a Exon 1 2 19kb 3 4 4I 40kb BDNF-LCPR 1kb 5

```
                                                                        9--->
                                                                   1 ggaaacag
  9 ctatgaccatgattacgccaagcgcgcaattaaccctcactaaagggaacaaaagctggagctccaccgc
                                                                      1--->
 79 ggtggcggccgctctagaactagtggatcccccgggctgcaggaattcgatatcaagcttTAGAGCAACC

149 CTCTGGCAAACAGGAAGAGATTAATTTTGTGGTATGCTTTTAAGGGACTTCCCAGGAAACTTCAAAAGCA
                                  3, 4--->                           7--->
219 GAAAAAGAAGCACTAGCTGCCTATTCCAAAATGTGTAAAACACCACTCAGCTTTTAAAAGTAGGATAAA
                           ①                ②           ③
289 CTCAGAGCGCGCG/CACACGCGCGCGCG/CACACACACACACACACACA/GAGAGA/ACATCTCTAGTAA
  <---8        <---5, 6
355 AAAGAAAAGTTGAGCTTTCTTAGCTAGATGTGTGTATTAGCCAGAAAAAGCCAAGGAGTGAAGGGTTTTA

425 GAGAACTGGAGGAGATAAAGTGGAGTCTGCATATGGAGGCATTTGAAATGGACTTAAATGTCTTTTTAA
                                                  <---2
495 TGCTGACTTTTTCAGTTTTCTCCTTACCAGACACATTGTTTTCATGACAaagcttatcgataccgtcgac

565 ctcgagggggggcccggtacccaattcgccctatagtgagtcgtattacgcgcgctcactggccgtcgtt
   <---10
635 ttacaac 641
```

b

5'-flanking	①	②	③	3'-flanking
AGAGCGCGCG	/ deletion /	(CA) 9	/ (GA) 2 /	ACATCTCTAG
	/ CACA (CG) 4 /	(CA) 10	/ (GA) 3 /	
	/ CACA (CG) 5 /	(CA) 11	/	
	/	(CA) 12	/	
	/	(CA) 13	/	
	/	(CA) 14	/	
	/	(CA) 15	/	

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]CACACACACACACACACACACACACACACACACAGAGAGAGAACAT-3′ and dispensation order was set as 5′-TCGCGCACAGCGCGCGCGTCACACACACACACACACACACACACACACACACATGAGAGAGAT-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′-CTCTCTGTGTGTGTGTGTGTGTGTG[TGTGTGTGTGTGTGTG]CGCGCGCGCGTGTGCGCGCGCTCTGAGTT-3′ and 5′-GCTCTCTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTACGCGCGCGCGTGTGCGCGCGCTCTC-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’.

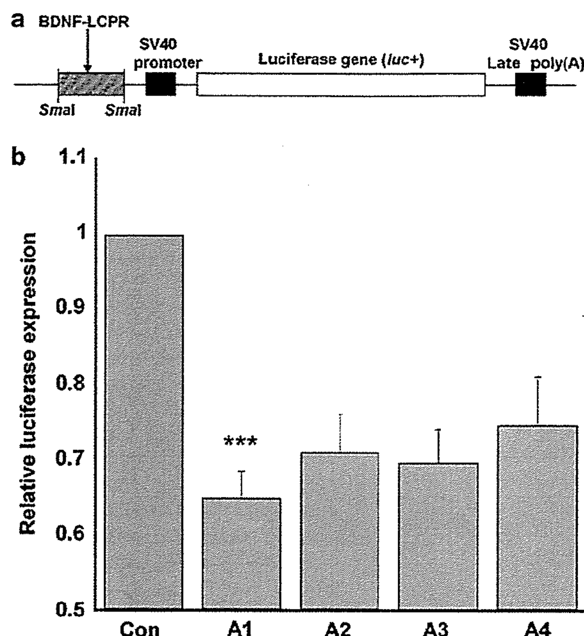

Figure 2 Luciferase reporter gene assay on the four major alleles of the BDNF-linked complex polymorphic region (BDNF-LCPR). (**a**) Schematic illustration of the luciferase assay construct for the BDNF-LCPR. (**b**) Relative luciferase expression (RLE) for pGL3-Promoter vector with insertion of each 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′*	*Fragment Size (bp)*[a]	*Allele counts (%)*		
			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	agagcgcgcgcg(del)(ca)$_{13}$(ga)$_3$acat	399	1 (0.3)	0 (0.0)	1 (0.2)
Variant 2	agagcgcgcgcacatg(cg)$_4$(ca)$_{12}$(ga)$_2$acat	405	0 (0.0)	1 (0.3)	1 (0.2)
Variant 3	agagcgcgcgcgcaca(cg)$_4$(ca)$_{13}$(ga)$_3$acat	411	1 (0.3)	0 (0.0)	1 (0.2)
Variant 4	agagtgcgcgcaca(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, $P=1.6\times10^{-90}$ 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[8–10] 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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Regular Article

Discrepancy of performance among working memory-related tasks in autism spectrum disorders was caused by task characteristics, apart from working memory, which could interfere with task execution

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Abstract Working memory performance has been inconsistently reported in autism spectrum disorders (ASD). Several studies in ASD have found normal performance in digit span and poor performance in digit symbol task although these are closely related with working memory. It is assumed that poor performance in digit symbol could be explained by confirmatory behavior, which is induced due to the vague memory representation of number–symbol association. Therefore it was hypothesized that the performance of working memory task, in which vagueness did not cause confirmatory behavior, would be normal in ASD. For this purpose, the Advanced Trail Making Test (ATMT) was used. The performance of digit span, digit symbol and ATMT was compared between ASD and normal control. The digit span, digit symbol and ATMT was given to 16 ASD subjects and 28 IQ-, age- and sex-matched control subjects. The scores of these tasks were compared. A significantly lower score for ASD was found only in digit symbol compared with control subjects. There were no significant difference in digit span and working memory estimated by ATMT. Discrepancy of scores among working memory-related tasks was demonstrated in ASD. Poor digit symbol performance, normal digit span and normal working memory in ATMT implied that ASD subjects would be intact in working memory itself, and that superficial working memory dysfunction might be observed due to confirmatory behavior in digit symbol. Therefore, to evaluate working memory in ASD, tasks that could stimulate psychopathology specific to ASD should be avoided.

Key words autism spectrum disorders, digit span, digit symbol, vagueness, working memory.

INTRODUCTION

Working memory refers to a cognitive function that provides concurrent temporary storage and manipulation of the information necessary for complex cognitive tasks.[1] For the past three decades, numerous studies have reported executive dysfunction in autism spectrum disorders (ASD).[2–5] Working memory is generally considered one of the executive functions,[2–4,6,7] but working memory performance in ASD has been inconsistently reported until now. Some studies found deficiency in working memory,[7–14] although others reported normal performance in ASD.[13–19] This inconsistency among the studies might be attributed to the

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task characteristics. Most of the working memory studies in ASD utilized original tasks not standardized, and the cognitive factors necessary for task performance, apart from working memory, were different among studies. For instance, sentence span by Bennetto *et al.* depended on verbal mediation strategies that could interrupt the task execution in ASD.[7] Also, in the study by Williams *et al.*, interpersonal and social elements could interfere with the execution, because pictures of face and family scene were used as stimuli to be memorized and sequences of spatial information were presented by tester's hand in spatial span test.[13]

Therefore we tried to estimate the cause of discrepancy of ASD working memory with prevalent tests in a clinical setting. The Wechsler Intelligence Scale is a widespread and standardized cognitive battery used for ASD.[20,21] This battery also demonstrates discrepant results among working memory-related subtests in ASD. These subtests are digit span and digit symbol. In the digit span test, the subject must memorize and repeat strings of digits in forward and backward order. This test assesses working memory mainly, and is also affected by attention, anxiety and preference of digit.[22,23] In the digit symbol test, memorization of number–symbol association could enhance task performance. This test assesses working memory, attention and visuomotor tracking.[23,24] Also, there are studies that propose that working memory and attention describe the same or a closely related mechanism.[25]

In ASD, several studies have found normal score in digit span and poor score in digit symbol.[7,26,27] Thus, even a standardized tool such as the Wechsler Intelligence Scale produced a discrepancy between the scores of subtests related with working memory. To date there have been no studies investigating the cause of deterioration of digit symbol in ASD.

To interpret the deterioration of digit symbol in ASD, we assumed that the poor digit symbol performance might be attributed not to disturbance of working memory itself but to a deficit in acting on the basis of retained vague information, because vagueness of working memory representation might cause repetitive confirmatory behavior that compels ASD subjects to check the correct number–symbol association, which would result in the delay of task performance.

According to this consideration, we hypothesized that a working memory task in which vague memory representation did not provoke confirmatory behavior, would yield normal performance in ASD. For this purpose we used a novel task: the Advanced Trail Making Test (ATMT). The ATMT, developed by Kajimoto,[28] is a computerized version of the Trail Making Test.[29,30] This task is carried out using visuomotor coordination, visual scanning as in the digit symbol test,[24] and is able to estimate quantitatively working memory, which would not be affected by vague memory representation because the vagueness of memory on the positions of subsequent numbers provokes no confirmatory behavior. Therefore we adopted the ATMT as a control task for digit symbol. These two tasks were different in regard to whether vagueness of memory could result in confirmatory behavior or not.

In the present study, we compared the performance of digit span, digit symbol and ATMT in ASD with that of normal controls to test the hypothesis that discrepancy of working memory task performance in ASD could be explained by task characteristics apart from working memory.

METHODS

Subjects

We classed one subject with autistic disorder and 15 with Asperger's disorder diagnosed by experienced psychiatrists with DSM-IV[31] as ASD group because no consistent evidence of etiologic differences was apparent for autistic disorder and Asperger's disorder, and they demonstrate similar clinical symptoms, except for language development in infancy.[32] One subject with autistic disorder in the present study demonstrated current symptoms that could not be differentiated from Asperger's disorder. The ASD subjects were diagnosed in adulthood after careful and multiple interviews with themselves and their parents, regarding developmental history and current state. Full-scale IQ (FSIQ) of the ASD subjects according to the Japanese version of the Wechsler Intelligence Scale–Revised (WAIS-R)[33] was >70. State of employment, marriage and education were used as indices estimating social adaptation in the ASD group. Six subjects were employed, one was married and one was a university student, and the other subjects were unemployed or withdrawing from school. We regarded the former eight subjects as the comparatively good social adaptation group, and the remainder as the comparatively poor social adaptation group.

We defined 28 normal volunteers as a control group. Full-scale IQ of control group was estimated from the four subtests of the Japanese version of the WAIS-R (information, similarities, picture completion, digit symbol), corresponding to the Japanese Wechsler Intelligence Scale-Revised Short Forms,[34] which did not estimate verbal IQ and performance IQ.

The sex ratio of the both groups was identical (male : female, 3 : 1). Also age and FSIQ were not significantly different between the ASD and control groups. Subject details are given in Table 1.

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Table 1. Subject data

	ASD group	Control group
Subjects (male/female)	16 (12/4)	28 (21/7)
Diagnosis (Autistic/ Asperger)	1/15	–
Age (years), mean (range)	28.0 (20–42)	28.3 (19–42)
FSIQ, mean (range)	101 (75–132)	103 (80–130)
VIQ, mean (range)†	107 (83–137)	
PIQ, mean (range)	91 (72–123)	

ASD, autism spectrum disorders; FSIQ, full-scale IQ; PIQ, performance IQ; VIQ, verbal IQ.
†In the ASD group VIQ was significantly higher than PIQ ($Z = 3.41$, $P = 0.0006$, Wilcoxon signed-ranks test).

Antipsychotics were prescribed to three subjects in the ASD group. Two subjects took antipsychotics at a dose of 100 mg and one took antipsychotics at a dose of 200 mg chlorpromazine-equivalent units per day. Low doses of antidepressants, hypnotics and antiparkinsonian agents were prescribed to several subjects in the ASD group.

This experimental procedure was approved by the ethics committee of Osaka University Graduate School of Medicine. All subjects gave written informed consent for his or her participation and patient anonymity was preserved.

Task

The ATMT was performed on a touchscreen monitor (Totoku-CV515PJ, Totoku Electric, Okubo, Shinjuku, Tokyo, Japan) connected to a laptop PC (Fig. 1).

Subjects were instructed to push the buttons as quickly as possible from one to 99. Then, subjects pushed 25 numbered black circles (buttons) displayed randomly on the 18 × 18-cm screen in numerical order by the dominant hand. A pushed button disappeared and a new button appeared simultaneously. The reaction time of a button press was measured as the time lag between a button press and next one. One trial included 99 button presses.

The ATMT had two types of tasks: random tasks (task R) and fixed tasks (task F). In task R, when a correct numbered button was pushed, the other buttons were rearranged randomly. In task F, when a correct numbered button was pushed, the location of the other buttons remained fixed. Task F allowed the subject to memorize the locations of the buttons found during visual scanning. Therefore in task F, the subject could shorten the reaction time compared with task R by using working memory of subsequent buttons. Vague

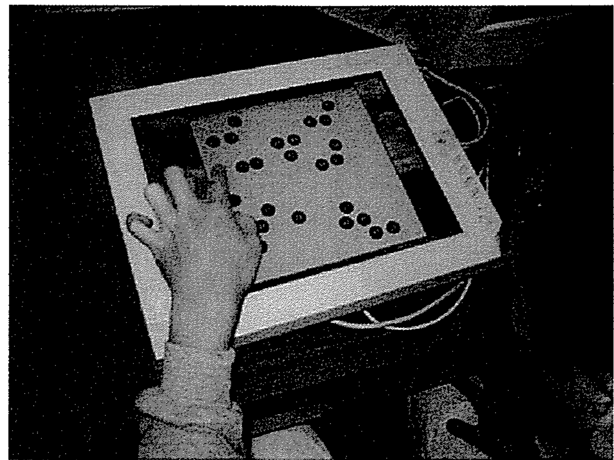

Figure 1. Execution of the Advanced Trail Making Test (ATMT).

memory representation might not disturb ATMT performance because no confirmatory behavior would be induced in this task design. Even if subjects were not confident with vague memory for the position, they could find the correct button, otherwise they could continue the task by visual scanning. Therefore this task design might not induce delay due to vague memory representation of a subsequent button position.

The utilization ratio of working memory (WMR) in ATMT is defined in Fig. 2. We defined the top 5% fastest reaction time in the distribution of all button presses in task R as the threshold of visual scanning. We considered that a reaction time in task F shorter than the threshold, was accomplished by utilization of working memory of button position. The WMR was calculated as the percentage of the button presses which had shorter reaction times than the threshold in task F.

In the process of WMR estimation, we used buttons with distances from the precedent button >300 pixels on the screen. Our previous study indicated that distances >300 pixels were considered to be outside the visual attention field.[35] We excluded the buttons inside the visual attention field because these buttons could be found by accident, and the short reaction time for these button presses should not be considered as working memory utilization. Also, buttons of single-figure numbers were excluded from the data analysis because they were easily found by subjects and produced shorter reaction times than double-figure numbers.

Procedure

The two types of ATMT tasks were presented in a pseudo-random order of R, F, F, R, and 30-s rests were

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inserted between each task. Total performance time was approximately 20 min.

In addition to ATMT, the digit symbol and digit span tests in the Japanese version of WAIS-R[33] (these two subtests were identical with the original WAIS-R[20]), were also carried out as tasks evaluating working memory.

Statistical analysis

All data are presented as mean ± SD. Two-sample t-test or Mann–Whitney U-test with two-tailed $P = 0.05$ set as the significance threshold was used to compare the reaction time and WMR in ATMT, and the raw score of digit span and digit symbol between the ASD and control groups.

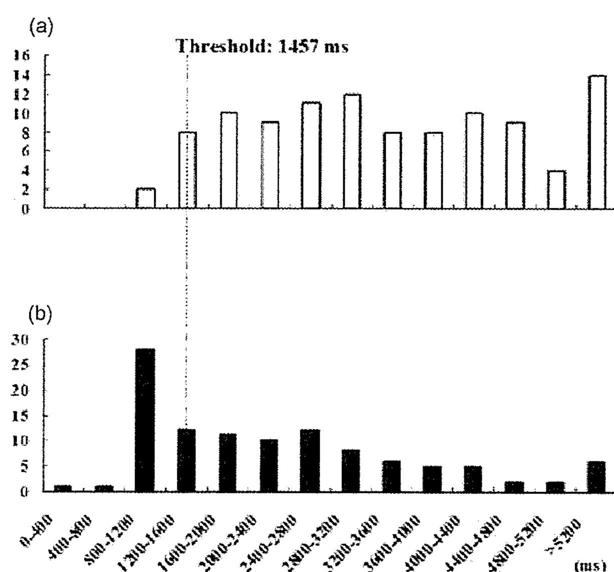

Figure 2. Number of button presses of (a) random task (task R) and (b) fixed task (task F) in two trials. The horizontal axes show response time and the vertical axes show frequencies of button presses. The threshold of this subject was 1457 ms. The utilization rate of working memory of this subject was 36.7% because within a total of 109 button presses in task F, 40 button presses with a response time shorter than 1457 ms were considered to utilize working memory.

RESULTS

The ATMT was given to 16 ASD subjects and 28 control subjects. In the ASD group, the mean reaction time in task R was 3957 ± 1051 ms, and the mean reaction time in task F was 3029 ± 943 ms. In the control group the mean reaction time in task R was 3610 ± 631 ms, and the mean reaction time in task F was 2499 ± 519 ms. Two-sample t-test showed no significant differences between the ASD and control groups in the reaction time of task R ($t = 1.20$, d.f. = 21, $P = 0.24$) and task F ($t = 2.07$, d.f. = 20, $P = 0.051$).

Next, WMR in ATMT and raw scores of digit span and digit symbol subtests were compared between the ASD and control groups with Mann–Whitney U-test. There was a significant difference only in the digit symbol subtest ($U = 83$, $P = 0.0006$) and no significant difference in WMR (U = 168, $P = 0.17$) or digit span (U = 202, $P = 0.59$). The results for these working memory tasks are given in Table 2.

In addition, we compared the eight ASD subjects of the comparatively good social adaptation group with the eight ASD subjects in the comparatively poor social adaptation group with regard to the aforementioned parameters, and we found no significant differences (reaction time in task R: $t = 0.31$, d.f. = 14, $P = 0.76$; reaction time in task F: $t = 0.76$, d.f. = 14, $P = 0.46$; WMR: $U = 17$, $P = 0.10$; digit symbol: $U = 32$, $P = 0.96$; digit span: $U = 17$, $P = 0.10$).

DISCUSSION

In the present study the ASD group demonstrated a discrepancy of working memory performance in the different tasks; that is, lower digit symbol and normal ATMT and normal digit span performance compared with the control group. This result was consistent with previous reports on digit span and digit symbol,[7,26,27]

Table 2. Working memory-related tasks

	ASD ($n = 16$)		Control ($n = 28$)			
	Mean	SD	Mean	SD	U	P
WMR in ATMT (%)	31.9	12.3	37.4	9.7	168	0.17
Digit span (raw score)	19	5	18	3	202	0.59
Digit symbol (raw score)	62	14	77	9	83	0.0006

ATMT, Advanced Trail Making Test; WMR, utilization rate of working memory.
U and P: significance after two-tailed Mann–Whitney U- test.

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and supported our hypothesis that ASD would be normal in working memory itself and that the apparent deterioration of working memory could be observed only in the tasks whose characteristics apart from working memory might interfere with the task execution.

The normal digit span and WMR in ATMT would indicate normal ability in retaining short-term information in the ASD group. The reason for the poor performance only in the digit symbol task was inferred as follows. Both digit symbol and ATMT require visuomotor coordination and visual scanning. The memory of number–symbol association in digit symbol as well as the memory of button position in ATMT could improve task performance. These tasks differ in that vagueness of working memory might induce confirmatory behavior and delay of task performance in the digit symbol test, although there might be no confirmatory behavior or delay in ATMT. In ATMT, no abnormalities were found in reaction time of task F, task R or WMR in the ASD group, which would mean no abnormalities in visuomotor coordination, visual scanning or working memory of button position. Therefore, the poor performance in the digit symbol test in the ASD group would not be explained by motor and memory components and could be interpreted by the difference of task characteristics as to whether vague memory representation might disturb the execution of the task or not.

This discussion is also consistent with clinical features of ASD exhibiting poor performance in vague situations and drastic change of adaptation level, depending on the environment.[5] For example, ASD patients have claimed: (i) 'I cannot go ahead unless the environment is put into place completely'; (ii) 'I cannot assent without investigating thoroughly'; and (iii) 'I can work if the task is defined'.

These statements reflect the characteristics of ASD patients, who cannot act on the basis of vague information and who require concrete information. This feature would be included in executive dysfunction in ASD such as cognitive inflexibility and rigidity.[3–5,16,36,37] Also, the repetitive confirmatory behavior of ASD, which might be induced in digit symbol subtest, could be attributed to cognitive inflexibility.[3–5,36,38]

Although several studies have been reported on working memory function in adolescent and adult patients with ASD, the results are totally discrepant. The studies by Russell *et al.*, Ozonoff and Strayer, and Koshino *et al.* reported normal working memory in ASD,[15,17,19] while the studies by Bennetto *et al.*, Minshew *et al.*, Morris *et al.*, Luna *et al.* and Williams *et al.* insisted on poor working memory in this disorder.[7,9–11,13] However, in these studies claiming working memory dysfunction, there was a possibility that the task characteristics apart from working memory could deteriorate the task performance. For example, Joseph *et al.* reported that patients with autism were deficient in the use of verbal mediation strategies to keep and monitor goal-related information in working memory.[14] Another possibility was that social elements in a task could interfere with the task performance, and moreover, computerized task presentation to minimize social elements could improve the task performance.[17,39] Furthermore, perseveration responses and task complexities could be the factors that led to the deterioration of task performance. In an executive golf task, Morris *et al.* used the number of error responses to choose the same target as an index of working memory dysfunction.[10] It is possible that the results of this task could be confounded by perseveration responses induced by cognitive inflexibility, one of the executive dysfunctions in ASD. Minshew *et al.*, and Luna *et al.*, evaluated working memory using an oculomotor delayed-response task.[9,11] This task required participants to determine the correct position of a visual target by memory-guided saccades. The complexity of response manner might affect the task performance.

In summary, we should be careful about concluding that ASD has working memory dysfunction because deteriorated performance of working memory-related task in ASD could be attributed to task characteristics such as verbal factors, social and interpersonal elements, perseveration and task complexities apart from working memory.

Examining the relationship between clinical features and cognitive functions in ASD is important. We also compared ASD subjects who had comparatively good social adaptation with those who had comparatively poor social adaptation with regard to performance of cognitive tasks, but we could find no significant differences between the two groups, probably due to the difficulty in appropriate evaluation of social adaptation. We could not confidently rely on the present results on social adaptation and cognitive function in ASD, and therefore this will require further study.

The present study had several limitations. First, we did not obtain direct evidence that the ASD group displayed repetitive confirmatory behavior in the digit symbol test. It has been noted that vagueness and perseveration are closely linked,[40] so confirmatory behavior induced in the ASD group by the digit symbol test could be adequately predicted. Although eye movement recording of the participants using a video camera or eye mark recorder would be needed to ascertain this objectively, this recording could spoil the natural execution of the experiment. In addition, our preliminary video camera recording could not assure evalua-

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tion of the number and duration of confirmatory behaviors from eye movement and head motion. Next, we assumed that ATMT was comparable to the digit symbol test regarding cognitive components such as working memory, visual scanning and visuomotor coordination, but the apparatus, movement and memory representation used to execute these tasks were somewhat different. In these tasks, the digit symbol test required visual memory and ATMT required both visual and spatial memory. Hence, it would be ideal if we used a task more similar to the digit symbol test.

In conclusion, the discrepancy of scores in working memory-related tasks in the present study implied that the ASD group had intact working memory per se but that superficial working memory dysfunction could be observed when the task characteristics apart from working memory might disturb the performance. Therefore, to evaluate working memory in ASD appropriately, the tasks stimulating psychopathology specific to ASD that could interfere with the task execution, should be avoided.

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Regular Article

Susceptibility genes for schizophrenia

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Abstract It is well known that genetic factors contribute to the susceptibility for schizophrenia. Recent advances in the molecular genetics of schizophrenia strongly suggest several susceptibility genes (e.g. dysbindin, neuregulin-1, DISC1, COMT, G72, RGS4 and Akt1). We discuss the evidence and biology of these genes. As glutamate transmission is especially implicated in these genes, neurobiological basis of schizophrenia might be elucidated by investigation of functional interactions between susceptibility genes for schizophrenia and the glutamatergic system.

Key words COMT, DISC1, dysbindin, neuregulin-1, schizophrenia, susceptibility gene.

INTRODUCTION

Schizophrenia is a major mental disorder that is one of the world's top 10 causes of long-term disability. This disease is characterized by psychosis and profound disturbances of cognition, emotion and social functioning. It affects approximately 1% of the general population across different countries and the cultural group worldwide. The fact that schizophrenia has a genetic component has long been established with high heritability estimates of 80%.[1,2] As the genetic transmission does not appear to follow simple mendelian single-gene inheritance pattern, this disease is a complex genetic disorder, like other common diseases. Many years of great effort has been devoted to identify susceptibility genes for schizophrenia. As a result, genome wide linkage studies suggested several positive linkage regions such as 1q, 5q, 6p, 6q, 8p, 10p, 13q, 22q.[3,4] A recent meta-analysis showed evidence for linkage at 8p, 13q and 22q,[5] and another meta-analysis at 1q, 2p, 2q, 3p, 5q, 6p, 8p, 11q, 14q, 20p, 22q.[6] The chromosomal abnormalities in schizophrenia have also added the evidence for susceptibility loci at 1q42[7,8] and 22q11.[9–11] A number of susceptibility genes for schizophrenia, including dysbindin, neuregulin-1, DISC1, COMT, and G72 and RGS4, have recently been identified in these loci.[12–17] The evidence for several genes becomes stronger now, as replication studies have achieved greater consistency than in the past.[18] Here we discuss the genetic evidence and biology of these susceptibility genes.

DYSBINDIN

A recent study implicated a gene on chromosome 6p, dysbindin (DTNBP1: dystrobrevin binding protein 1), as a susceptibility locus in Irish pedigrees.[12] Since then, a significant association between schizophrenia and genetic variation in dysbindin has been reported in various populations from Ireland, Wales, Germany/Hungary/Israel, Sweden, Bulgaria, USA, China, and Japan.[19–27] One study, which initially failed to replicate a positive association based on SNPs in an Irish population, became subsequently positive using a haplotype strategy.[28] Thus, genetic evidence for association with schizophrenia is quite strong. Talbot *et al.*[29] found that dysbindin protein levels were reduced in the hippocampal formation of patients with schizophrenia. This presynaptic reduction was observed especially in the

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inner molecular layer of the dentate gyrus. The expression levels of dysbindin mRNA and protein were also reduced in the prefrontal cortex in schizophrenic brains.[30,31]

Dysbindin is originally found as a binding partner of alpha- and beta-dystrobrevins, which are causative genes of Duchenne muscular dystrophy.[32] Dystrobrevins are parts of the dystrophin-associated protein complex which plays important roles in the normal function of muscle.[33] Cognitive impairments are commonly found in patients with Duchenne muscular dystrophy and it is thought to be due to an abnormality in the neuronal membrane that is caused by lack of dystrophin.[34] A model mouse of Hermansky–Pudlak syndrome, sandy mouse, is caused by a nonsense mutation in the dysbindin gene.[35] This disease is characterized by oculocutaneous albinism, prolonged bleeding and pulmonary fibrosis due to abnormal vesicle trafficking to lysosomes and related organelles.[36] Dysbindin is a component of the biogenesis of lysosome-related organelles complex (BLOC-1) and reduced expression of other proteins in this complex has been found in the sandy mouse.[35] Altered expression of dysbindin in the schizophrenic brain might affect the expression of BLOC-1, which could result in the abnormal protein trafficking in schizophrenia. Although several findings of function of dysbindin have been reported, little is known about the functions in neurons. Numakawa *et al.* have recently shown that dysbindin might influence exocytotic glutamate release via upregulation of the molecules in presynaptic machinery.[20] They also reported that dysbindin promotes neuronal viability through PI3K-Akt signaling.[20] Impairments of these functions of dysbindin could play an important role in the pathogenesis of schizophrenia.

NEUREGULIN-1

Neuregulin-1 (NRG-1), which maps to the 8p locus, has been shown as a susceptibility gene for schizophrenia by a combination of linkage and association analysis.[13] Additional evidence for association with schizophrenia has been reported by 10 independent groups,[37–46] while three studies failed to replicate it.[47–49] Notably, the majority of positive markers are located at the 5′ region of this gene, which is close to the first exons encoding type IV and type II of NRG-1. Quite strong evidence for the association with schizophrenia is suggested. Hashimoto *et al.* studied NRG-1 mRNA expression in dorsolateral prefrontal cortex (DLPFC) and found increased type I NRG1 mRNA in schizophrenia.[50] The elevation of type I expression was present relative to three house keeping genes and to other NRG-1 isoforms (type II and type III). However, type I NRG1 mRNA expression levels correlated with neuroleptic doses in patients with schizophrenia, thus it is unclear whether this finding reflected a neuroleptic effect or disease severity. It is notable that Law *et al.* replicated the increased mRNA expression of type I NRG-1 in a much larger and separate sample in hippocampus and did not find any correlation between medication and NRG1 mRNA.[51]

NRG-1 is one of the neuregulin family of proteins, which have a broad range of bioactivities in the central nervous system and contain an epidermal growth factor (EGF)-like motif that activates membrane-associated tyrosine kinase related to ErbB receptors.[52] NRG-1 regulates the expression and plasticity of N-methyl-d-aspartate (NMDA) receptors, of the $\beta 2$ subunit of the γ-amino butyric acid (GABA) receptor and of nicotinic acetylcholine receptor subtypes including $\alpha 5$, $\alpha 7$ and $\beta 4$ subunits.[53–56] A gene targeting approach for NRG-1-ErbB signaling revealed a behavioral phenotype in mice that overlaps with certain animal models for schizophrenia. For example, NRG-1 and ErbB4 mutant mice exhibit elevated activity levels in an open field, which was reversed by clozapine, and abnormal sensorimotor gating measured by prepulse inhibition of the startle reflex.[13,57] The NRG-1 gene generates multiple alternative splicing variants, classified into three primary isoform groups (types I: heregulin/acetylcholine receptor inducing activity/neu differentiation factor, II: glial growth factor, III: sensory and motor neuron-derived factor),[58] and recently additional 5′ exon containing transcripts (types IV, V, VI) have been found in human brain.[59] These NRG-1 isoforms play multiple and distinct functions in neuronal development, which may be relevant to neurodevelopmental abnormalities in schizophrenia.

DISC1

The Disrupted in Schizophrenia 1 (DISC1) gene has initially been identified at the breakpoint of a balanced translocation (1; 11)(q42.1; q14.3), which segregates with schizophrenia and related psychiatric disorders in a large Scottish family.[7,14] Five studies reported a significant association between schizophrenia and genetic variation in the DISC1 gene[60–64] and we also found such an association (Hashimoto *et al.*, unpubl. data, 2005). However, two studies failed to find the association.[65,66] There is evidence for association with bipolar disorder[62,64] and with major depression (Hashimoto *et al.*, unpubl. data, 2005). A frameshift mutation of the DISC1 gene has been found in an American family with schizophrenia and schizoaffective disorder.[67] These findings suggest that DISC1 may give a susceptibility to mood disorders as well as to schizophrenia.

The function of DISC1 is still unclear, however, increasing evidence suggests a role in cytoskeletal organization, as DISC1 interacting proteins are associated with the components of microtubule and actin.[68–71] DISC1 is likely to be involved in the neurite extension[68,70] and mitochondrial and nuclear related functions have also been suggested.[69,72–74]

COMT

Catechol O-methyltransferase (COMT) is a susceptibility gene for schizophrenia, which maps to 22q11 implicated in two meta-analyses of linkage studies.[5,6] Hemideletion of this region produces velo-cardio-facial syndrome (VCFS), a condition associated with increased risk of schizophrenia-like psychoses.[75] COMT is a key enzyme in the elimination of dopamine in the prefrontal cortex. A functional polymorphism of the COMT gene, Val158Met, affects prefrontal function, and the high-activity val allele has been reported to be a genetic risk factor for schizophrenia in at least eight studies.[18] Among the susceptibility genes for schizophrenia, only COMT has evidence for the association with functional polymorphism. As COMT val allele is associated with prefrontal abnormalities, COMT is linked more strongly with cognitive intermediate phenotypes (e.g. executive function, cortical processing and P300 evoked EEG response).[15,76,77] The mRNA expression levels of COMT in schizophrenia have been studied in DLPFC and they show only minor alterations.[78,79] Many negative results have also been reported and recent meta-analysis was inconclusive,[80] however, it is likely that the COMT Val158Met polymorphism is a part of the complex risk architecture of schizophrenia.[18]

G72

G72 was cloned from a 5 MB gene desert in the 13q linkage region.[16] Biochemical study revealed that G72 protein activated D-amino acid oxidase (DAAO), which was involved in the metabolism of D-serine, an agonist at the glycine modulately site of the NMDA receptor.[16] Chumakov *et al.* also reported that DAAO was associated with schizophrenia.[16] Subsequently, five studies suggested a significant association between schizophrenia and G72,[81–85] while one study did not support the association.[86] As the association with child-onset schizophrenia and with bipolar disorder has also been reported,[83,87,88] this gene is likely to be a susceptibility gene for psychosis. The increased expression of G72 mRNA was observed in DLPFC of postmortem brain in patients with schizophrenia, which is consistent with glutamatergic theory of schizophrenia.

RGS4

Regulator of G-protein signaling 4 (RGS4) has been discovered to be decreased in the prefrontal cortex of patients with schizophrenia using cDNA microarrays.[17] RGS4 maps to 1q, one of the suggestive linkage regions.[3,4,6] Five reports suggested the association with schizophrenia,[89–93] while two studies failed to replicate it.[94,95] RGS4-deficient mice showed normal behavior including intact prepulse inhibition, except subtle sensorimortor abnormality.[96] RGS4 accelerates the GTPase activities of G protein alpha-subunits and negatively modulates G protein-mediated signaling via dopamine, metabotropic glutamate, and muscarinic receptors. The evidence for genetic association between schizophrenia and RGS4 is suggestive.

AKT1

Akt1 (protein kinase B) is implicated as a susceptibility gene for schizophrenia using a combination of experiments.[97] Emamian *et al.* reported reduced expression of Akt1 protein in lymphocytes and postmortem brain tissue of patients with schizophrenia and genetic association between Akt1 and schizophrenia.[97] They also demonstrated higher sensitivity to amphetamine-induced PPI disruption in Akt1 knockout mouse.[97] Two subsequent studies supported the evidence for association of variants in the Akt1 gene with schizophrenia,[98,99] while one study failed to replicate it.[100] Akt has emerged as the focal point for many signal transduction pathways, regulating multiple cellular processes such as glucose metabolism, transcription, apoptosis, cell proliferation, angiogenesis, and cell motility.[101] In the central nervous system, the PI3K-Akt signaling pathway plays a critical role in mediating survival signals.[102,103] PI3-kinase-Akt signaling is also involved in the survival promoting effect of dysbindin.[20] Despite weak linkage evidence of Akt1 (14q) and the small number of positive association studies, biological evidence strengthens the candidacy of Akt1 as a susceptibility gene for schizophrenia.

CONCLUSION

Several studies have replicated the genetic association between polymorphisms in dysbindin, neuregulin-1, DISC1, COMT, G72, RGS4, and Akt1 and schizophrenia. However, no causative polymorphism has been described in schizophrenia, except for the val allele in the COMT gene. Discovery of the causative mutation is the next step of this field. As biological evidence of these genes accumulates in the glutamate transmission, further investigations of functional connectivity among

these susceptibility genes and the glutamatergic system should be conducted.

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