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ORIGINAL ARTICLE

A complex polymorphic region in the brain-derived neurotrophic factor (BDNF) gene confers susceptibility to bipolar disorder and affects transcriptional activity

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Previous studies have suggested that genetic variations in the brain-derived neurotrophic factor (BDNF) gene may be associated with several neuropsychiatric diseases including bipolar disorder. The present study examined a microsatellite polymorphism located approximately 1.0 kb upstream of the translation initiation site of the BDNF gene for novel sequence variations, association with bipolar disorder, and effects on transcriptional activity. Detailed sequencing analysis revealed that this polymorphism is not a simple dinucleotide repeat, but it is highly polymorphic with a complex structure containing three types of dinucleotide repeats, insertion/deletion, and nucleotide substitutions that gives rise to a total of 23 novel allelic variants. We obtained evidence supporting the association between this polymorphic region (designated as BDNF-linked complex polymorphic region (BDNF-LCPR)) and bipolar disorder. One of the major alleles ('A1' allele) was significantly more common in patients than in controls (odds ratio 2.8, 95% confidential interval 1.5–5.3, $P=0.001$). Furthermore, a luciferase reporter gene assay in rat primary cultured neurons suggests that this risk allele (A1) has a lower-transcription activity, compared to the other alleles. Our results suggest that the BDNF-LCPR is a functional variation that confers susceptibility to bipolar disorder and affects transcriptional activity of the BDNF gene.
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Keywords: association study; brain-derived neurotrophic factor (BDNF); bipolar disorder; polymorphism; susceptibility; transcriptional activity

Introduction

Brain-derived neurotrophic factor (BDNF) belongs to the neurotrophic factor family and promotes the development, regeneration, survival and maintenance of function of neurons.[1] It modulates synaptic plasticity and neurotransmitter release across multiple neurotransmitter systems, as well as the intracellular signal-transduction pathway.[2] BDNF has been implicated in the pathogenesis of mood disorders and in the mechanism of action of therapeutic agents such as mood stabilizers and antidepressants.[3] BDNF protein was reduced in postmortem brains of patients with bipolar disorder, compared to controls.[4] Chronic electroconvulsive seizure and antidepressant drug treatments increase mRNA of BDNF and its receptor trkB.[5] Lithium may also exert its neuroprotective effect through enhancing expression of BDNF and trkB.[6]

The BDNF gene is, therefore, an attractive candidate gene which may give susceptibility to bipolar disorder.[7] In accordance with this, at least three previous studies reported a significant association between the Val66Met polymorphism (NCBI dbSNP rs6265) of the BDNF gene and bipolar disorder in Caucasian populations.[8–10] In these studies, the Val66 allele was consistently found to have a risk-increasing effect on the development of bipolar disorder. However, this association was not replicated in other Caucasian[11–13] or Asian populations including ours.[14–16]

Another polymorphism of the BDNF gene that has been well studied as to the possible association with neuropsychiatric diseases is the 'GT repeat' located approximately 1.0 kb upstream of the translation initiation site of the gene.[17] With respect to the possible effect on mood disorders, a significant

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linkage disequilibrium with bipolar disorder[8] and a significant association with childhood onset mood disorder[18] have been reported in Caucasian populations, although one study failed to find such an association with bipolar disorder.[13] Furthermore, there is some evidence suggesting that this polymorphism plays a role in the pathogenesis of schizophrenia.[13,19,20] However, there is no study that examined whether this polymorphism has functional effects. Since micro- and minisatellite polymorphisms even located in intron have been shown to play a role in the expression of many genes,[21] it might be intriguing to examine whether this microsatellite polymorphism of the BDNF gene is associated with transcriptional activity in an allele-dependent manner.

The aim of the present study was to examine this microsatellite polymorphism (designated here as BDNF-linked complex polymorphic region (BDNF-LCPR) due to its complex structure) for novel sequence variations, association with bipolar disorder, and effects on transcriptional activity.

Materials and methods

Subjects

Subjects were 153 patients with bipolar disorder (71 males) and the same number of controls (71 males), matched for age, sex, ethnicity, and geographical area. These subjects, who were recruited from Showa University Hospital and Shiga University of Medical Science Hospital, Japan, were previously genotyped for the Val66Met polymorphism of the BDNF gene, yielding a result of no significant association.[16] Mean age (standard deviation (s.d.)) in the patients was 47.8 (s.d. 15.3) years and that in the controls 47.1 (11.0). All the patients and controls were biologically unrelated Japanese. Consensus diagnosis of bipolar disorder was made for each patient by at least two experienced psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders, 4th ed. (DSM-IV),[22] based on unstructured interviews and medical records. Among the patients, 94 individuals (61%) were diagnosed with bipolar I and the remaining 59 with bipolar II disorder. Patients who had one or more comorbid axis I disorders were excluded. The mean age of onset and number of episodes were 37.8 (s.d. 15.2) years and 3.9 (1.4) times, respectively. Thirty-four patients (22.2%) had at least one episode with psychotic features. Sixty-seven patients (43.8%) had a family history of major psychiatric illness (mood disorders or schizophrenia spectrum disorders) within their second-degree relatives. The controls were screened with a semi-structured interview and those individuals who had current or past contact to psychiatric services were excluded. In addition, those individuals who had a family history of major psychiatric illness or those who had a current or past history of regular use of psychotropic medication, including hypnotics, were excluded from the control group. After description of the study, written informed consent for the participation of the study was obtained from every subject. The study protocol was approved by ethics committee of each institution.

Sequence analysis

Venous blood was drawn and genomic DNA was extracted according to standard procedures. To determine accurate DNA sequences for the BDNF-LCPR, we cloned this polymorphic region and performed direct sequencing. An approximately 400 base-pair (bp) DNA fragment encompassing the polymorphic region was amplified by polymerase chain reaction (PCR) with primers of *Hind*III-tagged BDNF-LCPR-F1 and *Hind*III-tagged BDNF-LCPR-R1 (see Table 1 and Figure 1a). The purified PCR products were ligated into the *Hind*III site of the pBluescriptII SK (+) vector (Toyobo, Tokyo, Japan). The vector was transformed into *Escherichia coli*, DH5α and incubated. For sequencing, PCR amplification was performed with primers of GTTGTAAAACGACGGCCA GTG (Universal primer) and GGAAACAGCTATGAC CATG (Reverse primer). At least four clones were examined for each individual. Direct sequencing was performed with the CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA).

Cloning and sequencing analysis described above suggested that the polymorphic region is not a simple

Table 1 Primer sequences for sequencing the BDNF-linked complex polymorphic region (BDNF-LCPR)

Primer No.	*Primer Name*	*UCSC Chromosome11 5′→3′*	*Numbers in Fig. 1 5′→3′*	*Primer Sequence 5′→3′*
1	BDNF-LCPR-F1	27637949→27637930	139→158	TAGAGCAACCCTCTGGCAAA
2	BDNF-LCPR-R1	27637545→27637567	543→521	TGTCATGAAAACAATGTGTCTGG
3	BDNF-LCPR-F2	27637844→27637822	244→266	CCAAAATGTGTAAAACACCACTC
4	B-BDNF-LCPR-F2	27637844→27637822	244→266	**Biotin**-CCAAAATGTGTAAAACACCACTC
5	BDNF-LCPR-R2	27637715→27637741	373→347	GAAAGCTCAACTTTTCTTTTTACTAGA
6	B-BDNF-LCPR-R2	27637715→27637741	373→347	**Biotin**-GAAAGCTCAACTTTTCTTTTTACTAGA
7	BDNF-LCPR-F3	27637810→27637791	278→297	AGTAGGATAAACTCAGAGCG
8	BDNF-LCPR-R3	27637730→27637749	358→339	CTTTTTACTAGAGATGTTCT
9	Reverse		1→19	GGAAACAGCTATGACCATG
10	Unversal		641→621	GTTGTAAAACGACGGCCAGTG

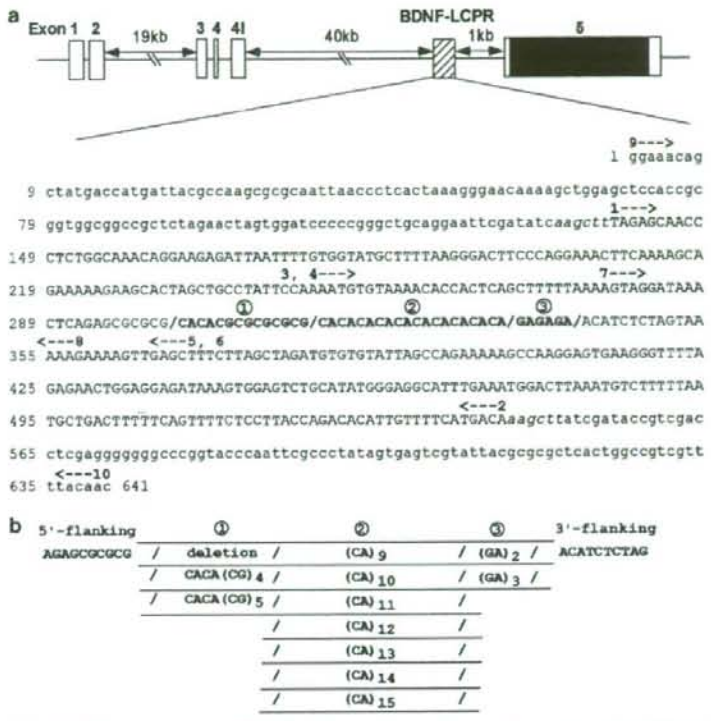

Figure 1 Structure of the BDNF gene and the BDNF-linked complex polymorphic region (BDNF-LCPR) cloned into a vector for sequence analysis. (**a**) In the schematic illustration of the BDNF gene,[25,26] coding region and non-coding exons are indicated with black and open boxes, respectively. Hatched box indicates BDNF-LCPR and its flanking region. An approximately 400 bp fragment encompassing the BDNF-LCPR is inserted into the *Hind*III site of the pBluescriptII SK (+) cloning vector. DNA sequence is according to the UCSC genome database. Genomic sequence, vector sequence, and the *Hind*III cloning sites are described in upper case, lower case, and Italic lower case, respectively. The three forms of dinucleotide repeats are described in bold upper case letters (①, ②, and ③) and separated by slashes. 5′ ends of the forward primers and 3′ends of reverse primers used for sequencing are shown in numbers with arrows that correspond to the primer numbers in Table 1. (**b**) Schematic illustration of the BDNF-LCPR.

dinucleotide repeat, but this polymorphism has a very complex structure. In addition, because of the stuttering effect in the PCR amplification, cloning and sequencing could not always determine the genotype of each individual. We then performed pyrosequencing that was able to differentiate true alleles from artifacts due to stuttering. The polymorphic region was amplified by PCR with primers of BDNF-LCPR-F2 and B-BDNF-LCPR-R2 for forward direction and B-BDNF-LCPR-F2 and BDNF-LCPR-R2 for reverse direction (Table 1). These primers were designed with the Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Pyrosequencing was performed with PSQ96MA System and PSQ96 SNP Reagent Kit (Pyrosequencing, AB, Uppsala, Sweden). Sequencing primers of both directions (forward: BDNF-LCPR-F3; reverse: BDNF-LCPR-R3, Table 1) were designed with the software supplied by Pyrosequencing, AB, Uppsala, Sweden (http://www.Pyrosequencing.com). On the basis of the sequences observed by cloning and direct sequence, described above, 'the sequence to analyze' was assumed to be 5′-CGCGCACA[CCGCGCGCG]CACACACACACACAC ACACACACACACACACAGAGAGAGAACAT-3′ and dispensation order was set as 5′-TCGCGCACAGCGCG CGCGTCACACACACACACACACACACACAC ACATGAGAGAGAT-3′ for sequencing in the forward direction. For sequencing in the reverse direction, 'the sequence to analyze' and the dispensation order was set as 5′-CTCTCTGTGTGTGTGTGTGTGTG [TGTGTGTGTGTGTGTG]CGCGCGCGCGTGTGCGCG CGCTCTGAGTT-3′ and 5′-GCTCTCTGTGTGTGTGTG TGTGTGTGTGTGTGTGTGTGTGTACGCGCGCGC GTGTGCGCGCGCTCTC-3′, respectively. Since the target sequence was rather long (~70 bp), we added single-stranded binding protein (SSB, Sigma-Aldrich, St Louis, MO, USA) to avoid wearing down of signal for sequencing.

DNA sequences of two chromosomes of each individual were determined by referring to results of both direct sequencing of cloned fragments and pyrosequencing. For ambiguous genotypic data, we repeated

experiments and determined genotype for every subject. Genotypes were read blind to affection status.

Association analysis with bipolar disorder

The presence of Hardy–Weinberg equilibrium in genotype distribution was examined by using the χ^2-test for goodness of fit. Allele frequencies of the BDNF-LCPR were compared between patients and controls by using the χ^2-test for independence. Then linkage disequilibrium and haplotype-based association analysis for the BDNF-LCPR and the Val66Met polymorphisms were carried out. These statistical analyses were performed by using the SPSS v11 (SPSS Japan Inc., Tokyo, Japan) and the COCAPHASE v2.403 program (http://www.hgmp.mrc.ac.uk/~fdudbrid/software/unphased/). All *P*-values reported are two-tailed.

Luciferase reporter gene assay in primary cultured neurons

Primary cultures were prepared from the cortex of postnatal 2 days old rats (SLC, Shizuoka, Japan) as described previously.[23] To generate plasmids for the luciferase gene reporter assay (Figure 2a), the BDNF-LCPR was amplified by PCR with primers of *Sma*I-tagged BDNF-LCPR-F1 and *Sma*I-tagged BDNF-LCPR-R1 (Table1 and Figure 1). The PCR products were inserted into the *Sma*I site upstream of the SV40 promoter in the pGL3-Promoter vector (Promega, Tokyo, Japan). The four major alleles were subject to the assay. Plasmid constructs were transfected at 5 days *in vitro*. Cells on 24-well plates were co-transfected with 800 ng of pGL3-Promoter firefly luciferase vectors that included major alleles of the BDNF-LCPR and 25 ng of phRL-TK renilla luciferase vector (Promega, Tokyo, Japan) as an internal control by using Lipofectamine 2000 reagent (Invitrogen, Tokyo, Japan). Empty pGL3-Promoter vector was transfected simultaneously.

At 24 h after transfection, luciferase activity was measured by using Dual-Luciferase Reporter Assay System (Promega, Tokyo, Japan) and a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany), as described previously.[24] Firefly and renilla luciferase activities were quantified sequentially as relative light units (RLU) by addition of their respective substrates. The ratio of firefly RLU to renilla RLU of each sample was automatically computed. Then the activity of each construct was expressed as the relative value compared to that of empty pGL3-Promoter vector (relative luciferase expression, RLE). Primary cultured cells were prepared three times and transfection was performed triplicate for each cell culture. Comparisons in RLE were carried out by analysis of variance (ANOVA) or *t*-test.

Results

Detection of novel variants

The structure of the BDNF gene[25,26] and DNA sequence of the cloned fragment according to the University of California, Santa Cruz (UCSC) genome database are illustrated in Figure 1a. We detected a total of 23 allelic variants in the BDNF-LCPR (registered to the DDBJ/EMBL/GenBank database, accession numbers AB212736 to AB212758). Sequences and allele frequencies in patients with bipolar disorder and controls are shown in Table 2. Allelic variants of the BDNF-LCPR consisted of three components of dinucleotide repeat of $(CA)_{del/2}(CG)_{del/4/5}$, $(CA)_{9-15}$, and $(GA)_{2/3}$, which were combined in succession (Figure 1b). In addition, there were four exceptional rare variants that contained a single nucleotide substitution (variants 2 and 4) or insertion of two nucleotides of cg (variants 1 and 3) immediately 5′ side of the repeats. The 'GT repeat' due to the original report[17] was CA, but not GT, repeat when the sequence was read in the forward direction of the BDNF gene. There were four major alleles of Del-12-3 (allele 1; A1), 4-12-3 (A2), 5-12-2 (A3), and 5-13-3 (A4). To perform statistical analyses, the remaining rare alleles were combined and considered to be 'allele 5 (A5)'. Supplementary figures S1 and S2 show images of direct sequencing of the major alleles, which were cloned and an example of pyrosequencing depicted in 'pyrogram'.

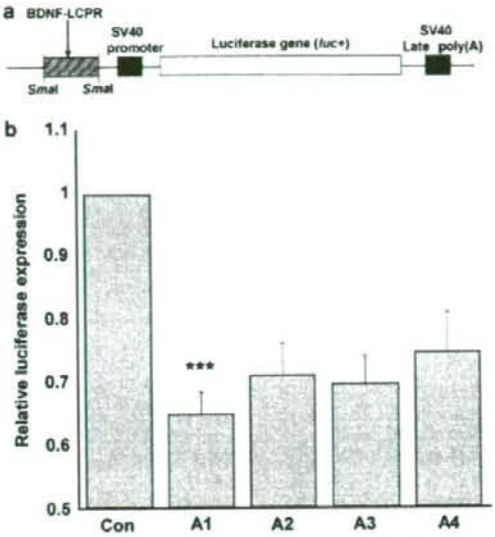

Figure 2 Luciferase reporter gene assay on the four major alleles of the BDNF-linked complex polymorphic region (BDNF-LCPR). (**a**) Schematic illustration of the luciferase assay construct for the BDNF-LCPR. (**b**) Relative luciferase expression (RLE) for pGL3-Promoter vector with insertion of each allele (A1, A2, A3, or A4) of the BDNF-LCPR in comparison with pGL3-Promoter vector without insertion of BDNF-LCPR (con). Error bars represent standard deviations (s.d.). **: RLE for the A1 allele was significantly lower than the remaining three alleles combined ($t = -3.4$, $df = 34$, $P = 0.002$).

Table 2 Detected alleles and their frequencies in patients with bipolar disorder and controls for the BDNF-linked complex polymorphic region (BDNF-LCPR)

Allele name	*Sequence 5′→3′*		*Allele counts (%)*		
		Fragment Size (bp)[a]	*Patients*	*Controls*	*Total*
Del-11-3	agagcgcgcg(del)$(ca)_{11}(ga)_3$acat	393	2 (0.7)	1 (0.3)	3 (0.5)
Del-12-2	agagcgcgcg(del)$(ca)_{12}(ga)_2$acat	393	5 (1.6)	8 (2.6)	13 (2.1)
Del-12-3 (A1)	agagcgcgcg(del)$(ca)_{12}(ga)_3$acat	395	36 (11.8)	14 (4.6)	50 (8.2)
Del-15-3	agagcgcgcg(del)$(ca)_{15}(ga)_3$acat	401	1 (0.3)	0 (0.0)	1 (0.2)
4-11-3	agagcgcgcgcaca$(cg)_4(ca)_{11}(ga)_3$acat	405	0 (0.0)	1 (0.3)	1 (0.2)
4-12-2	agagcgcgcgcaca$(cg)_4(ca)_{12}(ga)_2$acat	405	0 (0.0)	1 (0.3)	1 (0.2)
4-12-3 (A2)	agagcgcgcgcaca$(cg)_4(ca)_{12}(ga)_3$acat	407	32 (10.5)	48 (15.7)	80 (13.1)
4-13-2	agagcgcgcgcaca$(cg)_4(ca)_{13}(ga)_2$acat	407	3 (1.0)	3 (1.0)	6 (1.0)
4-13-3	agagcgcgcgcaca$(cg)_4(ca)_{13}(ga)_3$acat	409	1 (0.3)	3 (1.0)	4 (0.7)
5-9-2	agagcgcgcgcaca$(cg)_5(ca)_9(ga)_2$acat	401	1 (0.3)	0 (0.0)	1 (0.2)
5-10-3	agagcgcgcgcaca$(cg)_5(ca)_{10}(ga)_3$acat	405	6 (2.0)	6 (2.0)	12 (2.0)
5-11-2	agagcgcgcgcaca$(cg)_5(ca)_{11}(ga)_2$acat	405	4 (1.3)	2 (0.7)	6 (1.0)
5-11-3	agagcgcgcgcaca$(cg)_5(ca)_{11}(ga)_3$acat	407	1 (0.3)	0 (0.0)	1 (0.2)
5-12-2 (A3)	agagcgcgcgcaca$(cg)_5(ca)_{12}(ga)_2$acat	407	82 (26.8)	89 (29.1)	171 (27.9)
5-12-3	agagcgcgcgcaca$(cg)_5(ca)_{12}(ga)_3$acat	409	5 (1.6)	6 (2.0)	11 (1.8)
5-13-2	agagcgcgcgcaca$(cg)_5(ca)_{13}(ga)_2$acat	409	12 (3.9)	11 (3.6)	23 (3.8)
5-13-3 (A4)	agagcgcgcgcaca$(cg)_5(ca)_{13}(ga)_3$acat	411	110 (35.9)	105 (34.3)	215 (35.1)
5-14-2	agagcgcgcgcaca$(cg)_5(ca)_{14}(ga)_2$acat	411	0 (0.0)	1 (0.3)	1 (0.2)
5-14-3	agagcgcgcgcaca$(cg)_5(ca)_{14}(ga)_3$acat	413	3 (1.0)	5 (1.6)	8 (1.3)
Exceptional variants					
Variant 1	agagcgcgcg**cg**(del)$(ca)_{13}(ga)_3$acat	399	1 (0.3)	0 (0.0)	1 (0.2)
Variant 2	agagcgcgcgcaca**tg**$(cg)_4(ca)_{12}(ga)_2$acat	405	0 (0.0)	1 (0.3)	1 (0.2)
Variant 3	agagcgcgcg**cg**caca$(cg)_4(ca)_{13}(ga)_3$acat	411	1 (0.3)	0 (0.0)	1 (0.2)
Variant 4	agag**tg**cgcgcaca$(cg)_5(ca)_{12}(ga)_2$acat	407	0 (0.0)	1 (0.3)	1 (0.2)
Total chromosomes			306 (100.0)	306 (100.0)	612 (100.0)

[a]Fragment size of PCR product amplified by primers of BDNF-LCPR-F1 and BDNF-LCPR-R1 (see Table 1).

Association analysis with bipolar disorder

Genotype and allele distributions in patients and controls are shown in Table 3. The genotype distributions were in Hardy–Weinberg equilibrium (for the patients: $\chi^2 = 4.5$, df = 13, $P = 0.98$; for the controls: $\chi^2 = 8.2$, df = 13, $P = 0.83$). The overall allele frequencies differed significantly between patients and controls ($\chi^2 = 13.4$, df = 4, $P = 0.0093$). The global-P-value estimated by the permutation test of 10 000 simulations, correcting for multiple testing, yielded a similar result ($P = 0.010$). The A1 allele was clearly more common in patients than in controls (11.8 vs 4.6%, odds ratio (OR) 2.8, 95% confidential interval (CI) 1.5–5.3, $\chi^2 = 10.5$, df = 1, $P = 0.001$). When the three components of the BDNF-LCPR, that is, $(CA)_{del/2}$ $(CG)_{del/4/5}$, $(CA)_{9-15}$, and $(GA)_{2/3}$, were examined separately, only the first component showed a significant association with bipolar disorder (Table 4). Deletion of the first component, as seen in the A1 allele, was significantly more common in patients than in controls (14.6 vs 8.3%, OR 1.9, 95% CI 1.1–3.2, $\chi^2 = 5.9$, df = 1, $P = 0.015$).

Then we examined linkage disequilibrium and haplotype-based association for the BDNF-LCPR and the Val66Met polymorphism. As reported previously,[16] there was no significant association between the Val66Met polymorphism and bipolar disorder in the current sample; the frequencies of the Val66 allele were 0.60 and 0.62 in patients and comparison groups, respectively ($\chi^2 = 0.25$, df = 1, $P = 0.62$). Results of haplotype-based analysis for these two polymorphisms are shown in Table 5. There was a very tight linkage disequilibrium between the BDNF-LCPR and Val66Met polymorphism ($D' = 0.91$ for patients and $D' = 0.90$ for cotrols; $\chi^2 = 512$, df = 28, $P = 1.6 \times 10^{-90}$ in total subjects). The Val66 allele was linked to the A1, A2, or A3 allele, while the Met66 allele was to the A4 allele. The haplotype-based association analysis yielded a significant result (global $P = 0.0069$) estimated by the permutation test, correcting for multiple testing. Since the A1 allele was completely linked to the Val66 allele, the most significant individual P-value of 0.001 was obtained when the A1-Val66 was assumed to be the risk. When pairwise linkage disequilibrium across three components of the BDNF-LCPR and Val66Met was examined individually, there was a tight linkage disequilibrium between

Table 3 Genotype and allele distributions in patients with bipolar disorder and controls for the BDNF-linked complex polymorphic region (BDNF-LCPR)

Genotype/allele	*Counts (%)*		
	Patients	*Controls*	*Total*
Genotype			
A1/A1	2 (1.3)	0 (0.0)	2 (0.7)
A1/A2	5 (3.2)	1 (0.7)	6 (2.0)
A1/A3	12 (7.8)	3 (2.0)	15 (4.9)
A1/A4	9 (5.9)	9 (5.9)	18 (5.9)
A1/A5	6 (3.9)	1 (0.6)	7 (2.3)
A2/A2	3 (2.0)	4 (2.6)	7 (2.3)
A2/A3	7 (4.6)	18 (11.8)	25 (8.2)
A2/A4	9 (5.9)	13 (8.5)	22 (7.2)
A2/A5	5 (3.3)	8 (5.2)	13 (4.2)
A3/A3	11 (7.2)	11 (7.2)	22 (7.2)
A3/A4	29 (19.0)	32 (20.9)	61 (19.9)
A3/A5	12 (7.8)	14 (9.1)	26 (8.5)
A4/A4	23 (15.0)	17 (11.1)	40 (13.1)
A4/A5	17 (11.1)	17 (11.1)	34 (11.1)
A5/A5	3 (2.0)	5 (3.2)	8 (2.6)
Total subjects	153 (100.0)	153 (100.0)	306 (100.0)
Allele			
A1	36 (11.8)	14 (4.6)	50 (8.2)
A2	32 (10.5)	48 (15.7)	80 (13.1)
A3	82 (26.8)	89 (29.1)	171 (27.9)
A4	110 (35.9)	105 (34.3)	215 (35.1)
A5	46 (15.0)	50 (16.3)	96 (15.7)
Total chromosomes	306 (100.0)	306 (100.0)	612 (100.0)

each component of the BDNF-LCPR and the Val66-Met, while linkage disequilibrium within the three components were much weaker (Supplementary Table S1). The deletion of the first component of the BDNF-LCPR was completely linked to the Val66 allele; the $(CA)_{del}(CG)_{del}$ allele was completely linked to the Val 66 allele, while the Val66 allele was linked to any of the $(CA)_{del/2}(CG)_{del/4/5}$ alleles.

Luciferase reporter gene assay in primary cultured neurons

Figure 2b shows observed RLEs for the major four alleles of the BDNF-LCPR, compared to RLE without insertion of such alleles (empty pGL3-Promoter vector). RLE decreased due to insertion of the polymorphic region for all the alleles compared to the empty pGL3-Promoter vector, suggesting that the BDNF-LCPR and its flanking region may have a silencer-like effect on transcriptional activity. When RLE was compared among the four alleles, there was a significant difference ($F=5.9$, df = 3, 32, $P=0.003$, ANOVA). RLE for the A1 allele was the smallest among the four alleles. When RLE for the A1 allele was compared to that for the remaining three alleles combined, the difference was significant ($t=-3.4$, df = 34, $P=0.002$), providing evidence suggesting that the A1 allele is associated with lower transcriptional activity.

Discussion

The present study demonstrated that a microsatellite polymorphism of the BDNF gene originally reported as a 'GT repeat'[17] is not a simple dinucleotide repeat, but a very complex structure of polymorphism, containing three types of dinucleotide repeats, insertion/deletion, and nucleotide substitutions, which is consistent in part with a recent report.[27] We therefore designated this region as BDNF-linked complex polymorphic region (BDNF-LCPR). The nucleotide sequences were determined by combination of pyrosequencing together with direct sequencing after cloning. Thus sequencing errors are unlikely. As a result, a total of 23 novel allelic variants were detected, although only five alleles had been identified in the original report.[17] We obtained evidence suggesting an association between the BDNF-LCPR and bipolar disorder. This is in accordance with a previous study[8] that reported a significant association between this polymorphism and bipolar disorder. However, detected alleles and their distribution considerably differ between this previous study[8] and the current study since the former genotyped the polymorphism by fragment-size analysis. We detected multiple alleles for each fragment size; for example, the A2 and A3 alleles had the same fragment size (407 bp, see Table 2). Therefore, fragment size analysis is not enough to perform an association study on the BDNF-LCPR.

Of note, the microsatellite corresponding to the BDNF-LCPR and its flanking region are conserved in rodents at similar location relative to the translation initiation site of the BDNF gene (1065 bp upstream in humans, 921 bp in rats, and 963 bp in mice). The nucleotide sequences flanking the microsatellite were highly homologous between humans and rodents (rat: 68% and mouse 66%, according to our calculation based on sequences from GenBank accession number AABR03134358.1 for rat and AY057907 for mouse). We then examined whether the BDNF-LCPR is associated with transcriptional activity in an allele-dependent manner, using luciferase reporter gene assay on primary cultured neurons from the rat brain cortex. The results provided evidence that the A1 allele is associated with lower transcriptional activity, compared to the other major alleles. This is interesting because the A1 allele, which is 12 or 16 bp shorter than the other major alleles (see Table 2), were found to be increased in patients with bipolar disorder, compared to controls. These results suggest that the A1 allele plays a role in giving susceptibility to bipolar disorder by reducing transcriptional activity of the BDNF gene. Since the A1 allele has deletion of the first component of the BDNF-LCPR, and this deletion was significantly more common in patients than in controls, it is possible that such deletion might be responsible for altering transcriptional

Table 4 Allelic association analysis of each of the three components of the BDNF-LCPR with bipolar disorder

Allele	*Counts (%)*		
	Patients	*Controls*	*Significance (P-value)*
1. $(CA)_{del/2}(CG)_{del/4/5}$			
Del–del	44 (14.6)	25 (8.3)	0.015
2–4	38 (12.6)	54 (17.9)	0.07
2–5	220 (72.8)	223 (73.8)	0.78
Total chromosomes[a]	302 (100)	302 (100)	0.044[b]
2. $(CA)_{9-15}$			
9	1 (0.3)	0 (0.0)	0.24
10	6 (2.0)	6 (1.9)	1.00
11	7 (2.3)	4 (1.3)	0.36
12	160 (52.3)	168 (54.9)	0.52
13	128 (41.8)	122 (39.9)	0.62
14	3 (1.0)	6 (2.0)	0.31
15	1 (0.3)	0 (0.0)	0.24
Total chromosomes	306 (100)	306 (100)	1.00[b]
3. $(GA)_{2/3}$			
2	107 (35.0)	117 (38.2)	0.40
3	199 (65.0)	189 (61.8)	0.40
Total chromosomes	306 (100)	306 (100)	0.44[b]

Individual alleles were tested for association by grouping all others together and applying the χ^2-test (df = 1).
[a]For the $(CA)_{del/2}(CG)_{del/4/5}$, four individuals who carried an exceptionally rare variant (see Table 2) were excluded from the analysis.
[b]Global *P*-values were estimated by the permutation test with 10 000 simulations, correcting for multiple testing.

Table 5 Haplotype-based association analysis for the BDNF-linked complex polymorphic region (BDNF-LCPR) and Val66Met polymorphism in patients with bipolar disorder and controls

Haplotype	*Counts (%)*		
	Patients	*Controls*	*Significance (P-value)*
A1-Val	36 (11.8)	14 (4.6)	0.001
A2-Val	32 (10.4)	48 (15.7)	0.054
A3-Val	82 (26.8)	89 (29.1)	0.53
A4-Val	0 (0.0)	1 (0.3)	0.23
A4-Met	110 (36.0)	104 (34.0)	0.61
A5-Val	35 (11.4)	39 (12.7)	0.62
A5-Met	11 (3.6)	11 (3.6)	1.0
Total chromosomes	306 (100)	306 (100)	0.0069[a]

Individual haplotypes were tested for association by grouping all others together and applying the χ^2 test (df = 1).
[a]Global *P*-value was estimated by the permutation test with 10 000 simulations.

activity and conferring the susceptibility. Our result is in line with a recent finding that BDNF protein was reduced in postmortem brains of patients with bipolar disorder, compared to controls.[4]

In previous studies[6–10] that reported a positive association between the Val66met polymorphism of the BDNF gene and bipolar disorder, the Val66 allele was consistently found to be the risk allele. However, other studies[11–16] failed to find such an association. The Val66Met polymorphism has been found to have functional effects. The Met66 allele was associated with poorer episodic memory, abnormal hippocampal activation, and lower hippocampal *n*-acetyl aspartate in humans and that the Met66 allele showed lower

depolarization-induced secretion and failed to localize to secretory granules or synapses in neurons.[28] The relationship between the Met66 allele and poorer episodic memory has been further demonstrated.[29] Since impairment in verbal episodic memory is one of the most consistently reported cognitive problems in individuals with bipolar disorder,[30,31] it is not feasible that the Val66 allele, but not the Met66 one, has consistently been reported to be the risk allele for bipolar disorder.[8–10] In our linkage disequilibrium analysis between the BDNF-LCPR and the Val66Met polymorphisms, the A1 allele was completely linked to the Val66 allele, which may explain, at least in part, the inconsistent results in the previous studies. That is, the A1 allele might be a responsible allele; however, its linkage to the Val66 allele have made the Val66 allele over-represented in some samples but not in the other samples, since the Val66 allele is linked to not only the A1 allele but also A2, A3, and A5 alleles. To demonstrate this hypothesis, the association between the BDNF-LCPR and bipolar disorder should be reevaluated based on the current findings. In addition, studies examining the possible association of the BDNF-LCPR with brain structure and functions are warranted.

Several studies have performed an association study between the 'GT repeat' and schizophrenia, which have also yielded conflicting results.[13,19,20,32–35] To resolve the inconsistent findings, further studies based on the current information are required.

In conclusion, we demonstrated that a microsatellite of the BDNF gene, which was originally reported as a 'GT repeat'[17] is not a simple dinucleotide repeat, but has a complex structure of polymorphism. Association analysis and luciferase reporter gene assay suggest that the BDNF-LCPR is a functional polymorphism that confers susceptibility to bipolar disorder and affects transcriptional activity.

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The association between the Val158Met polymorphism of the catechol-*O*-methyl transferase gene and morphological abnormalities of the brain in chronic schizophrenia

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The catechol-O-methyl transferase (COMT) gene is considered to be a promising schizophrenia susceptibility gene. A common functional polymorphism (Val158Met) in the COMT gene affects dopamine regulation in the prefrontal cortex (PFC). Recent studies suggest that this polymorphism contributes to poor prefrontal functions, particularly working memory, in both normal individuals and patients with schizophrenia. However, possible morphological changes underlying such functional impairments remain to be clarified. The aim of this study was to examine whether the Val158Met polymorphism of the COMT gene has an impact on brain morphology in normal individuals and patients with schizophrenia. The Val158Met COMT genotype was obtained for 76 healthy controls and 47 schizophrenics. The diagnostic effects, the effects of COMT genotype and the genotype-diagnosis interaction on brain morphology were evaluated by using a voxel-by-voxel statistical analysis for high-resolution MRI, a tensor-based morphometry. Patients with schizophrenia demonstrated a significant reduction of volumes in the limbic and paralimbic systems, neocortical areas and the subcortical regions. Individuals homozygous for the Val-COMT allele demonstrated significant reduction of volumes in the left anterior cingulate cortex (ACC) and the right middle temporal gyrus (MTG) compared to Met-COMT carriers. Significant genotype-diagnosis interaction effects on brain morphology were noted in the left ACC, the left parahippocampal gyrus and the left amygdala-uncus. No significant genotype effects or genotype-diagnosis interaction effects on morphology in the dorsolateral PFC (DLPFC) were found. In the control group, no significant genotype effects on brain morphology were found. Schizophrenics homozygous for the Val-COMT showed a significant reduction of volumes in the bilateral ACC, left amygdala-uncus, right MTG and left thalamus compared to Met-COMT schizophrenics. Our findings suggest that the Val158Met polymorphism of the COMT gene might contribute to morphological abnormalities in schizophrenia.

Keywords: schizophrenia; polymorphism; COMT; ACC; DLPFC

Abbreviations: ACC = anterior cingulate cortex; COMT = catechol-*O*-methyl transferase; DLPFC = dorsolateral prefrontal cortex; FDR = false discovery rate; IQ = intelligence quotient; JART = Japanese version of National Adult Reading Test; ROI = region of interest; SPM = statistical parametric mapping; TBM = tensor-based morphometry

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Introduction

Schizophrenia is a severe neuropsychiatric disorder with deficits of multiple domains of cognitive functions, volition and emotion. Family and twin studies have provided cumulative evidence for a genetic basis of schizophrenia (Kendler, 1983; McGue *et al.*, 1983; Sullivan *et al.*, 2003); however, identification of the underlying susceptibility loci has been limited. Collective data have suggested that the aetiology of schizophrenia involves the interplay of complex polygenic influences and environmental risk factors operating on brain maturational processes (Harrison *et al.*, 2005).

In vivo neuroimaging studies have demonstrated that brain abnormalities should play an important role in the pathophysiology of schizophrenia. Structural MRI studies have demonstrated relatively consistent brain abnormalities in patients with schizophrenia, such as enlargement of the ventricular system and regional volume decrease in the temporal lobe structures (Gaser *et al.*, 2001; Okubo *et al.*, 2001; Shenton *et al.*, 2001; Davidson and Heinrichs, 2003). Studies with schizophrenics and their healthy siblings demonstrate that even healthy siblings share some of morphological abnormalities observed in schizophrenia (Steel *et al.*, 2002; Gogtay *et al.*, 2003). A recent morphological MR study revealed that a common polymorphism of the brain-derived neurotrophic factor, one of the well-known schizophrenia susceptibility genes, affected the anatomy of the hippocampus and prefrontal cortex (PFC) in healthy individuals (Pezawas *et al.*, 2004). Furthermore, some studies have suggested that environmental factors interact with genetic factors (Cannon *et al.*, 1993; Nelson *et al.*, 2004). For example, obstetric complications are well known non-genetic risk factors of schizophrenia. However, a previous study suggested that obstetric complications might induce brain morphological abnormalities in schizophrenics and their siblings, but not in comparison with subjects at low genetic risk for schizophrenia (Cannon *et al.*, 1993). These facts suggest that genetic factors should have considerable impact on brain morphology in patients with schizophrenia.

Catechol-*O*-methyl transferase (COMT) is a promising schizophrenia susceptibility gene because of its role in monoamine metabolism (Goldberg *et al.*, 2003; Stefanis *et al.*, 2004; Harrison *et al.*, 2005). A common single nucleotide polymorphism (SNP) of the COMT gene producing an amino acid substitution of methionine (met) to valine (val) at position 108/158 (Val158Met) affects dopamine regulation in the PFC (Palmatier *et al.*, 1999). This polymorphism impacts on the stability of the enzyme, such that the Val-COMT allele has significantly lower enzyme activity than the Met-COMT allele (Weinberger *et al.*, 2001; Chen *et al.*, 2004). Several studies have revealed that the Val-COMT allele is associated with poorer performances, compared to the Met-COMT allele, in cognitive tasks of frontal function such as the Wisconsin Card Sorting Test (WCST) and N-back task (Egan *et al.*, 2001; Weinberger *et al.*, 2001; Goldberg *et al.*, 2003). The underlying mechanism of such behavioural differences may be related to lower prefrontal dopamine levels arising from higher dopamine catabolism mediated by the Val-COMT allele (Chen *et al.*, 2004; Tunbridge *et al.*, 2004).

The results of studies on the association between the Val158Met polymorphism and schizophrenia have, however, been controversial (Daniels *et al.*, 1996; Kunugi *et al.*, 1997; Ohmori *et al.*, 1998; Norton *et al.*, 2002; Galderisi *et al.*, 2005; Ho *et al.*, 2005). The result of a meta-analysis was even more inconclusive (Fan *et al.*, 2005). Such inconsistency was also found in associations between frontal functions and the Val158Met polymorphism (Egan *et al.*, 2001; Weinberger *et al.*, 2001; Goldberg *et al.*, 2003; Ho *et al.*, 2005). The possible morphological changes due to the COMT gene might be present and play a role in susceptibility to schizophrenia and in giving rise to impaired frontal functions. However, morphological changes underlying functional impairments remain to be clarified.

A recent advancement of methods for MR volumetry, such as voxel-based morphometry and deformation-based morphometry [or tensor-based morphometry (TBM)], allows us to explore and analyse brain structures of schizophrenics (Wright *et al.*, 1995; Gaser *et al.*, 2001). Using TBM techniques, we investigated the association between the Val158-Met polymorphism of the COMT gene and brain morphology in normal individuals and patients with schizophrenia. The aim of this study was to clarify whether there are significant genotype and/or genotype-disease interaction effects on brain morphology.

Methods

Subjects

Seventy-six healthy subjects and forty-seven patients with schizophrenia participated in the study. All the subjects were biologically unrelated Japanese. Written informed consent was obtained from all the subjects in accordance with ethical guidelines set by a local ethical committee. All normal subjects were screened using a questionnaire on medical history and excluded if they had neurological, psychiatric or medical conditions that could potentially affect the CNS, such as substance abuse or dependence, atypical headache, head trauma with loss of consciousness, asymptomatic or symptomatic cerebral infarctions detected by T_2-weighted MRI, hypertension, chronic lung

disease, kidney disease, chronic hepatic disease, cancer, or diabetes mellitus. The patients were diagnosed on the basis of DSM-IV criteria, information from medical records and a clinical interview. All patients were stable and/or partially remitted at the time of MR measurement and neuropsychological tests.

According to genotypes, each group (control and schizophrenia) was categorized into three groups; the homozygous Val-COMT group (control: n = 38, two were left-handed, schizophrenia: n = 19, one was left-handed), the Val/Met-COMT group (control: n = 25, three were left-handed, schizophrenia: n = 22, all were right-handed) and the remaining homozygous Met-COMT group (control: n = 13, all were right-handed, schizophrenia: n = 6, all were right-handed). Because of the small number of subjects with homozygous Met-COMT, the Val/Met-COMT and homozygous Met-COMT groups were combined and treated as one group, the Met-COMT carriers. Table 1 shows the characteristics of each group. All groups were of comparable age, gender (χ^2 test, df = 3, P = 0.38) and handedness (χ^2-test, df = 3, P = 0.53). No genotype effects and genotype-diagnosis interaction effects were found in years of education, scores of full scale Intelligence Quotient (IQ) and scores of premorbid IQ [Japanese version of National Adult Reading Test (JART) score], however, patients who had fewer years of education ($P < 0.0001$), had lower scores of both full scale IQ and JART ($P < 0.001$). The duration of illness, medication and hospitalization, the age at disease onset and drug dose (chlorpromazine equivalent) of those homozygous for the Val-COMT did not differ from the Met-COMT carriers.

SNP genotyping

Venous blood was drawn from subjects and genomic DNA was extracted from whole blood according to the standard procedures. The Val158Met polymorphism of the COMT gene (dbSNP accession: rs4680) was genotyped using the TaqMan 5′-exonuclease allelic discrimination assay, described previously (Hashimoto *et al.*, 2004, 2005). Briefly, primers and probes for detection of the SNP are: forward primer 5′-GACTGTGCCGCCATCAC-3′, reverse primer 5′-CAGGCATGCACACCTTGTC-3′, probe 1 5′-VIC-TTTCGCTG-GCGTGAAG-MGB-3′ and probe 2 5′-FAM-CGCTGGCATGAAG-MGB-3′. PCR cycling conditions were: at 95°C for 10 min, 50 cycles of 92°C for 15 s and 60°C for 1 min.

MRI procedures

All MR studies were performed on a 1.5 tesla Siemens Magnetom Vision plus system. A three dimensional (3D) volumetric acquisition of a T_1-weighted gradient echo sequence produced a gapless series of thin sagittal sections using an MPRage sequence (TE/TR, 4.4/11.4 ms; flip angle, 15°; acquisition matrix, 256 × 256; 1 NEX, field of view, 31.5 cm; slice thickness, 1.23 mm).

Image analysis (TBM)

The basic principle of TBM is to analyse the local deformations of an image and to infer local differences in brain structure. In TBM, MRI scans of individual subjects are mapped to a template image with three-dimensional (3D) non-linear normalization routines. Local deformations were estimated by a univariate Jacobian approach. The basic principle of TBM is the same as a method used in a previous report described as deformation-based morphometry (Gaser *et al.*, 2001). Firstly, inhomogeneities in MR images were corrected using a bias correction function in statistical parametric mapping (SPM2), then the corrected image was scalp-edited by masking with a probability image of brain tissue obtained from each image using a segmentation function in SPM2. Using a linear normalization algorithm in SPM2, all brains were resized to a voxel size of 1.5 mm and adjusted for orientation and overall width, length and height (Fig. 1A). Therefore, brains were transformed to the anatomical space of a template brain whose space is based on Talairach space (Talairach and Tournoux, 1988). Subsequent non-linear normalization introduced local deformations to each brain to match it to the same scalp-edited template brain (Fig. 1C). The non-linear transformation was done using the high-dimension-warping algorithm (Ashburner and Friston, 2004). After the high dimensional warping, each image (Fig. 1B) looks similar to the template (Fig. 1C). Figure 2 demonstrated a mean MR image of 76 controls (left) and a mean MR image of 47 schizophrenics after high dimensional warping (Fig. 2). We obtained 3D deformation fields for every brain (Fig. 1D). Each of these 3D deformation fields consists of displacement vectors for every voxel, which describe the 3D displacement needed to locally deform the brain to match it to the template. We calculated the Jacobian determinants to obtain voxel by voxel parametric maps of local volume change relative to the template brain (Fig. 1E). The local Jacobian determinant is a parameter commonly used in continuum mechanics (Gurtin, 1987), which characterizes volume changes, such as local shrinkage or enlargement caused by warping. The parametric maps of Jacobian determinants were analysed using SPM2, which implements a 'general linear model'. To test hypotheses about regional population effects and interaction, data were analysed by an analysis of covariance (ANCOVA) without global normalization. There was no significant difference in age among the four groups, however, patients with schizophrenia, particularly those homozygous for the Val-COMT allele, were older than controls. Therefore, we treated age and years of education and scores of JART as nuisance variables. Since TBM explores the entire brain (grey matter, CSF space and white matter) at once, the search volume of TBM has a large number of voxels and since our interest was in morphological changes in the grey matter and CSF space, we excluded white matter tissue from analyses by using an explicit mask (Fig. 1F). We used $P < 0.001$, corrected for multiple comparisons with false discovery rate (FDR) <0.05 as a statistical threshold. The resulting sets of t values constituted the statistical parametric maps {SPM (t)}. Firstly, we estimated the main effects, the genotype effect in total subjects (the Val/Val-COMT versus the Met-COMT carriers) and the diagnostic effect (schizophrenia versus controls) and then the genotype-diagnosis interaction effect was estimated. Furthermore, the effects of genotypes in each group (controls carrying the Val/Val-COMT gene versus controls carrying the Met-COMT gene and schizophrenics carrying the Val/Val-COMT gene versus schizophrenics carrying the Met-COMT gene) were estimated within the ANCOVA design matrix. Anatomical localization accorded both to MNI coordinates and Talairach coordinates obtained from M. Brett's transformations (www.mrc-cbu.cam.ac.uk/Imaging/mnispace.html) and are presented as Talairach coordinates (Talairach and Tournoux, 1988). Since previous studies have demonstrated the association between the Val158Met polymorphism and the dorsolateral PFC (DLPFC), we applied an additional hypothesis-driven region of interest (ROI) method to test regional population effects in the DLPFC. For this ROI analysis, we used the Wake Forest University PickAtlas (Maldjian *et al.*, 2003) within the ANCOVA design matrix for SPM analysis. We set $P < 0.05$ (uncorrected) with a small volume correction ($P < 0.05$ within the ROI) to assess grey matter volume changes in the DLPFC (Brodmann area 46, 9 and 8).

Table 1 Subject characteristics

	Control Val/Val	Met carriers	Schizophrenia Val/Val	Met carriers	Diagnosis F (P)	Genotype F (P)*	Genotype by diagnosis F (P)
Number of subjects	38	38	19	28			
Gender (M/F)	16 out of 22	14 out of 24	11 out of 8	13 out of 15			
Handedness (R/L)	36 out of 2	35 out of 3	18 out of 1	28 out of 0			
Age (years)	41.47 (13.42)	39.26 (10.6)	45.98 (15.29)	43.05 (10.57)	3.633 (0.059)	1.7 (0.195)	0.21 (0.647)
Education (years)	17 (3.16)	16.06 (2.57)	12.67 (2.43)	13.33 (3.31)	30.855 (<0.0001)	0.047 (0.828)	1.61 (0.208)
Full scale IQ (WAIS-R)	113.42 (12.05)	108.93 (13.58)	80.69 (17.68)	88.958 (22.08)	57.9 (<0.001)	0.29 (0.59)	3.41 (0.068)
JART	78.8 (10.45)	75.42 (13.65)	54.69 (20.74)	62.25 (27.06)	23.366 (<0.001)	0.292 (0.59)	2.014 (0.159)
Wechsler Memory Scale—Revised							
Verbal memory	111.78 (15.001)	111.061 (12.89)	78.0 (21.623)	81.33 (18.57)	86.93 (<0.001)	0.147 (0.702)	0.354 (0.553)
Visual memory	112.1 (8.51)	106.55 (11.99)	74.78 (24.32)	83.29 (20.613)	85.51 (<0.001)	0.204 (0.65)	4.605 (0.03)
General memory	113.31 (13.92)	110.85 (12.22)	74.43 (21.3)	79.33 (19.14)	111.93 (<0.001)	0.135 (0.715)	1.226 (0.27)
Attention/concentration	104.47 (13.25)	102.94 (16.51)	87.79 (19.09)	92.54 (17.38)	16.08 (0.001)	0.228 (0.634)	0.866 (0.14)
Delayed recall	111.88 (15.46)	112.48 (10.08)	77.07 (20.92)	81.21 (19.19)	99.74 (<0.001)	0.52 (0.475)	0.284 (0.59)
WCST (preservative error)	2.5 (3.89)	3.14 (3.90)	12.08 (11.54)	8.52 (10.63)	24.5 (<0.0001)	0.93 (0.34)	1.93 (0.17)
Digit span	11.12 (3.25)	10.77 (3.34)	7.83 (3.93)	9.09 (2.74)	12.165 (0.0007)	0.415 (0.52)	1.28 (0.261)
Onset age			25.38 (10.34)	23.74 (7.992)		0.52	
Duration of illness (years)			19.86 (14.93)	18.84 (9.8)		0.77	
Duration of hospitalization (months)			66 (153.41)	59.59 (91.18)		0.86	
Duration of medication (years)			12.86 (14.21)	16.4 (9.89)		0.29	
Drug dose of typical antipyschotic drugs (mg/day, chlorpromazine equivalent)			617.9 (720.18)	700.38 (752.67)		0.69	
Drug dose of atypical antipyschotic drugs (mg/day, chlorpromazine equivalent)			282.3 (428.29)	340.23 (482.19)		0.66	

Mean (standard deviation); WAIS-R = Wechsler Adult Intelligence Scale—Revised; JART = Japanese version of National Adult Reading Test; WCST = Wisconsin Card Sorting Test.

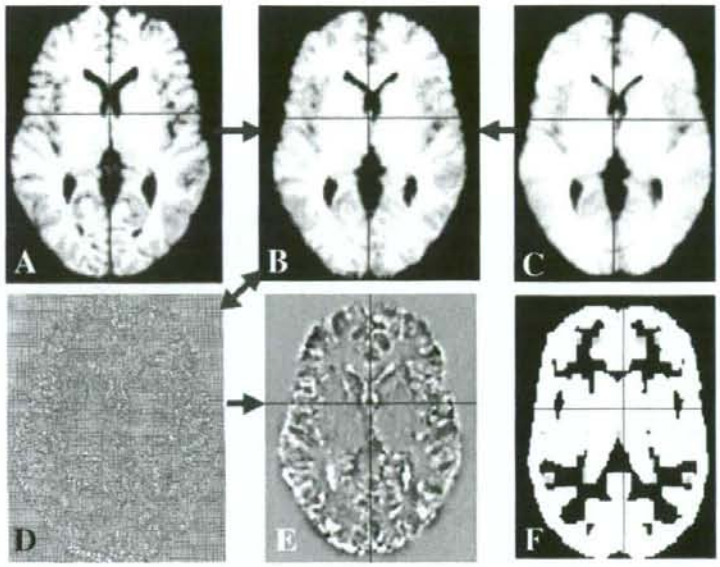

Fig. 1 Steps of analysis for tensor-based morphometry. An example is shown for a single subject in one axial slice. The single object brain (**A**) has been corrected for orientation and overall size to the template brain (**C**). Non-linear spatial normalization removes most of the anatomical differences between the two brains by introducing local deformations to the object brain, which then (**B**) looks as similar as possible to the template. Image (**D**) shows the deformations applied to the object brain by a deformed grid. Statistical analysis can be done univariate using the local Jacobian determinant as a derivative of the field (**E**). An explicit mask image (**F**) was used to explore morphology in the grey matter and CSF space.

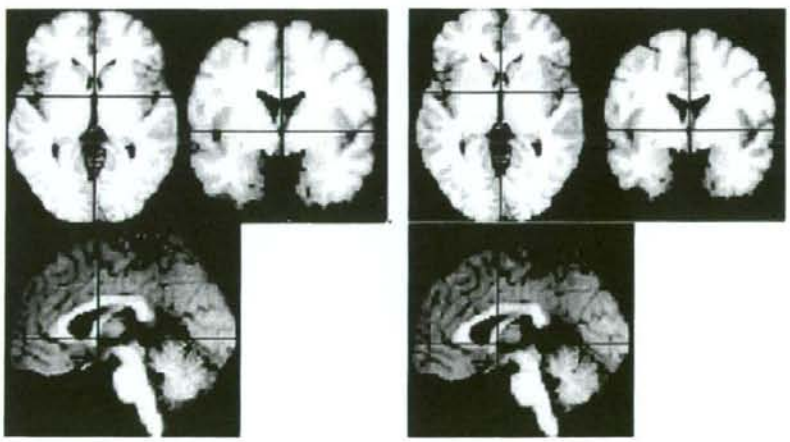

Fig. 2 Mean images after high dimensional warping control subjects and schizophrenics. *Left*: The mean image of warped MR images obtained from 76 controls. Even after averaging, the mean image is not blurred. *Right*: The mean image of warped MR images obtained from 47 schizophrenics. The mean image of schizophrenic looks similar to that of controls.

Results

Behavioural data

Patients had a lower full scale IQ, measured by the Wechsler Adult Intelligence Scale—Revised, than controls. They also had a lower expected premorbid IQ measured by a JART, lower scores of Wechsler Memory Scale—Revised and demonstrated poorer performance of working memory measures such as the number of preservative errors in the WCST and digit span (Table 1). No genotype or genotype-diagnosis interaction effects were found in working memory measures

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).

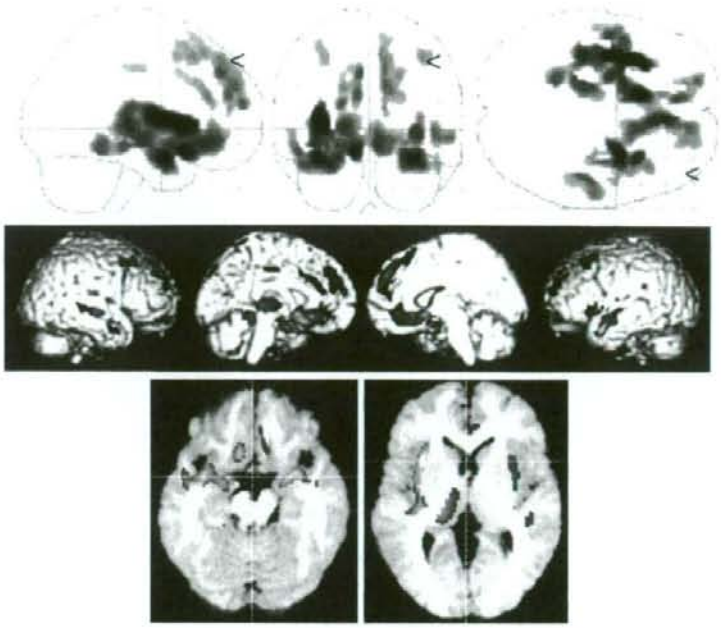

Fig. 3 Decreased volumes in schizophrenics ($n = 47$) as compared to controls ($n = 76$). *Top*: The SPM {t} is displayed in a standard format as a maximum-intensity projection (MIP) viewed from the right, the back and the top of the brain. The anatomical space corresponds to the atlas of Talairach and 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.