minor allele [b]BP: bipolar disorder, CON: control [c]global p value: haplotypic analysis.

Table 3-1
PPP1R1B copy number variations detected by qPCR[a] (first sample set)

	SCZ[b] (n=24)	BP[b] (n=24)	CON[b] (n=24)
Relative quantity	0.81 ± 0.14	0.87 ± 0.10	1.11 ± 0.34
Decrease	6	3	2
Normal	17	21	15
Increase	1	0	7

[a]qPCR: quantitative real-time PCR.
[b]SCZ: schizophrenia, BP: bipolar disorder, CON: control.

results in a second independent sample set as shown in Table 2-2. When the two sample sets were merged, power analysis showed the level for detecting association was higher than 80% for bipolar disorder at the genotype relative risk of 1.24 to 1.39 under a multiplicative model of inheritance.

DNA copy number analysis revealed aberrations in the copy number in the schizophrenic patients (increase=1 patient, decrease=6), the bipolar patients (decrease=3), and the control subjects (increase=7, decrease=2; Table 3-1). In the second independent sample set, we observed aberrations in the schizophrenic patients (increase=2, decrease=1), the bipolar patients (increase=2, decrease=1), and the control subjects (increase=2, decrease=5; Table 3-2). All analyses were performed in duplicate.

4. Discussion

According to the common disease-common variants hypothesis (Chakravarti, 1999), the present study showed that *PPP1R1B* was unlikely to be related to be the development of schizophrenia and bipolar disorder in Japanese patients. These results were consistent with a recently published study that examined Chinese patients (Li et al., 2006).

The SNPs used in the association analysis, which covered the entire gene, included all of the common SNPs (more than 5% frequency) listed in the dbSNP database; therefore, it is unlikely that there are other common variants related to these disorders. Because we did not perform mutation screening of this gene, however, the possibility that rare variants could be causal to the development of these disorders cannot be excluded. The GRR value calculated using power analysis was appropriate when compared to other promising candidate genes for schizophrenia (Schwab et al., 2005; Schwab et al., 2003; Shifman et al., 2002).

Recently, Meyer-Lindenberg et al. (2007) tested for an association in schizophrenia with SNPs in this gene using a relatively small sample of Caucasian families, and found a strong association with rs879606 and mild association with two other SNPs (rs3764352 and rs3794712). Two of these three SNPs (rs879606 and rs3764352), however, did not show any significant association in our samples, suggesting that ethnic differences might play a role in these associations.

rs3794712 was not further pursued for two reasons. *Firstly*, this SNP is not validated and the frequency of this SNP is not reported in the dbSNP database. *Secondly*, this SNP is unlikely to affect the function of this gene considering its genomic position in the middle of an intron and the results from *in silico* analysis (RegRNA; http://regrna.mbc.nctu.edu.tw/index.html).

In the present study, we found that copy number differences in the region that includes the SNPs deviated from the HWE and were significantly associated with bipolar disorder (Tables 3-1 and 3-2), suggesting that the presence of copy number alterations gives rise to the deviation from the HWE due to a high frequency of heterozygotes. Therefore, caution must be taken when interpreting results from SNP analysis. These results, however, must be regarded as preliminary because we did not observe a definite association between copy number differences and the disorders. Because accumulating evidence has highlighted that CNVs are observed in many chromosome regions, *including 17q12* (Sharp et al., 2006), and copy number alterations can affect gene expression (Redon et al., 2006), further investigations of the CNVs as well as the SNPs may be an effective complementary approach to elucidate the genetic risk factors underlying the complex phenotypes associated with psychiatric disorders, including schizophrenia and bipolar disorder (Lee and Lupski, 2006).

A couple of limitations should be considered in the present study. Firstly, we observed a strong association between rs12601930 and bipolar disorder in the first sample set even after a Bonferroni correction (corrected genotype p=0.000588); the genotype frequency of the minor allele homozygotes (TT) in the control subjects, however, was only 2% (Table 2-1). This low frequency may have resulted in a false-positive association. When we used a Cochran–Armitage trend test (Balding, 2006)

Table 3-2
PPP1R1B copy number variations detected by qPCR[a] (second sample set)

	SCZ[b] (n=24)	BP[b] (n=24)	CON[b] (n=24)
Relative quantity	0.95 ± 0.10	0.98 ± 0.14	1.03 ± 0.27
Decrease	2	2	2
Normal	69	69	65
Increase	1	1	5

[a]qPCR: quantitative real-time PCR.
[b]SCZ: schizophrenia, BP: bipolar disorder, CON: control.

to correct the observed association with rs12601930, which deviated from the HWE, there was no significance in the genotype frequency (Armitage's $p=0.804$). Thus, the possibility of a type I error derived from an insufficient sample size should be considered. Secondly, although the method used for copy number analysis has been widely used and is thought to be highly reliable due to the consistency of results obtained in our duplicate experiments, supplementary methods, such as Southern blotting, fluorescence in situ hybridization, or array comparative genomic hybridization, would have provided further confirmation of our results. Finally, the male/female ratios and average ages were not completely consistent between the schizophrenic patients and the control subjects. Based on results from an exploratory analysis using a logistic regression model, however, these variables do not appear to contribute to the results obtained in the present association study (data not shown).

In conclusion, our findings suggest that SNPs within *PPP1R1B* do not elevate the risk for either schizophrenia or bipolar disorder in the Japanese population. Further functional analysis of the CNVs and association studies using other endophenotypes including cognitive function should be needed to clarify the exact role of this gene in the pathophysiology of these disorders.

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Contributors

Author Akira Yoshimi, Nagahide Takahashi, and Shinichi Saito designed the study and wrote the protocol. Author Norio Ozaki and Yukihiro Noda performed the literature searches and analyses. Author Akira Yoshimi wrote the first draft of the manuscript and Nagahide Takahashi and Shinichi Saito revised it. All of the authors contributed to and have approved the final version of the manuscript.

Conflict of interest

All authors declare that they have no conflicts of interests.

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Association between the brain-derived neurotrophic factor Val66Met polymorphism and brain morphology in a Japanese sample of schizophrenia and healthy comparisons

Tsutomu Takahashi [a,d], Michio Suzuki [a,d,*], Masahiko Tsunoda [a], Yukiko Kawamura [f,g], Nagahide Takahashi [f,h], Hiroshi Tsuneki [b], Yasuhiro Kawasaki [a,d], Shi-Yu Zhou [a,d,e], Soushi Kobayashi [b], Toshiyasu Sasaoka [b], Hikaru Seto [c], Masayoshi Kurachi [a,d], Norio Ozaki [f]

[a] *Department of Neuropsychiatry, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan*
[b] *Department of Clinical Pharmacology, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan*
[c] *Department of Radiology, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan*
[d] *Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Tokyo, Japan*
[e] *Department of Psychiatry and Medical Psychology, Dalian Medical University, Dalian, China*
[f] *Department of Psychiatry, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[g] *Division of Clinical Science and Neuropsychopharmacology, Faculty of Pharmacy, Meijo University, Nagoya, Japan*
[h] *Laboratory of Molecular Neuropsychiatry, Department of Psychiatry, Mount Sinai School of Medicine, New York, USA*

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Abstract

Magnetic resonance imaging was used to investigate the relation between the brain-derived neurotrophic factor (BDNF) Val66Met polymorphism and volumetric measurements for the medial temporal lobe structures (amygdala, hippocampus, and parahippocampal gyrus) and prefrontal subregions (the superior frontal gyrus, middle frontal gyrus, inferior frontal gyrus, ventral medial prefrontal cortex, orbitofrontal cortex, and straight gyrus) in a Japanese sample of 33 schizophrenia patients and 29 healthy subjects. For the controls, the Met carriers had significantly smaller parahippocampal and left superior frontal gyri than the Val homozygotes. The schizophrenia patients carrying the Met allele had a significantly smaller right parahippocampal gyrus than those with the Val/Val genotype, but the genotype did not affect the prefrontal regions in schizophrenia patients. These findings might reflect different genotypic effects of BDNF on brain morphology in schizophrenia patients and healthy controls, implicating the possible role of the brain morphology as an endophenotype for future genetic studies in schizophrenia.
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Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family of growth factors, mediates differentiation and survival of neurons during development as well as synaptic plasticity in the mature nervous system [17,18,25]. A single nucleotide polymorphism (SNP) in the BDNF gene that produces an amino acid substitution (valine to methionine) at codon 66 (Val66Met) has been reported to affect the activity-dependent secretion of BDNF in cultured hippocampal neurons [4] and also human cognitive functioning [4,9,37]. Moreover, recent magnetic resonance imaging (MRI) studies in healthy subjects reported the effect of this functional SNP on brain morphology, with BDNF Met carriers having a reduced volume of the hippocampus [3,24,33], parahippocampal gyrus [20], or prefrontal cortex [24].

Several lines of evidence suggest a role for BDNF in the pathogenesis of schizophrenia. Recent postmortem studies in schizophrenia demonstrated decreased BDNF levels in the prefrontal cortex [10,40], and changes in the plasma BDNF levels were also reported in schizophrenia patients [8]. The association between the BDNF Val66Met polymorphism and treatment responses or clinical symptoms in schizophrenia [13,16,22]

* Corresponding author at: Department of Neuropsychiatry, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan. Tel.: +81 76 434 2281; fax: +81 76 434 5030.

E-mail address: suzukim@med.u-toyama.ac.jp (M. Suzuki).

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implies that the BDNF gene would be a plausible candidate for a schizophrenia-susceptibility gene.

Although previous genetic studies have not supported a significant role of the BDNF gene variants in the development of schizophrenia [15,19], several MRI studies have suggested different genotypic effects of BDNF on brain morphology in schizophrenia patients and healthy controls [1,11,12,33]. These studies have suggested that the effect of BDNF Val66Met polymorphism on medial temporal lobe [12,33] or frontal gray matter [11] volume is pronounced among schizophrenia patients as compared to healthy individuals, although the data are not entirely consistent [1]. These findings suggest a potential role for the investigation of the brain morphology as a neurobiological endophenotype in future genetic studies of schizophrenia [7], but first require further replication.

In this study, we used MRI to investigate the association between the BDNF Val66Met polymorphism and brain morphology in schizophrenia patients and healthy subjects. Based on previous MRI observations [3,20,24,33], regions of interest (ROIs) for the volumetric measurements were placed in the medial temporal and frontal lobe structures. We predicted from previous reports [11,12,33] that variation in the BDNF gene (Val66Met), a candidate locus for schizophrenia, would affect brain morphology differently in schizophrenia patients versus healthy subjects.

Thirty-three schizophrenia patients [20 males and 13 females, mean age = 25.6 ± 4.5 (S.D.) years (range, 19–36)] who met the ICD-10 diagnostic criteria for research [41] were recruited from the inpatient and outpatient clinics of the Department of Neuropsychiatry, Toyama University Hospital. All patients were receiving neuroleptic medication at the time of scanning [mean haloperidol equivalent dose = 12.2 ± 8.6 (S.D.) mg/day], with a mean duration of medication of 2.7 years (S.D. = 2.8). Fifteen patients were being treated with typical neuroleptics and 18 patients were receiving atypical neuroleptics. At the time of the MRI study, their mean scores on the scale for the assessment of negative symptoms (SANS) and the scale for the assessment of positive symptoms (SAPS) [2] were 49.7 (S.D. = 22.9) and 26.1 (S.D. = 24.7), respectively. The control subjects consisted of 29 healthy volunteers (17 males and 12 females) recruited from members of the community, hospital staff, and university students. Their mean age was 24.2 ± 6.1 (S.D.) years (range, 18–38). They were given a questionnaire consisting of 15 items concerning their family and past histories, and present illness. They did not have any personal or family history of psychiatric illness in their first-degree relatives. The minnesota multiphasic personality inventory (MMPI) was administered to all the control candidates, and they were excluded if any T-score for the validity scales or the clinical scales exceeded 70. This cohort is largely included in our previous MRI studies, which investigated the morphology in the temporal and frontal lobe structures in schizophrenia [21,32,35]. All subjects were right-handed Japanese and physically healthy at the time of the study, and none had a lifetime history of serious head trauma, neurological illness, serious medical or surgical illness, or substance abuse. The two diagnostic groups were matched for age, gender, height, and parental education. This study was approved by the ethical committees of Toyama University and Nagoya University. Written informed consent was obtained from all subjects.

Magnetic resonance images were obtained by utilizing a 1.5-T magnetom vision (Siemens Medical System, Inc., Erlangen, Germany) with a three-dimensional gradient-echo sequence fast low-angle shots (FLASH) yielding 160–180 contiguous T1-weighted slices of 1.0-mm thickness in the sagittal plane. The imaging parameters were: repetition time = 24 ms; echo time = 5 ms; flip angle = 40°; field of view = 256 mm; and matrix size = 256×256 pixels. The voxel size was $1.0\,mm \times 1.0\,mm \times 1.0\,mm$. Image processing for volumetric analysis has been described in detail elsewhere [34]. Briefly, on a Unix workstation (Silicon Graphics, Inc., Mountain View, CA), the image data were processed using the software package Dr. View 5.3 (Asahi Kasei Joho System Co., Ltd., Tokyo, Japan). Brain images were realigned in three dimensions to standardize for differences in head tilt during image acquisition and were then reconstructed into entire contiguous coronal images, with a 1-mm thickness, perpendicular to the anterior commissure-posterior commissure line. The whole cerebrum was manually separated from the brainstem and cerebellum. The signal-intensity histogram distributions from the T1-weighted images across the whole cerebrum were then used to semi-automatically segment the voxels into gray matter, white matter, and cerebrospinal fluid. The intracranial volume (ICV) was measured to correct for differences in head size as previously described [42]; the groups did not significantly differ in their ICV volumes.

The medial temporal structures (amygdala, hippocampus, and parahippocampal gyrus) and prefrontal sub-regions (the superior frontal gyrus, middle frontal gyrus, inferior frontal gyrus, ventral medial prefrontal cortex, orbitofrontal cortex, and straight gyrus) were manually traced on consecutive 1-mm coronal slices with the corresponding sagittal and axial planes simultaneously presented for reference. The procedures for delineation of these structures were described in detail previously [21,32]. The gray matter volumes of the prefrontal sub-regions were obtained by using the above-mentioned segmentation procedure. For the medial temporal lobe structures, volumes of gray and white matter were measured together. Three trained raters (S.Z., H.H., and L.N) measured the volumes of each ROI without knowledge of the subjects' identity, gender, and diagnosis. Intra- and inter-rater intraclass correlation coefficients in a subset of five randomly selected brains were over 0.92 for all ROIs.

Genomic DNA was extracted from EDTA-containing venous blood samples according to standard procedures. The genotyping of the Val66Met SNP (rs6265) of the BDNF gene was carried out using the polymerase chain reaction-restriction fragment length polymorphism assay. Sequences of primer pairs are Fw: 5′-ACTCTGGAGAGCGTGAATGG-3′ and Rv: 5′-CCGAACTTTCTGGTCCTCAT-3′. NlaIII was used for digestion.

Genotypic distribution and allelic frequencies were compared between schizophrenia patients and healthy subjects using the chi-square test. For the genotypic effects on brain morphology, each diagnostic group was examined separately based on the hypothesis that variation in the BDNF gene would affect brain

morphology differently in schizophrenia patients and healthy subjects. The relative volume [(absolute volume/ICV) × 100] for each region was analyzed using the repeated measures analysis of covariance (ANCOVA) with age and gender as covariates, genotype [Val homozygotes versus Met carriers (heterozygotes and Met homozygotes combined)] as a between-subject factor, and side (left versus right) as a within-subject variable. Heterozygotes and Met homozygotes were combined and categorized as Met carriers following the strategy used by Ho et al. [12]. The volumetric measurements for all ROIs in this study were normally distributed (Kolmogorov–Smirnov test). Post hoc Spjotvoll and Stoline tests, modified Tukey's tests for unequal sample size, were carried out to follow up the significant main effects or interactions yielded by ANCOVAs. Statistical significance was defined as $p < 0.05$.

The observed genotypic frequency of SNP was within the distribution expected according to Hardy–Weinberg equilibrium. As for the genotypic distribution, 12/33 schizophrenia patients (36.4%) and 13/29 controls (44.8%) were Val homozygotes, 6/33 patients (18.2%) and 5/29 controls (17.2%) were Met homozygotes, and 15/33 patients (45.5%) and 11/29 controls (37.9%) were heterozygotes, with no significant group difference (chi-square = 0.49, $p = 0.783$). No significant difference was observed in allelic frequencies between the patients and controls either (chi-square = 0.29, $p = 0.592$). For schizophrenia patients, no differences between the genotype groups were observed in age, education, parental education, numbers of hospitalization, onset age, illness duration, total score for SANS and SAPS, or medication status (dose, duration, and typical versus atypical). Although an association between smoking behavior and BDNF Val66Met polymorphism has been previously reported in schizophrenia [38], assessment of cigarette smoking behavior was not comprehensively undertaken in this sample.

Table 1 shows a comparison of the relative ROI volumes between the subjects with the Val/Val genotype and Met carriers. For the healthy controls, ANCOVA showed a significant main effect of genotype for the parahippocampal gyrus and a significant genotype-by-side interaction for the superior frontal gyrus, with the Met carriers having a smaller parahippocampal gyrus (post hoc test, $p = 0.014$) and smaller left superior frontal gyrus (post hoc test, $p = 0.010$) than the Val homozygotes (Fig. 1). For the schizophrenia patients, ANCOVA demonstrated a significant genotype-by-side interaction for the parahippocampal gyrus, showing that the Met carriers had a smaller right parahippocampal gyrus than the Val homozygotes (post hoc test, $p = 0.022$) (Fig. 1).

This volumetric MRI study investigated the effect of the BDNF Val66Met polymorphism on the prefrontal and medial temporal lobe structures in schizophrenia patients and healthy controls. We demonstrated that the Met carriers had a significantly smaller left superior frontal gyrus than the Val homozygotes among the controls but not the patients. For the parahippocampal gyrus, our results demonstrated that the Met allele is related to a reduction in volume bilaterally for controls, but only in the right hemisphere for the schizophrenia patients.

The present finding is consistent with the result of a previous MRI study using voxel-based morphometry that showed a similar association between the BDNF Val66Met polymorphism and the left superior frontal gyrus only for healthy controls [12]. The prefrontal cortex is an area that has received much attention in the search for the neural substrate of schizophrenia. In addition to a significant volume reduction [29,30,32], prefrontal abnormalities in schizophrenia have been implicated in negative symptoms and several cognitive impairments [6]. Although we did not find a specific genotypic effect of the BDNF on brain morphology in schizophrenia, a recent longitudinal MRI study demonstrated that BDNF Met allele contributed to frontal gray matter reduction over time in schizophrenia patients [11]. BDNF is known to support the survival and differentiation of dopaminergic neurons, and regulates the structural and functional maturation of the prefrontal cortex during young adulthood in the human brain [8,39]. Impairment of BDNF and related dopaminergic functions at this critical period in neurodevelopment, which could be influenced by variation in the BDNF gene, has important implications for the prefrontal pathology of schizophrenia. In addition, the findings of Ho et al. [11] support ongoing neuroplastic effects of BDNF beyond neurodevelopment. Our failure to find genotypic effects of BDNF on prefrontal gray matter volume in schizophrenia can possibly be explained by the fact

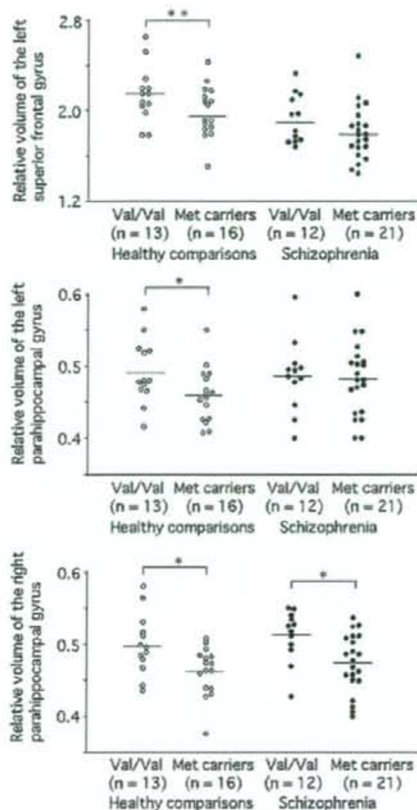

Fig. 1. Scatter plots of relative volumes (100 × absolute volume/intracranial volume) for the left superior frontal gyrus gray matter and the parahippocampal gyrus in healthy controls and schizophrenia patients. Horizontal lines indicate means. Post hoc comparisons: $^{*}p < 0.05$, $^{**}p < 0.01$.

Table 1
BDNF genotypic differences in relative volume for regions of interest in schizophrenia patients and healthy controls

Brain region	Healthy comparisons		Analysis of covariance[a]				Schizophrenia patients		Analysis of covariance[b]			
	Val/Val ($N = 13$)	Met carriers ($N = 16$)	Genotype		Genotype × side		Val/Val ($N = 12$)	Met carriers ($N = 21$)	Genotype		Genotype × side	
			F	p	F	p			F	p	F	p
Amygdala			0.63	0.436	0.80	0.379			0.52	0.478	<0.01	0.965
Left	0.074 ± 0.011	0.078 ± 0.008					0.065 ± 0.006	0.066 ± 0.011				
Right	0.081 ± 0.007	0.075 ± 0.012					0.070 ± 0.006	0.071 ± 0.010				
Hippocampus			1.72	0.201	2.21	0.148			0.92	0.346	1.19	0.283
Left	0.208 ± 0.026	0.204 ± 0.020					0.201 ± 0.027	0.193 ± 0.025				
Right	0.224 ± 0.021	0.210 ± 0.018					0.218 ± 0.030	0.203 ± 0.024				
Parahippocampal gyrus			**5.22**	**0.031**	0.09	0.772			0.68	0.415	**4.93**	**0.034**
Left	0.492 ± 0.045	0.459 ± 0.038					0.487 ± 0.050	0.483 ± 0.052				
Right	0.499 ± 0.042	0.461 ± 0.034					0.512 ± 0.036	0.474 ± 0.041				
Superior frontal gyrus			0.70	0.410	**8.24**	**0.008**			1.30	0.264	0.06	0.811
Left	2.132 ± 0.249	1.980 ± 0.228					1.909 ± 0.214	1.775 ± 0.245				
Right	1.937 ± 0.273	1.956 ± 0.233					1.795 ± 0.226	1.679 ± 0.239				
Middle frontal gyrus			0.35	0.560	1.72	0.201			0.05	0.825	<0.01	0.968
Left	1.762 ± 0.340	1.867 ± 0.265					1.836 ± 0.246	1.764 ± 0.299				
Right	1.753 ± 0.361	1.766 ± 0.279					1.777 ± 0.184	1.708 ± 0.276				
Inferior frontal gyrus			0.14	0.716	0.08	0.781			0.43	0.518	0.47	0.498
Left	0.941 ± 0.174	0.915 ± 0.126					0.856 ± 0.111	0.840 ± 0.126				
Right	0.881 ± 0.140	0.865 ± 0.113					0.826 ± 0.134	0.782 ± 0.115				
Ventral medial PFC			0.30	0.590	2.73	0.110			0.35	0.558	2.44	0.129
Left	0.402 ± 0.083	0.399 ± 0.074					0.384 ± 0.064	0.364 ± 0.060				
Right	0.376 ± 0.051	0.417 ± 0.057					0.349 ± 0.061	0.364 ± 0.063				
Orbitofrontal cortex			0.01	0.905	0.23	0.636			1.37	0.252	1.28	0.267
Left	1.032 ± 0.112	1.016 ± 0.113					1.033 ± 0.095	0.986 ± 0.113				
Right	1.051 ± 0.086	1.024 ± 0.091					1.048 ± 0.063	0.969 ± 0.116				
Straight gyrus			1.15	0.294	2.13	0.156			0.32	0.576	0.55	0.466
Left	0.212 ± 0.032	0.210 ± 0.024					0.196 ± 0.022	0.192 ± 0.027				
Right	0.228 ± 0.033	0.205 ± 0.025					0.209 ± 0.032	0.196 ± 0.027				

PFC, prefrontal cortex; values represent means ± S.D.s. Relative volumes were calculated as follows: 100 × absolute volume/intracranial volume. Absolute volumes for the medial temporal structures and frontal regions in a larger sample were published elsewhere [32].

[a] Df = 1, 25 for the effect of genotype, and 1, 27 for genotype-by-side interaction.

[b] Df = 1, 29 for the effect of genotype, and 1, 31 for genotype-by-side interaction.

that the present cross-sectional study did not examine progressive brain morphologic changes. The possibility also exists that brain morphology in schizophrenia is more liable to be influenced by non-genetic factors such as antipsychotic medication [11,27]. This medication issue will be further discussed below.

For the parahippocampal gyrus, which is another structure that has been implicated in the pathophysiology of schizophrenia [5,26], we found a significant relationship between the BDNF genotype and the volume of this region only in the right hemisphere in schizophrenia patients. Our findings may be partly consistent with a previous MRI study showing that schizophrenia patients and nonpsychotic relatives from families with multiply affected ill members had significantly smaller right parahippocampal gyrus than controls [28]. However, our results did not accord with the previous MRI study [12], which showed a genotypic effect of BDNF only on the left parahippocampal gyrus in schizophrenia. The timing and course of brain morphologic changes in schizophrenia are not well understood [23] and brain changes related to genetic vulnerability of the illness are not necessarily static over time [11]. Thus, further longitudinal studies are needed to clarify the contribution of genetic and non-genetic factors to the neurobiology of schizophrenia.

As discussed above, the role of the BDNF Val66Met polymorphism in the prefrontal and medial temporal lobe structures in schizophrenia remains unclear. Additionally, we investigated only a single polymorphism in this study, though schizophrenia is thought to have a multifactorial etiology in which multiple susceptibility genes interact with environmental insults. Nevertheless, the present findings support the possibility that BDNF affects brain morphology differently in schizophrenia patients and healthy subjects, implicating brain morphology as a potential endophenotype for future genetic studies in schizophrenia.

In this study, we did not find a significant association between the BDNF Val66Met polymorphism and hippocampal volume in schizophrenia patients or healthy controls. In contrast, several MRI studies reported a smaller hippocampal volume in Met carriers [3,24,33], supporting the notion that the BDNF expressed abundantly in the hippocampus plays an important role in human memory formation [17,18,25]. This discrepancy might be partly explained by ethnic differences. In a recent MRI study, Inoue et al. [14] showed a lack of association between the BDNF Val66Met genotype and manually measured volumes of the amygdala and hippocampus in 116 healthy Japanese individuals. Another MRI study in healthy Japanese found no genotypic effect of the BDNF Val66Met polymorphism on hippocampal volume either, but demonstrated an association between this polymorphism and the volume of the parahippocampal gyrus as in the present study [20]. Furthermore, there seem to be ethnic differences in brain morphology [43] and also in the frequency of the BDNF Val66Met polymorphism, with healthy Japanese individuals carrying Met significantly more often than healthy Caucasians [31,36]. Thus, both present and previous findings suggest possible interethnic differences in the variation of the BDNF gene as well as in its genotypic effects on brain morphology especially for the medial temporal lobe structures.

A few possible confounding factors in the present study need to be addressed. First, all the schizophrenia patients were on antipsychotic medication, which might directly affect brain morphology [27]. Although duration of antipsychotic exposure in our sample was relatively short and there was no difference in medication status between the genotype groups, pharmacotherapy, particularly with typical antipsychotics, may cause gray matter reduction in cortical regions even over short periods [11,27]. Thus, medication effects may have influenced our findings, with particular regard to comparisons between healthy subjects and schizophrenia patients. Secondly, the relatively small number of subjects limited our ability to generalize the findings of the study. Failure to replicate previous findings of genotypic effect of the BDNF on hippocampal volume may be a result of the limited sample size of this study. Additionally, the weak association between the BDNF genotypes and brain morphology did not allow for statistical correction for multiple comparisons, representing a further limitation of the study. Thus, the results of the present study should be confirmed by an additional study with a large number of subjects without sustained antipsychotic treatment.

In conclusion, our preliminary findings suggest that a variation of the BDNF Val66Met polymorphism may affect brain morphology differently in schizophrenia patients and healthy controls in the left superior frontal and parahippocampal gyri. Although we did not observe a genotypic effect of this polymorphism on brain morphology specific to schizophrenia, our findings imply the possible role of brain morphology as an endophenotype for future genetic studies in schizophrenia.

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The association of genotypic combination of the DRD3 and BDNF polymorphisms on the adhesio interthalamica and medial temporal lobe structures

Tsutomu Takahashi [a,d], Michio Suzuki [a,d,*], Masahiko Tsunoda [a], Yukiko Kawamura [f,g], Nagahide Takahashi [f,h], Nobuhisa Maeno [f,i], Yasuhiro Kawasaki [a,d], Shi-Yu Zhou [a,d,e], Hirofumi Hagino [a], Lisha Niu [a], Hiroshi Tsuneki [b], Soushi Kobayashi [b], Toshiyasu Sasaoka [b], Hikaru Seto [c], Masayoshi Kurachi [a,d], Norio Ozaki [f]

[a] *Department of Neuropsychiatry, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan*
[b] *Department of Clinical Pharmacology, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan*
[c] *Department of Radiology, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan*
[d] *Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Tokyo, Japan*
[e] *Department of Psychiatry and Medical Psychology, Dalian Medical University, Dalian, China*
[f] *Department of Psychiatry, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[g] *Division of Clinical Science and Neuropsychopharmacology, Faculty of Pharmacy, Meijo University, Nagoya, Japan*
[h] *Laboratory of Molecular Neuropsychiatry, Department of Psychiatry, Mount Sinai School of Medicine, New York, USA*
[i] *Department of Brain Science and Molecular Imaging, National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, Obu, Japan*

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ABSTRACT

Abnormal neurodevelopment in midline structures such as the adhesio interthalamica (AI), as well as in the medial temporal lobe structures has been implicated in schizophrenia, while its genetic mechanism is unknown. This magnetic resonance imaging study investigated the effect of the genotypic combination of the dopamine D3 receptor (DRD3) Ser9Gly and brain-derived neurotrophic factor (BDNF) Val66Met polymorphisms on the AI length and volumetric measures of the medial temporal lobe structures (amygdala, hippocampus, and parahippocampal gyrus) in 33 schizophrenia patients and 29 healthy controls. The subjects with a combination of the Ser/Ser genotype of DRD3 and Met-containing genotypes of BDNF (high-risk combination) had a shorter AI than those without it in the healthy controls, but not in the schizophrenia patients. The subjects carrying the high-risk combination had a smaller posterior hippocampus than those without it for both diagnostic groups. These genotypic combination effects on brain morphology were not explained by the independent effect of each polymorphism. These findings suggest the effect of gene–gene interaction between the DRD3 and BDNF variations on brain morphology in midline and medial temporal lobe structures, but do not support its specific role in the pathogenesis of schizophrenia.

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1. Introduction

The adhesio interthalamica (AI), a narrow bridge of glial cells connecting the medial surfaces of the thalamus on each side, is variable in size among individuals and missing in about 20–30% of human brains (Kretschmann and Weinrich, 1992; Percheron, 2004). Although its role in humans is unclear, the AI develops during early gestation (O'Rahilly and Muller, 1990; Rosales et al., 1968) and is likely to be involved in dopaminergic regulation (Cheramy et al., 1984; Romo et al., 1984). While not consistently replicated (Ettinger et al., 2007; de Souza Crippa et al., 2006; Meisenzahl et al., 2000), the AI was found to be more often absent and shorter in schizophrenia compared with healthy subjects (Erbagci et al., 2002; Nopoulos et al., 2001; Snyder et al., 1998; Shimizu et al., in press; Takahashi et al., 2008). In addition, several magnetic resonance imaging (MRI) studies reported an association between the midline brain structures and the medial temporal morphology in schizophrenia (Kasai et al., 2004; Kwon et al., 1998; Takahashi et al., 2007, 2008). Thus, the AI findings in schizophrenia could be a marker of early developmental and dopaminergic abnormalities in the midline and associated medial temporal lobe structures. However, the genetic mechanism underlying this neurodevelopmental process is largely unknown.

The dopamine D3 receptor (DRD3) gene could be a strong candidate gene for schizophrenia-susceptibility considering the dopamine hypothesis of the illness (Meltzer and Stahl, 1976). Increased DRD3

Abbreviations: AI, adhesio interthalamica; ANOVA, Analysis of variance; ANCOVA, Analysis of covariance; BDNF, Brain-derived neurotrophic factor; CASH, Comprehensive Assessment of Symptoms and History; DRD3, Dopamine D3 receptor; ICC, Intraclass correlation coefficient; ICD-10, International Classification of Diseases, 10th edition; ICV, Intracranial volume; MRI, Magnetic resonance imaging; SANS, Scale for the Assessment of Negative Symptoms; SAPS, Scale for the Assessment of Positive Symptoms; SNP, single nucleotide polymorphism.

* Corresponding author. Department of Neuropsychiatry, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan. Tel.: +81 76 434 2281; fax: +81 76 434 5030.
E-mail address: suzukim@med.u-toyama.ac.jp (M. Suzuki).

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expression in the brain of drug-free schizophrenia patients also implicates its role in the pathophysiology of the illness (Gurevich et al., 1997). The DRD3 Ser9Gly single nucleotide polymorphism (SNP), which causes higher dopamine binding affinity (Lundstrom and Turpin, 1996), has been shown to affect the response to antipsychotics (Scharfetter et al., 1999; Szekeres et al., 2004) or susceptibility to tardive dyskinesia (Bakker et al., 2006) in schizophrenia, although it is not consistently replicated (Liou et al., 2004; Malhotra et al., 1998). Several (Dubertret et al., 1998; Jonsson et al., 2003; Shaikh et al., 1996), but not all (Jonsson et al., 2004), meta-analyses have demonstrated the association between the Ser allele or Ser/Ser genotype and schizophrenia. Given the complex genetic background of schizophrenia (Sawa and Snyder, 2002), these inconsistencies might reflect interactions with other genes that regulate the expression of DRD3.

The genotypic interaction effect of DRD3 and brain-derived neurotrophic factor (BDNF) may be of interest in the search for the pathogenesis of schizophrenia because the BDNF supports the survival and differentiation of dopaminergic neurons by regulating DRD3 expression (Guillin et al., 2004, 2007). Although previous genetic studies testing associations between BDNF gene variants and schizophrenia have produced contradictory results (Jonsson et al., 2006), BDNF Val66Met SNP may weakly impact on clinical variables such as clinical symptoms or treatment response (Hong et al., 2003; Krebs et al., 2000; Numata et al., 2006) as well as on brain morphology (Ho et al., 2006; Szeszko et al., 2005) in schizophrenia. Interestingly, Gourion et al. (2005) demonstrated a significant interaction effect of the DRD3 Ser9Gly and BDNF Val66Met SNPs on the onset age of schizophrenia. To our knowledge, however, no studies in schizophrenia have examined the gene–gene interaction effect of these SNPs on quantitative neurobiological endophenotypes.

In summary, the current evidence suggests that 1) the genotypic combination of DRD3 and BDNF may play a role in the dopaminergic pathology of schizophrenia and that 2) the morphology of the AI and related medial temporal lobe structures may be a marker of early neurodevelopmental and dopaminergic deficits in schizophrenia. Based on these notions, we hypothesized that the interaction of DRD3 and BDNF would affect these brain structures specifically in schizophrenia. The current MRI study sought to test this hypothesis by examining the genotypic interaction effect of the DRD3 Ser9Gly and BDNF Val66Met SNPs on the length of the AI and volumetric measures of the medial temporal lobe structures (amygdala, hippocampus, and parahippocampal gyrus) in schizophrenia patients and healthy controls.

2. Methods

2.1. Subjects

Demographic and clinical data of the subjects in this study are shown in Table 1. This cohort was largely included in that in our previous MRI study, which investigated the morphology of the AI and medial temporal lobe structures in schizophrenia (Takahashi et al., 2008). All subjects were right-handed Japanese and physically healthy at the time of the study, and none had a lifetime history of serious head trauma, neurological illness, serious medical or surgical illness, or substance abuse. The two diagnostic groups were matched for age, gender, height, and parental education (Table 1).

Thirty-three schizophrenia patients who met the ICD-10 diagnostic criteria for research (World Health Organization, 1993) were recruited from the inpatient and outpatient clinics of the Department of Neuropsychiatry, Toyama University Hospital. Diagnoses were made following structured clinical interviews by psychiatrists with the Comprehensive Assessment of Symptoms and History (CASH; Andreasen et al., 1992). Clinical symptoms were rated at the time of scanning using the Scale for the Assessment of Negative Symptoms (SANS) and the Scale for the Assessment of Positive Symptoms (SAPS) (Andreasen, 1984). All patients were receiving neuroleptic medication at the time of scanning; 15 patients were treated with typical neuroleptics and 18 patients were receiving atypical neuroleptics. Patients were also receiving anticolinergic drugs ($N=28$), benzodiazepines ($N=25$), antidepressant ($N=1$), and/or carbamazepine ($N=2$).

Table 1
Clinical and genetic descriptions of healthy comparisons and patients with schizophrenia

Variable	Healthy comparisons	Schizophrenia patients	Group comparisons (one-way ANOVA or chi-square test)
	$N=29$	$N=33$	
Male/female	17/12	20/13	Chi-square=0.03, $p=0.874$
Age (years)	24.2±6.1	25.6±4.5	$F(1, 60)=1.01$, $p=0.319$
Height (cm)	166.0±7.0	165.5±7.8	$F(1, 60)=0.05$, $p=0.819$
Education (years)	15.4[a]±2.4	13.5±1.7	$F(1, 60)=13.45$, $p=0.001$
Parental education (years)	12.2±2.3	12.3±2.2	$F(1, 60)=0.10$, $p=0.759$
Age at onset (years)	–	22.2±4.4	–
Duration of illness (years)	–	3.5±3.6	–
Duration of medication (years)	–	2.7±2.8	–
Drug (mg/day, haloperidol equivalent)	–	12.2±8.6	–
Total SAPS score	–	26.1±24.7	–
Total SANS score	–	49.7±22.9	–
DRD3 genotypes (*N*: Ser homo/hetero/ Gly homo)	12/16/1	15/15/2[b]	Chi-square=0.55, $p=0.759$
BDNF genotypes (*N*: Val homo/hetero/ Met homo)	13/11/5	12/15/6	Chi-square=0.49, $p=0.783$

Values represent means±SDs. SANS, scale for the assessment of negative symptoms; SAPS, scale for the assessment of positive symptoms.
[a] $p<0.01$: compared to schizophrenia patients (Scheffé's test).
[b] DRD3 gene was not detected for one patient.

The control subjects consisted of 29 healthy volunteers recruited from members of the community, hospital staff, and university students. They were given a questionnaire consisting of 15 items concerning their personal (13 items; e.g., a history of obstetric complications, substantial head injury, seizures, neurological or psychiatric diseases, impaired thyroid function, hypertension, diabetes, and substance use) and family (2 items) histories of illness. They did not have any personal or family history of psychiatric illness in their first-degree relatives. The Minnesota Multiphasic Personality Inventory (New Japanese MMPI Committee, 1997) was administered to all the control candidates, and they were excluded if any *T*-score for the validity scales or the clinical scales exceeded 70. This study was approved by the ethical committees of Toyama University and Nagoya University. Written informed consent was obtained from all subjects.

2.2. Magnetic resonance imaging procedures

Magnetic resonance images were obtained by utilizing a 1.5-T Magnetom Vision (Siemens Medical System, Inc, Erlangen, Germany) with a three-dimensional gradient-echo sequence FLASH (fast low-angle shots) yielding 160–180 contiguous T1-weighted slices of 1.0-mm thickness in the sagittal plane. The number of slices acquired varied between subjects according to the head size of each case. The imaging parameters were: repetition time=24 ms; echo time=5 ms; flip angle=40°; field of view=256 mm; and matrix size=256×256 pixels. The voxel size was 1.0 mm^3. Image processing for volumetric analysis has been described in detail elsewhere (Takahashi et al., 2002). Briefly, on a Unix workstation (Silicon Graphics, Inc, Mountain View, CA, USA), the image data were processed using the software package Dr View 5.3

(Asahi Kasei Joho System Co, Ltd, Tokyo, Japan). Brain images were realigned in three dimensions to standardize for differences in head tilt during image acquisition and were then reconstructed into entire contiguous coronal images of 1-mm thickness, perpendicular to the anterior commissure–posterior commissure line. The whole cerebrum was manually separated from the brainstem and cerebellum. The signal-intensity histogram distributions from the T1-weighted images across the whole cerebrum were then used to semi-automatically segment the voxels into gray matter, white matter, and cerebrospinal fluid. The intracranial volume (ICV) was measured to correct for differences in head size as previously described (Zhou et al., 2003).

2.3. Assessment of regions of interest

To assess the length of the AI, the number of slices where an AI was clearly seen was counted on consecutive 1-mm coronal slices (Takahashi et al., 2008). Both intra- and inter (TT and KN)-rater intra-class correlation coefficients (ICCs) for the length of the AI in a subset of 30 randomly selected brains were over 0.97.

The medial temporal lobe structures (amygdala, hippocampus, and parahippocampal gyrus) were manually traced on consecutive 1-mm coronal slices with the corresponding sagittal and axial planes simultaneously presented for reference. Detailed delineation methods for these structures have been described elsewhere (Niu et al., 2004; Suzuki et al., 2005a,b). The volumes of gray and white matter in each of these structures including the parahippocampal gyrus were measured together. The inferior border of the amygdala in contact with the hippocampus head was determined by reference to the sagittal plane; the alveus was used to differentiate these structures. The hippocampus and the parahippocampal gyrus were subdivided into anterior and posterior parts at the level of the posterior edge of the mammillary body. Two trained raters (HH and LN) measured the volumes of the medial temporal lobe structures without knowledge of the subjects' identity, gender, or diagnosis. Inter- and intra-rater ICCs in five randomly selected brains were over 0.93.

2.4. DNA procedures

Genomic DNA was extracted from EDTA-containing venous blood samples according to standard procedures. The genotyping of the Val66Met SNP (rs6265) of the BDNF gene and the Ser9Gly SNP (rs6280) of the DRD3 gene were carried out using polymerase chain reaction-restriction fragment length polymorphism assays. Sequences of primer pairs and restriction enzymes were as follows; Fw: 5′-ACTCTGGAGAGCGTGAATGG-3′, Rv: 5′-CCGAACTTTCTGGTCCTCAT-3′, Nla III (rs6265); Fw: 5′-CTCTGCCCCACAGGTGTAGT-3′, Rv: 5′-CAAGCCCCAAAGAGTCTGAT-3′, Hae III (rs6280).

2.5. Statistical analysis

Statistical analysis was carried out using the software package STATISTICA for Macintosh (StatSoft, Tulsa, OK, USA). Genotype frequencies between schizophrenia patients and healthy comparisons were compared using chi-square test to test for Hardy–Weinberg equilibrium. Based on the previous observation (Gourion et al., 2005), the genotypic combination of the Ser/Ser genotype of the DRD3 gene and Met-containing genotypes of the BDNF gene was categorized as a "high-risk genotypic combination". The other combinations (i.e. Gly-containing genotypes of the DRD3 gene and/or Val/Val genotype of the BDNF gene) were categorized as "non-high-risk genotypic combinations".

In order to examine these genotype effects on AI, the absolute length of AI was analyzed by analysis of covariance (ANCOVA) with age, gender, and ICV as covariates, with diagnosis (schizophrenia patients versus controls) and genotypic combination (high-risk versus non-high-risk) as between-subject factors.

For the medial temporal lobe structures, the relative volumes [(absolute volume / ICV) × 100] for each ROI were analyzed using the repeated measures ANCOVA with age and gender as covariates, with diagnosis and genotypic combination as between-subject factors, and hemisphere (left versus right) as a within-subject variable. We used relative volumes for these ANCOVAs, but we considered main effects or interactions to be significant if the results did not change when the absolute volumes were used with age, gender, and ICV as covariates. The AI length and volumetric measures for all ROIs in this study were normally distributed (tested by the Kolmogorov–Smirnov test). The post hoc Scheffé's test was employed to follow up the significant main effects or interactions yielded by ANCOVAs. Statistical significance was defined as $p<0.05$.

3. Results

The observed genotype frequency of the SNPs was within the distribution as expected according to the Hardy–Weinberg equilibrium. No significant difference was observed in the genotypic distribution of the BDNF Val66Met SNP or the DRD3 Ser9Gly SNP between the healthy controls and schizophrenia patients (Table 1). The DRD3 gene was not detected for one schizophrenia patient, and this case was removed from subsequent analyses. As for the genotypic combination, 10/32 schizophrenia patients (31.3%) and 7/29 controls (24.1%) were categorized as having the high-risk combination, showing no significant group difference (chi-square = 0.38, $p=0.536$).

For schizophrenia patients, no differences between the genotypic combination groups (high-risk versus non-high-risk) were observed for age, onset age, illness duration, total SANS or SAPS score, or medication status (dose, duration, and typical versus atypical).

Table 2 shows a comparison of the AI length and relative ROI volumes between the subjects with and without the high-risk genotypic combination. ANCOVA of the AI length showed a significant diagnosis-by-genotypic combination interaction, with subjects with the high-risk genotypic combination having a shorter AI than those without it for the healthy controls (post hoc test, $p=0.048$), but not for the schizophrenia patients (post hoc test, $p=0.981$) (Fig. 1).

For the medial temporal lobe structures, we found significant main effects of genotypic combination for the posterior hippocampus and posterior parahippocampal gyrus, showing that the subjects with high-risk genotypic combination had a smaller posterior hippocampus (post hoc test, $p=0.010$) and posterior parahippocampal gyrus (post hoc test, $p=0.013$) than those without it. When the absolute volumes were used for the statistical analyses, however, the genotypic combination effect was significant for the posterior hippocampus ($F=5.38$, $p=0.024$) but not for the posterior parahippocampal gyrus (Table 2). Thus, we considered only the effect on the posterior hippocampus to be significant.

We further tested whether these genotypic combination effects on the AI or posterior hippocampus could be explained by the independent effect of each SNP. No genotype effect was seen on these structures when the effect of DRD3 Ser9Gly SNP alone (Ser homozygotes versus Gly carriers) or BDNF Val66Met SNP alone (Met carriers versus Val homozygotes) was tested.

4. Discussion

To our knowledge, this is the first volumetric MRI study to report the gene–gene interaction effect of the DRD3 Ser9Gly and BDNF Val66Met SNPs on the brain morphology in schizophrenia patients and healthy controls. The subjects carrying the combination of the Ser/Ser genotype of DRD3 and Met-containing genotypes of BDNF had a shorter AI than those without it in the healthy controls, but not in the schizophrenia patients. On the other hand, the subjects with the same genotypic combination had a significantly smaller posterior hippocampus than those without it in both diagnostic groups. Of note, these

Table 2

Absolute AI length and relative volume for medial temporal lobe structures in schizophrenia patients and healthy comparisons with and without the high-risk genotypic combination[a]

Brain region	Healthy comparisons		Schizophrenia patients		Analysis of covariance[b]					
	Non-high-risk	High-risk	Non-high-risk	High-risk	Genotype		Diagnosis		Diagnosis × genotype	
	(N=22)	(N=7)	(N=22)	(N=10)	F	p	F	p	F	p
ICV (cm³)[c]	1469±123	1525±123	1498±159	1616±110	2.59	0.113	3.02	0.088	0.63	0.432
AI length (mm)	10.0±2.9	6.4±4.0	6.5±3.3	6.0±3.6	0.45	0.504	2.08	0.155	**5.33**	**0.025**
Amygdala					0.04	0.846	14.09	<0.001	0.11	0.741
Left	0.076±0.010	0.076±0.008	0.067±0.006	0.064±0.014						
Right	0.077±0.011	0.082±0.008	0.070±0.007	0.073±0.012						
Anterior hippocampus					<0.01	0.963	<0.01	0.958	0.32	0.573
Left	0.058±0.017	0.054±0.018	0.051±0.021	0.049±0.014						
Right	0.061±0.022	0.060±0.016	0.062±0.022	0.070±0.028						
Posterior hippocampus					6.01	0.017	2.05	0.158	0.19	0.664
Left	0.151±0.022	0.143±0.015	0.147±0.023	0.137±0.020						
Right	0.159±0.024	0.143±0.015	0.150±0.026	0.127±0.023						
Anterior PHC					<0.01	0.957	6.72	0.012	0.75	0.390
Left	0.156±0.032	0.154±0.024	0.167±0.028	0.171±0.028						
Right	0.178±0.030	0.163±0.020	0.186±0.028	0.185±0.022						
Posterior PHC[d]					4.30	0.043	0.07	0.787	0.52	0.472
Left	0.326±0.031	0.294±0.025	0.318±0.035	0.308±0.046						
Right	0.309±0.030	0.287±0.044	0.307±0.031	0.285±0.036						

Values represent means±SDs.

AI, adhesio interthalamica; ICV, intracranial volume; PHG, parahippocampal gyrus.

[a] Relative volumes were calculated as follows: 100×absolute volume/intracranial volume. The genotypic combination of the Ser/Ser genotype of DRD3 gene and Met-containing genotypes of BDNF gene was categorized as "high-risk genotypic combination".

[b] $df=1, 54$ for the AI length and 1, 55 for the ICV and the volume of the medial temporal lobe structures.

[c] Age and gender were used as covariates.

[d] Main effect of genotype was not significant when the absolute volume was used with age, gender, and ICV as covariates ($F=2.65$, $p=0.109$).

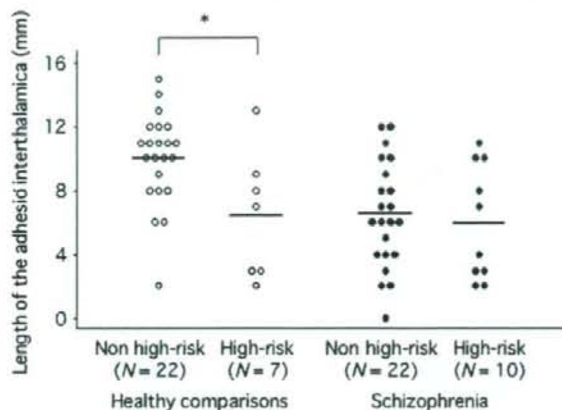

Fig. 1. Scatter plots of length of the adhesion interthalamica in healthy comparisons and schizophrenia patients. Horizontal lines indicate means. Post hoc comparisons: $^*p<0.05$.

genotypic interaction effects were not explained by the independent effect of the DRD3 or BDNF. Thus, our findings suggest the effect of gene–gene interaction between the DRD3 and BDNF variations on brain morphology in midline and medial temporal lobe structures. Contrary to our prediction, however, we did not find a specific interaction effect of these genes on brain morphology in schizophrenia.

With regard to the association between these genotypic variations and schizophrenia or its clinical characteristics such as age at onset of psychosis, large-scale meta-analyses of the BDNF and DRD3 suggested each genotype effect was of small magnitude (e.g., Jonsson et al., 2003, 2006; Xu et al., 2007). Nevertheless, Gourion et al. (2005) reported a significant association between the combination of the Ser/Ser genotype of DRD3 and Met-containing genotypes of BDNF and earlier onset of schizophrenia. Although we did not find a significant genotypic interaction effect on the clinical variables in schizophrenia possibly due to the small sample size, these previous findings support the notion that schizophrenia is multifactorial in origin and cannot be easily explained by a single genetic component (Harrison and Weinberger, 2005; Sawa and Snyder, 2002).

The reported malformation of the AI in schizophrenia is likely to reflect early neurodevelopment and consequent dopaminergic abnormalities in the neural network including the thalamic region (Takahashi et al., 2008). The functional significance of the AI in human brain is obscure, but animal studies have shown that the AI is involved in regulating the release of dopamine in the basal ganglia (Cheramy et al., 1984; Romo et al., 1984). It is interesting to note that DRD3 mRNA is preferentially expressed in limbic-related and basal ganglia regions, as well as in the thalamus in the human brain (Suzuki et al., 1998). Low thalamic D2/D3 receptor binding in drug-naïve patients with schizophrenia demonstrated by positron emission tomography studies (Talvik et al., 2003; Buchsbaum et al., 2006) implied deficits in the thalamic dopaminergic system (Sanchez-Gonzalez et al., 2005; Garcia-Cabezas et al., 2007) in schizophrenia. Given the role of BDNF as a modulator of dopaminergic function during development by regulating DRD3 expression (Guillin et al., 2004, 2007), it can be hypothesized that the genotypic interaction effect of these genes is at least partly related to the thalamic dopaminergic pathology of schizophrenia, which could result in the differences in the prevalence or length of the AI in schizophrenia patients. In this study, however, we did not provide direct evidence for the genotypic combination effect of the DRD3 and BDNF SNPs on the AI in schizophrenia. A recent MRI study in monozygotic twins concordant or discordant for schizophrenia also failed to find genetic contribution to the AI abnormalities (Ettinger et al., 2007). Thus, the possible interaction effect of these genes on brain morphology, including the midline structures, should be further examined in schizophrenia.

Regarding the medial temporal lobe structures, our findings demonstrated that the combination of the Ser/Ser genotype of DRD3 and Met-containing genotypes of BDNF may contribute to the volume reduction of the hippocampus in both schizophrenia patients and healthy comparisons. This finding is largely in line with previous MRI studies that demonstrated the association between the Met allele of the BDNF gene and a reduced volume of the hippocampus in schizophrenia (Szeszko et al., 2005) or healthy subjects (Bueller et al., 2006; Pezawas et al., 2004; Szeszko et al., 2005), and further supports the role of BDNF as a regulating factor of DRD3. The medial temporal lobe has already decreased in volume by the onset of schizophrenia (Shenton et al., 2001; Vita et al., 2006) and several (Boos et al., 2007; Lawrie et al., 2001; Seidman et al., 2003) but not all (Goldman et al., 2008; Honea et al., 2008) MRI studies have demonstrated a similar reduction in subjects at genetic high-risk for developing schizophrenia. These findings suggest the heritability of the medial temporal lobe changes in schizophrenia, but these changes might represent only a weak intermediate phenotype for schizophrenia (Goldman et al., 2008; Honea et al., 2008). In fact, the previous MRI study (Szeszko et al., 2005) as well as the present study did not reveal statistically significant genotype-by-diagnosis interaction regarding the effect of the BDNF Val66Met SNP on the medial temporal morphology in schizophrenia and healthy controls. As for the DRD3 Ser9Gly SNP, to our knowledge, no volumetric MRI studies have investigated its genotype effect on the brain morphology in schizophrenia patients or in healthy subjects, but the Ser/Ser genotype was reported to contribute to the quality of eye tracking performance, another potential phenotypic marker of schizophrenia, in both schizophrenia patients and healthy subjects (Rybakowski et al., 2001). Taken together, the genetic effect of DRD3, BDNF, or a combination of these genes could contribute to the neurobiological phenotypic markers observed in schizophrenia such as the medial temporal abnormalities, but it seems likely that each effect on the pathogenesis of schizophrenia is weak and cannot explain all the differences in these phenotypic expressions between schizophrenia patients and healthy controls.

A few possible confounding factors in the present study need to be addressed. First, all schizophrenia patients in this study were on neuroleptic medication, which could have affected the brain morphology. Although there was no difference in medication status between the genotype groups (Ser/Ser versus Gly carrier for the DRD3 gene, Val/Val versus Met carrier for the BDNF gene, and high-risk versus non-high-risk genotypic combinations), the effects of medication may have biased our results, with particular regard to comparisons between healthy subjects and schizophrenia patients. In fact, our preliminary data showed a negative correlation between the AI length and daily medication dosage in a different sample of psychotic disorder patients (unpublished data). Secondly, this cross-sectional study did not examine progressive brain morphologic changes. The AI has been implicated in early neurodevelopment, but it also undergoes atrophy with age (Rosales et al., 1968), which we found to be accelerated in schizophrenia (unpublished data). Although the present study failed to find a specific interaction effect of the BDNF and DRD3 polymorphisms on brain morphology in schizophrenia, the possibility exists that these genetic variations contribute to brain changes over time in the illness (Ho et al., 2007). In addition to these potential confounding factors, this study was limited by the relatively small number of subjects carrying the combination of the Ser/Ser genotype of DRD3 and Met-containing genotypes of BDNF. Our unexpected finding of the interaction effect of these genes on the AI only in healthy comparisons may be partly related to the lower statistical power due to the small sample size. Thus, the present findings require replication in a larger sample without sustained neuroleptic medication in a longitudinal design. Furthermore, other genes for schizophrenia-susceptibility should be

included in future studies for gene–gene interaction effects on the pathogenesis of schizophrenia.

5. Conclusion

Our preliminary results demonstrated a significant genotypic interaction effect between the DRD3 Ser9Gly and BDNF Val66Met polymorphisms on the midline and associated medial temporal morphology. However, the present study did not find a specific genotypic effect on brain morphology in schizophrenia, implicating that the independent or interaction effects of these genetic variations are unlikely to play a major role in the pathogenesis of schizophrenia.

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