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A polymorphism of the metabotropic glutamate receptor mGluR7 (*GRM7*) gene is associated with schizophrenia

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Abstract

Introduction: Glutamate dysfunction has been implicated in the pathophysiology of schizophrenia. The metabotropic glutamate receptors (mGluRs) are G-protein-coupled receptors. *GRM7*, the gene that encodes mGluR7, is expressed in many regions of the human central nervous system. The *GRM7* gene is located on human chromosome 3p26, which has been suggested by linkage analysis to contain a susceptibility locus for schizophrenia.
Methods: We screened for mutations in all exons, exon/intron junctions, and promoter regions of the *GRM7* gene in Japanese patients with schizophrenia and evaluated associations between the detected polymorphisms and schizophrenia. We examined the influence of one polymorphism associated with schizophrenia on the expression of *GRM7* by dual-luciferase assay in transfected cells.
Results: Twenty-five polymorphisms/mutations were detected in *GRM7*. Case-control analysis revealed a potential association of a synonymous polymorphism (371T/C, rs3749380) in exon 1 with schizophrenia in our case-control study of 2293 Japanese patients with schizophrenia and 2382 Japanese control subjects (allelic $p=0.009$). Dual-luciferase assay revealed suppression of

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transcription activity by exon 1 containing this polymorphism and a statistically significant difference in the promoter activity between the T and C alleles.
Conclusions: Our results support the possible association of a *GRM7* gene polymorphism with genetic susceptibility to schizophrenia.

Keywords: Glutamate; Expression; Luciferase; Prefrontal

1. Introduction

Schizophrenia is a severe psychiatric disorder, which is equally prevalent in men and women and affects approximately 1% of the population worldwide. Several neurotransmitter systems and functional networks within the brain have been found to be affected in patients with schizophrenia. The glutamatergic neuronal dysfunction hypothesis is one of the main explanatory hypotheses (Carlsson et al., 1997). Glutamate is the primary excitatory neurotransmitter in brain. It is contained as a neurotransmitter in approximately 60% of brain neurons, including almost all cortical pyramidal neurons. Further, virtually 100% of brain neurons contain some type of glutamate receptor. Glutamate mediates its effects on the central nervous system via both ionotropic and metabotropic receptors. The metabotropic glutamate receptors (mGluRs), which are G-protein-coupled receptors, are divided into 3 groups on the basis of sequence homology, putative signal transduction mechanisms, and pharmacologic properties (Nakanishi, 1994; Pin and Duvoisin, 1995). The mGluRs in group I are mGluR1 and mGluR5, those in group II are mGluR2 and mGluR3, and those in group III are mGluRs 4, 6, 7, and 8. Group II and group III mGluRs are linked to inhibition of the cyclic AMP cascade but differ in their agonist selectivities.

mGluR7 is the most highly conserved mGluR subtype across mammalian species (Flor et al., 1997). Makoff et al. (1996) observed by in situ hybridization that *GRM7* is expressed in many areas of the human brain, especially the cerebral cortex, hippocampus, and cerebellum. mGluR7 is localized directly in the presynaptic zone of the synaptic cleft of glutamatergic synapses (Kinoshita et al., 1998; Kosinski et al., 1999), where it is thought to act as an autoreceptor that is activated by glutamate released from the presynaptic terminal during action potentials. Furthermore, mGluR7 is thought to be a key player in shaping synaptic responses at glutamatergic synapses as well as in regulating key aspects of inhibitory GABAergic transmission (Kinoshita et al., 1998; Kosinski et al., 1999).

mGluR7 has putative roles in anxiety, emotional responses, and spatial working memory (Callaerts-Vegh et al., 2006; Cryan et al., 2003; Mitsukawa et al., 2006). Cognitive dysfunction is estimated to occur in 75%–85% of patients with schizophrenia, often precedes the onset of other symptoms (Reichenberg et al., 2006). Working memory is one of primary cognitive domains that are crucial for developing targets for the treatment of cognition in schizophrenia (Nuechterlein et al., 2004).

mGluR7 ablation causes dysregulation of the hypothalamic–pituitary–adrenal axis and increases hippocampal BDNF protein levels (Mitsukawa et al., 2005). Dysregulation of BDNF production or release is associated with neuropsychiatric disorders, such as schizophrenia (Harrison and Weinberger, 2005). Association between the val66met polymorphism of the *BDNF* gene and hippocampal volume in human, particularly in patients with schizophrenia (Szeszko et al., 2005).

In the present study, we examined the *GRM7* gene as a candidate for schizophrenia.

2. Materials and methods

2.1. Subjects

All subjects were of Japanese descent and were recruited from the main island of Japan. Patients with schizophrenia were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV). Control subjects were mentally healthy, unrelated subjects with no self-reported family history of mental illness within second-degree relatives. We sequenced the 5′ region, exons and exon–intron boundaries of the *GRM7* gene in 32 patients (mutation screening patients) with schizophrenia (mean age±SD, 46.5±10.9 years; 17 men and 15 women) to identify polymorphisms. We then genotyped 576 patients including the mutation screening patients (mean age±SD, 46.6±14.8 years; 322 men and 254 women) and 576 control subjects (mean age±SD, 46.8±12.9 years; 268 men and 308 women) (1st association population) with Predesigned TaqMan single nucleotide polymorphism (SNP) genotyping assays. When Predesigned TaqMan SNP genotyping assays were not available, we performed direct sequencing of DNAs from 96 patients including the mutation screening

patients (mean age±SD, 50.3±13.1 years; 55 men and 40 women) and 96 control subjects (mean age±SD, 53.6±9.1 years; 42 men and 54 women). For SNPs for which an association with schizophrenia was suggested in the first association population, we performed genotyping in an independent sample of 1817 patients (mean age± SD, 45.5±14.1 years; 962 men and 855 women) and 1728 control subjects (mean age±SD, 46.2±13.6 years; 958 men and 770 women) (confirmation population). The present study was approved by the ethics committees of the University of Tsukuba and participating institutes. All participants provided written informed consent.

2.2. DNA isolation and genotyping

DNAs were extracted from peripheral lymphocytes by standard phenol–chloroform extraction. The genomic structure of *GRM7* was determined from the University of California at Santa Cruz (UCSC) database (http://genome.ucsc.edu/cgi-bin/hgGateway) and the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). To screen for polymorphisms, we performed direct sequencing of genomic DNAs with a Big Dye Terminator Cycle Sequencing Kit and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). All exons, exon–intron junctions, and 1.6 kb of the 5′ flanking region of *GRM7* were amplified from the genomic DNAs of 24 randomly selected patients. The sequences of primers and conditions used for amplification for the mutation screening are available upon request. We genotyped polymorphisms with the TaqMan SNP Genotyping Assay (Applied Biosystems) and ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).

2.3. Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cultured cells with the SV Total RNA Isolation System (Promega, Madison, WI, USA). cDNA was synthesized from RNA with Revertra Ace (Toyobo, Tokyo, Japan) and oligo dT primer. Expression of *GRM7* was quantified by real-time quantitative RT-PCR with the TaqMan Gene Expression Assay and ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) per the manufacturer's instructions. Primers and probes were purchased from Applied Biosystems (Assays-on-Demand Assay ID: Hs00179051_m1). *GAPDH* was used as an internal control. Data were collected and analyzed with Sequence Detector Software (SDS) version 2.1 (Applied Biosystems) and the standard curve method.

2.4. Luciferase reporter assay

To assay promoter activity of the 5′-flanking region and exon 1 of the *GRM7* gene, 9 fragments of the 5′ region were cloned into the pGL3-Basic plasmid with and without a Simian virus 40 enhancer sequence (Promega, Madison, WI, USA). The day before transfection, NH-12 cells (Japanese Collection of Research Bioresources Gene Bank, http://genebank.nibio.go.jp/gbank/index_e.html) were plated at 1×10^5 cells/well in a 24-well plate and grown in DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Equitech-Bio, Kerrville, TX, USA). One microgram of each test plasmid was transiently cotransfected into the cells with 0.1 μg of pRL-TK plasmid (an internal standard reporter) (Promega) with Lipofectamine 2000 (Invitrogen) per manufacturer's protocol. After 48 h, the dual-luciferase assay was performed with a PicaGene Dual SeaPansy Kit (Toyo Ink, Tokyo, Japan) according to the manufacturer's instructions.

2.5. Statistics

Deviation from predicted Hardy–Weinberg frequencies was examined by chi-square test. Individual allelic associations were examined by Fisher's exact test. Genotypic associations were examined by Armitage's Trend Test for the reasons discussed by Devlin and Roeder (1999). A significant association was defined when the given p value for allelic or genotypic tests was less than 5% (uncorrected $p<0.05$) and the same association was confirmed in an independent population with $p<0.05$. Linkage disequilibrium (LD) between polymorphisms and haplotype block structures was evaluated with Haploview software version 3.11 (Barrett et al., 2005). Haplotype blocks were generated with the default algorithm taken from Gabriel et al. (2002). Haplotypic associations with disorders were examined with Haploview software, which performs association tests on the set of blocks selected by obtaining counts for case-control association tests by summing the fractional likelihoods of each individual for each haplotype by the EM algorithm.

3. Results

Twenty-five polymorphisms/mutations were detected in the exons, exon–intron junctions, and 5′-flanking region of the *GRM7* gene (Fig. 1). Genotyping was carried out for all detected polymorphisms except rare variants with allele frequencies < 0.05 and polymorphisms in LD with each other ($r^2=1$). Among these SNPs, 1724A/G (rs34373930), 1938C/T (rs7614915), IVS8+

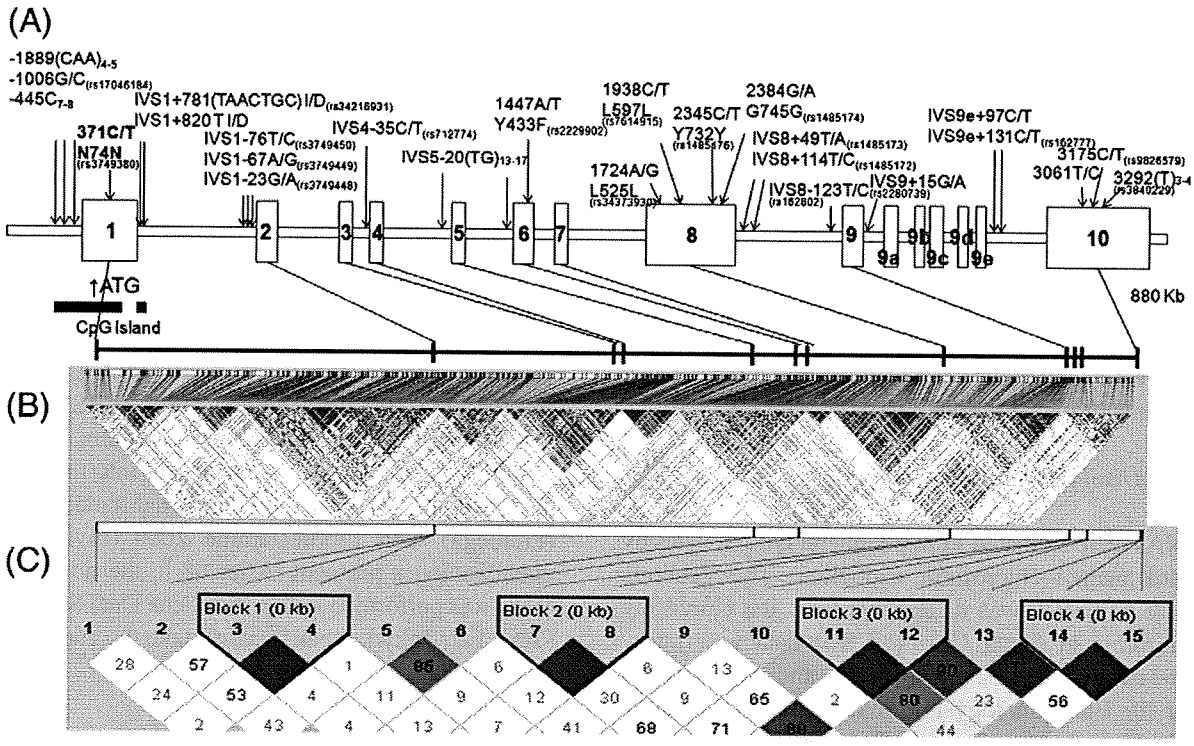

Fig. 1. Schematic representation of the *GRM7* gene and relevant mutations/polymorphisms (A), linkage disequilibrium plot of the Japanese population from HapMap database (B), and between SNPs genotyped in this study shown in Table 1 (C). Figures in rhombus are D' between SNPs and filled rhombus without figures indicates $D'=1$ in (C).

49T/A (T/A at the position of 49 base-pair starting from the G of the donor site of intron 8), and 2345C/T (rs1485175) were in complete LD ($r^2=1$), and IVS8+114T/C and 2384G/A (rs1485174) were also in complete LD ($r^2=1$) in 24 screening samples. Therefore, we genotyped the 2345C/T and 2384G/A polymorphisms as representative SNPs. The IVS9+97C/T, 2912T/C, and 3292(A)3-4 (rs3840229) SNPs were genotyped in 96 patients and 96 controls by direct sequencing, and the other 12 SNPs were genotyped in 576 patients and 575 control subjects by TaqMan SNP Genotyping Assay (Applied Biosystems). The genotypic distributions of these 15 SNPs did not deviate significantly from Hardy–Weinberg equilibrium ($p>0.05$). A synonymous polymorphism (371T/C) in exon 1 of *GRM7* showed a potential association for schizophrenia (allelic uncorrected $p=0.04$) (Table 1). We did not detect a significant association with schizophrenia of any of the other 14 SNPs, including the Tyr433Phe polymorphism (allelic uncorrected $p=0.33$; genotypic uncorrected $p=0.63$), which was previously reported to be associated (Bolonna et al., 2001). The haplotype blocks consisted of IVS1-76T/C, IVS1-67A/G, and IVS1-23G/A and of 2345C/T and 2384A/G. No significant haplotypic association was suggested for these 2 haplotype blocks ($p>0.05$). We confirmed the association of the 371T/C polymorphism with schizophrenia in an independent population of 1717 patients and 1807 control subjects and confirmed the association (allelic $p=0.03$, one-sided) (Table 2). The allelic p value of the association in the total population of 2293 patients with schizophrenia and 2382 control subjects was 0.009 (Table 2). According to the HapMap database (http://www.hapmap.org/index.html), the 371T/C polymorphism (rs3749380) was not in the LD block and was not in LD with other SNPs within 80 kb ($r^2<0.7$). Weak LD was observed with rs458351 ($D'=0.89$ and $r^2=0.69$), which is 30 kb upstream of rs3749380.

Expression of *GRM7* mRNA was assessed by RT-PCR in 7 different human cell lines (IMR-32, NH-12, TN-2, NB-1, SCCH-26, A172, and T98G). *GRM7* was expressed in NH-12 and SCCH-26 cells (data not shown). Because expression in NH-12 cells was higher than in SCCH-26 cells, we used NH-12 cells, a human cell line derived from neuroblastoma, for luciferase assays. Dual-luciferase assay revealed that the strongest promoter activity for *GRM7* was contained in the 1-kb fragment upstream of the ATG site of exon 1. However, a promoter construct containing the sequence from the ATG to the end of exon 1 showed significantly lower activity, indicating that the 371T/C polymorphism is in a regulatory region. When an SV40

Table 1
Genotypic and allelic distributions of polymorphisms in the *GRM7* gene

Polymorphism	Population	*n*	Genotype count (frequency)						*p*	Allele count (frequency)				*p*
371T/C (rs3749380)			CC		TC		TT			C		T		
	Patients	576	241	(0.42)	256	(0.44)	79	(0.14)		738	(0.64)	414	(0.36)	
	Controls	575	274	(0.48)	236	(0.41)	65	(0.11)	0.04	784	(0.68)	366	(0.32)	0.04
IVS1-76T/C (rs3749450)			CC		TC		TT			C		T		
	Patients	568	10	(0.02)	148	(0.26)	410	(0.72)		168	(0.15)	968	(0.85)	
	Controls	561	18	(0.03)	129	(0.23)	414	(0.74)	0.95	165	(0.15)	957	(0.85)	0.96
IVS1-67A/G (rs3749449)			GG		AG		AA			G		A		
	Patients	570	8	(0.01)	95	(0.17)	467	(0.82)		111	(0.10)	1029	(0.90)	
	Controls	567	7	(0.01)	89	(0.16)	471	(0.83)	0.60	103	(0.09)	1031	(0.91)	0.59
IVS1-23G/A (rs3749448)			AA		GA		GG			A		G		
	Patients	569	26	(0.05)	181	(0.32)	362	(0.64)		233	(0.20)	905	(0.80)	
	Controls	566	30	(0.05)	178	(0.31)	358	(0.63)	0.75	238	(0.21)	894	(0.79)	0.75
IVS4-35C/T (rs712774)			CC		CT		TT			C		T		
	Patients	571	121	(0.21)	260	(0.46)	190	(0.33)		502	(0.44)	640	(0.56)	
	Controls	559	117	(0.21)	268	(0.48)	174	(0.31)	0.66	502	(0.45)	616	(0.55)	0.65
1447T/A (rs2229902, Phe433Tyr)			AA		AT		TT			A		T		
	Patients	575	488	(0.85)	81	(0.14)	6	(0.01)		1057	(0.92)	93	(0.08)	
	Controls	569	484	(0.85)	82	(0.14)	3	(0.01)	0.76	1050	(0.92)	88	(0.08)	0.75
2345C/T (rs1485175)			CC		CT		TT			C		T		
	Patients	569	120	(0.21)	275	(0.48)	174	(0.31)		515	(0.45)	623	(0.55)	
	Controls	562	118	(0.21)	282	(0.50)	162	(0.29)	0.69	518	(0.46)	606	(0.54)	0.69
2384A/G (rs1485174)			AA		GA		GG			A		G		
	Patients	569	18	(0.03)	150	(0.26)	401	(0.70)		186	(0.16)	952	(0.84)	
	Controls	561	24	(0.04)	162	(0.29)	375	(0.67)	0.15	210	(0.19)	912	(0.81)	0.14
IVS8-123T/C (rs162802)			CC		TC		TT			C		T		
	Patients	569	3	(0.01)	91	(0.16)	475	(0.83)		97	(0.09)	1041	(0.91)	
	Controls	566	9	(0.02)	88	(0.16)	469	(0.83)	0.49	106	(0.09)	1026	(0.91)	0.48
IVS9+15G/A (rs2280739)			AA		GA		GG			A		G		
	Patients	570	4	(0.01)	84	(0.15)	482	(0.85)		92	(0.08)	1048	(0.92)	
	Controls	564	3	(0.01)	86	(0.15)	475	(0.84)	0.94	92	(0.08)	1036	(0.92)	0.94
IVS9e+97C/T			CC		CT		TT			C		T		
	Patients	96	75	(0.78)	20	(0.21)	1	(0.01)		170	(0.89)	22	(0.11)	
	Controls	95	78	(0.82)	16	(0.17)	1	(0.01)	0.53	172	(0.91)	18	(0.09)	0.62
IVS9e+131C/T (rs162777)			CC		CT		TT			C		T		
	Patients	570	22	(0.04)	186	(0.33)	362	(0.64)		230	(0.20)	910	(0.80)	
	Controls	566	23	(0.04)	178	(0.31)	365	(0.64)	0.82	224	(0.20)	908	(0.80)	0.82
2912T/C			TT		TC		CC			T		C		
	Patients	96	78	(0.81)	18	(0.19)	0	(0.00)		174	(0.91)	18	(0.09)	
	Controls	95	83	(0.87)	12	(0.13)	0	(0.00)	0.32	178	(0.94)	12	(0.06)	0.34
3175C/T (rs9826579)			CC		CT		TT			C		T		
	Patients	574	25	(0.04)	203	(0.35)	346	(0.60)		253	(0.22)	895	(0.78)	
	Controls	567	23	(0.04)	189	(0.33)	355	(0.63)	0.44	235	(0.21)	899	(0.79)	0.44
3292(A)3–4 (rs3840229)			33		34		44			3		4		
	Patients	96	80	(0.83)	16	(0.17)	0	(0.00)		176	(0.92)	16	(0.08)	
	Controls	95	78	(0.82)	17	(0.18)	0	(0.00)	0.85	173	(0.91)	17	(0.09)	0.86

enhancer was added downstream of the luciferase gene, the 371C allele showed significantly higher promoter activity than the 371T allele (Fig. 2).

4. Discussion

In the present study, we examined associations between polymorphisms in the *GRM7* gene and schizophrenia. Weak association was found for a synonymous SNP (371T/C) in exon 1 in the first association population, and this association was replicated in the confirmation population. The T allele, which is associated with schizophrenia, has lower promoter activity than the C allele. On the basis of this finding, we hypothesized that lower expression of mGluR7 may increase risk of developing schizophrenia, though studies of the expression of GRM7 in brains of patients with schizophrenia have not been reported.

Table 2
Association of the 371T/C polymorphism (rs3749380) in the *GRM7* gene with schizophrenia

Polymorphism population	*n*	Genotype count (frequency)						*p*	Allele count (frequency)				*p*
		CC		CT		TT			C		T		
Screening population													
Patients	576	241	(0.42)	256	(0.44)	79	(0.14)		738	(0.64)	414	(0.36)	
Controls	575	274	(0.48)	236	(0.41)	65	(0.11)	0.04	784	(0.68)	366	(0.32)	0.04
Confirmatory population													
Patients	1717	715	(0.42)	771	(0.45)	231	(0.13)		2201	(0.64)	1233	(0.36)	
Controls	1807	799	(0.44)	794	(0.44)	214	(0.12)	0.07	2392	(0.66)	1222	(0.34)	0.03
Total													
Patients	2293	956	(0.42)	1027	(0.45)	310	(0.14)		2939	(0.64)	1647	(0.36)	
Controls	2382	1073	(0.45)	1030	(0.43)	279	(0.12)	0.01	3176	(0.67)	1588	(0.33)	0.009[a]

Genotypic *p* was calculated by Armitage's Trend Test and allelic *p* was calculated by Fisher's exact test.
[a] Odd ratio=1.12, 95% confidence interval=1.03–1.22.

mGluR7 was the first group III mGluR found to be enriched presynaptically at active zones of hippocampal pyramidal cells (Shigemoto et al., 1996). The low affinity of mGluR7 for glutamate suggests that mGluR7 might act as a "low-pass filter" that suppresses release of glutamate only when action potentials arriving at a high frequency produce massive glutamate release. The interaction with PICK1 (protein interacting with PRKCA 1) is crucial for

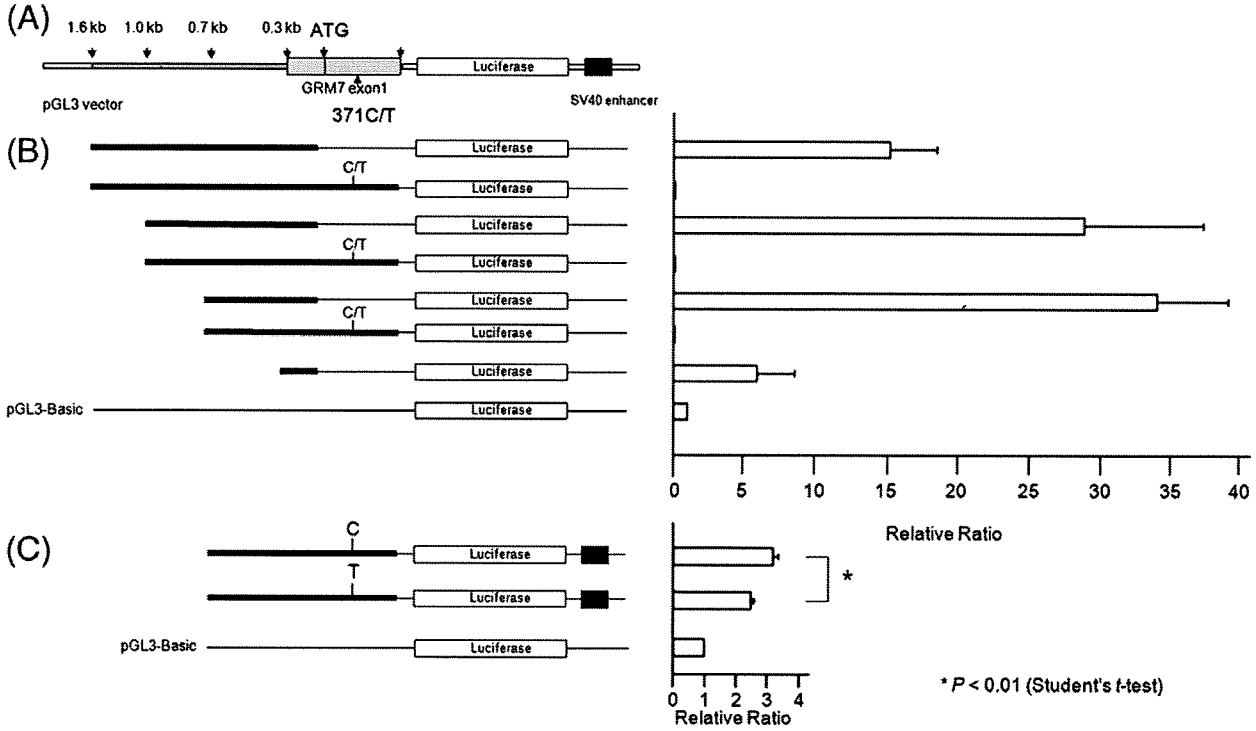

Fig. 2. Luciferase assays. (A) Schematic representation of the *GRM7* gene and reporter gene constructs. (B) Transcriptional activity of various constructs of the 5′ region and exon 1 of the human *GRM7* gene in NH-12 cells. The region indicated by fine lines were not included in the constructs. Cotransfections were performed with pRL-TK (*Renilla* luciferase) to normalize transfection efficiency. Luciferase activity was assayed 48 h after transfection. Relative ratio of expression is shown as relative to that of pGL3-Basic, which was set at 1. The mean±SEM was calculated from triplicate assays. (C) Effect of the 371C/T polymorphism on *GRM7* promoter activity. This assay was performed with pGL3-enhancer vector, because the constructs that contain *GRM7* exon 1 show extremely low luciferase activity.

the clustering of mGluR7 at presynaptic release sites (Boudin et al., 2000). *PICK1* is reported to be associated with schizophrenia (Dev and Henley, 2006; Fujii et al., 2006).

In the present study, we found an association between a functional SNP, 371T/C, in the gene encoding mGluR7 and schizophrenia. Recently, a genome-wide association study of 2000 individuals with bipolar disorder and 3000 control subjects found a strong association of SNP marker rs1485171 ($p=9.7\times10^{-5}$) in *GRM7* with bipolar disorder (Consortium, 2007). Therefore, genetic variations in the *GRM7* gene may be involved in both schizophrenia and bipolar disorder.

An association between Tyr433Phe polymorphism of *GRM7* and schizophrenia was reported (Bolonna et al., 2001). However, we failed to detect the association (allele, $p=0.33$; genotype, $p=0.63$). Recently, a copy number variation of the *GRM7* locus has been reported in patients with schizophrenia (Wilson et al., 2006). In the present study, we did not observe significant deviation from Hardy–Weinberg expectancy of the genotypic distributions of SNPs, indicating that copy number variations at the SNP examined in the present study are not common and are unlikely.

In the present study, the association of 371T/C with schizophrenia was observed in two independent case-control populations. However, its weak association (OR=1.12) requires replication studies in large sample populations of more than 2000 cases and 2000 control subjects with a power greater than 0.8. We believe that *GRM7* is an interesting target worth such studies for schizophrenia and other psychiatric disorders.

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Contributors

Author Ohtsuki ran the experiment and wrote the manuscript. Author Horiuchi and Koga prepared the sample analyzed. Author Arai, Niizato, Itokawa, Inada, Iwata, Iritani, Ozaki, Kunugi, Ujike, Watanabe, Someya, managed the sample collection. Author Ishiguro and Arinami designed this study and undertook the statistical analysis and supervised this study.

Conflict of interest

No author has conflict of interest.

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References

Barrett, J.C., Fry, B., Maller, J., Daly, M.J., 2005. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21 (2), 263–265.

Bolonna, A.A., Kerwin, R.W., Munro, J., Arranz, M.J., Makoff, A.J., 2001. Polymorphisms in the genes for mGluR types 7 and 8: association studies with schizophrenia. Schizophr. Res. 47 (1), 99–103.

Boudin, H., Doan, A., Xia, J., Shigemoto, R., Huganir, R.L., Worley, P., Craig, A.M., 2000. Presynaptic clustering of mGluR7a requires the PICK1 PDZ domain binding site. Neuron 28 (2), 485–497.

Callaerts-Vegh, Z., Beckers, T., Ball, S.M., Baeyens, F., Callaerts, P.F., Cryan, J.F., Molnar, E., D'Hooge, R., 2006. Concomitant deficits in working memory and fear extinction are functionally dissociated from reduced anxiety in metabotropic glutamate receptor 7-deficient mice. J. Neurosci. 26 (24), 6573–6582.

Carlsson, A., Hansson, L.O., Waters, N., Carlsson, M.L., 1997. Neurotransmitter aberrations in schizophrenia: new perspectives and therapeutic implications. Life Sci. 61 (2), 75–94.

Consortium WTC C, 2007. Genome-wide association study of 14,000 cases of seven common diseases and 3000 shared controls. Nature 447 (7145), 661–678.

Cryan, J.F., Kelly, P.H., Neijt, H.C., Sansig, G., Flor, P.J., van Der Putten, H., 2003. Antidepressant and anxiolytic-like effects in mice lacking the group III metabotropic glutamate receptor mGluR7. Eur. J. Neurosci. 17 (11), 2409–2417.

Dev, K.K., Henley, J.M., 2006. The schizophrenic faces of PICK1. Trends Pharmacol. Sci. 27 (11), 574–579.

Devlin, B., Roeder, K., 1999. Genomic control for association studies. Biometrics 55 (4), 997–1004.

Flor, P.J., Van Der Putten, H., Ruegg, D., Lukic, S., Leonhardt, T., Bence, M., Sansig, G., Knopfel, T., Kuhn, R., 1997. A novel splice variant of a metabotropic glutamate receptor, human mGluR 7b. Neuropharmacology 36 (2), 153–159.

Fujii, K., Maeda, K., Hikida, T., Mustafa, A.K., Balkissoon, R., Xia, J., Yamada, T., Ozeki, Y., Kawahara, R., Okawa, M., Huganir, R.L., Ujike, H., Snyder, S.H., Sawa, A., 2006. Serine racemase binds to PICK1: potential relevance to schizophrenia. Mol. Psychiatry 11 (2), 150–157.

Gabriel, S.B., Schaffner, S.F., Nguyen, H., Moore, J.M., Roy, J., Blumenstiel, B., Higgins, J., DeFelice, M., Lochner, A., Faggart, M., Liu-Cordero, S.N., Rotimi, C., Adeyemo, A., Cooper, R., Ward, R., Lander, E.S., Daly, M.J., Altshuler, D., 2002. The structure of haplotype blocks in the human genome. Science 296 (5576), 2225–2229.

Harrison, P.J., Weinberger, D.R., 2005. Schizophrenia genes, gene expression, and neuropathology: on the matter of their convergence. Mol. Psychiatry 10 (1), 40–68.

Kinoshita, A., Shigemoto, R., Ohishi, H., van der Putten, H., Mizuno, N., 1998. Immunohistochemical localization of metabotropic glutamate receptors, mGluR7a and mGluR7b, in the central nervous system of the adult rat and mouse: a light and electron microscopic study. J. Comp. Neurol. 393 (3), 332–352.

Kosinski, C.M., Risso Bradley, S., Conn, P.J., Levey, A.I., Landwehrmeyer, G.B., Penney Jr., J.B., Young, A.B., Standaert, D.G., 1999. Localization of metabotropic glutamate receptor 7 mRNA and mGluR7a protein in the rat basal ganglia. J. Comp. Neurol. 415 (2), 266–284.

Makoff, A., Pilling, C., Harrington, K., Emson, P., 1996. Human metabotropic glutamate receptor type 7: molecular cloning and mRNA distribution in the CNS. Brain Res. 40 (1), 165–170.

Mitsukawa, K., Yamamoto, R., Ofner, S., Nozulak, J., Pescott, O., Lukic, S., Stoehr, N., Mombereau, C., Kuhn, R., McAllister, K.H.,

van der Putten, H., Cryan, J.F., Flor, P.J., 2005. A selective metabotropic glutamate receptor 7 agonist: activation of receptor signaling via an allosteric site modulates stress parameters in vivo. Proc. Natl. Acad. Sci. U. S. A. 102 (51), 18712–18717.

Mitsukawa, K., Mombereau, C., Lotscher, E., Uzunov, D.P., van der Putten, H., Flor, P.J., Cryan, J.F., 2006. Metabotropic glutamate receptor subtype 7 ablation causes dysregulation of the HPA axis and increases hippocampal BDNF protein levels: implications for stress-related psychiatric disorders. Neuropsychopharmacology 31 (6), 1112–1122.

Nakanishi, S., 1994. Metabotropic glutamate receptors: synaptic transmission, modulation, and plasticity. Neuron 13 (5), 1031–1037.

Nuechterlein, K.H., Barch, D.M., Gold, J.M., Goldberg, T.E., Green, M.F., Heaton, R.K., 2004. Identification of separable cognitive factors in schizophrenia. Schizophr. Res. 72 (1), 29–39.

Pin, J.P., Duvoisin, R., 1995. The metabotropic glutamate receptors: structure and functions. Neuropharmacology 34 (1), 1–26.

Reichenberg, A., Weiser, M., Caspi, A., Knobler, H.Y., Lubin, G., Harvey, P.D., Rabinowitz, J., Davidson, M., 2006. Premorbid intellectual functioning and risk of schizophrenia and spectrum disorders. J. Clin. Exp. Neuropsychol. 28 (2), 193–207.

Shigemoto, R., Kulik, A., Roberts, J.D., Ohishi, H., Nusser, Z., Kaneko, T., Somogyi, P., 1996. Target-cell-specific concentration of a metabotropic glutamate receptor in the presynaptic active zone. Nature 381 (6582), 523–525.

Szeszko, P.R., Lipsky, R., Mentschel, C., Robinson, D., Gunduz-Bruce, H., Sevy, S., Ashtari, M., Napolitano, B., Bilder, R.M., Kane, J.M., Goldman, D., Malhotra, A.K., 2005. Brain-derived neurotrophic factor val66met polymorphism and volume of the hippocampal formation. Mol. Psychiatry 10 (7), 631–636.

Wilson, G.M., Flibotte, S., Chopra, V., Melnyk, B.L., Honer, W.G., Holt, R.A., 2006. DNA copy-number analysis in bipolar disorder and schizophrenia reveals aberrations in genes involved in glutamate signaling. Hum. Mol. Genet. 15 (5), 743–749.

Impaired Secretion of Brain-Derived Neurotrophic Factor and Neuropsychiatric Diseases

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Abstract: Recent studies have elucidated mechanisms of brain-derived neurotrophic factor (BDNF) secretion, and impaired secretion of BDNF may be involved in the pathogenesis of several neuropsychiatric diseases. The huntingtin gene, for example, has been shown to regulate vesicular transport of BDNF, which may play a role in the neurodegeneration present in Huntington's disease. In animal studies, mice lacking calcium-dependent activator protein for secretion 2 (CADPS2), which is involved in the activity-dependent release of BDNF, showed several phenotypes including autistic behavior. A single nucleotide polymorphism that results in an amino-acid change (Val66Met) in the BDNF gene has been shown to cause a decline in the function of BDNF vesicular sorting and has been reported to be associated with behavioral and intermediate phenotypes (e.g., episodic memory) in humans. In this review, we introduce recent progress in the molecular mechanisms of BDNF secretion and discuss its possible role in the pathophysiology and treatment of neuropsychiatric diseases.

INTRODUCTION

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, has been implicated in a broad range of processes that are important for neuronal survival and synaptic plasticity in the central nervous system (CNS) [1-3]. Early in the 1950s, nerve growth factor (NGF) was discovered by Levi-Montalcini and Hamburger and Cohen [4,5] as a soluble factor that induced fiber outgrowth of chicken sympathetic neurons. Subsequently, Barde *et al.* [6] isolated BDNF, which was later found to be homologous to NGF [7], from pig brain as a neuronal survival factor. These discoveries motivated homology-based searches for additional family members of which there are currently a total of four in mammals – NGF, BDNF, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). Additional members are conserved in fish – neurotrophin-6 (NT-6) and neurotrophin-7 (NT-7) [8,9]. All neurotrophins are secreted from neuronal (partially glial) cells and bind to their receptors in an autocrine/paracrine manner. In the last two decades, a bulk of studies have suggested that neurotrophins, especially BDNF, are involved in the pathophysiology of neuropsychiatric diseases through their role in the regulation of synaptic efficacy (synaptic plasticity) and synaptogenesis in the CNS. In this review, we focus on recent findings of secretion mechanisms of BDNF and their relationship with neuropsychiatric diseases.

I. BIOLOGICAL FUNCTIONS OF BDNF

i. Survival and Synaptic Plasticity

Neurotrophins exert their biological effects through the binding of secreted homodimeric neurotrophins to two types of transmembrane receptor proteins: the tyrosine kinase Trk (tropomyosin-related kinase) receptors and the low affinity common neurotrophin receptor (p75NTR). Neurotrophins are expressed in a precursor form (pro-neurotrophins) and are proteolytically processed to a mature form. Mature neurotrophins preferentially bind to their specific Trk receptor: NGF to TrkA, BDNF and NT-4/5 to TrkB and NT-3 to TrkC. Pro-neurotrophins, however, bind to p75NTR with higher affinity than mature neurotrophins, although they have a lower affinity for Trk receptors [10]. Binding of neurotrophins to Trk receptors immediately generates receptor dimerization and autophosphorylation of tyrosine residues in the intracellular kinase domain. Trk receptor phosphorylation activates intracellular signaling regulated by mitogen-activated protein kinase (MAPK), phosphoinositide-3 (PI3)-kinase/Akt and phospholipase C-γ (PLC-γ) pathways as well as several small G proteins, including Ras, Rap-1, and the CDC-42-Rac-Rho family [11-13]. These intracellular signaling cascades modulate expression of genes and are responsible for most of the long-term effects of neurotrophins related to neuronal growth, survival, and differentiation [14]. On the other hand, binding of pro-neurotrophins to p75NTR leads to antagonistic effects to Trk receptor signaling. Several of these p75NTR-dependent signaling are pro-apoptotic and can be suppressed by Trk receptor-initiated signaling [15,16]. The first evidence of a significant relationship between neurotrophins and synaptic plasticity was obtained by Lohof *et al.* [17]; exogenous BDNF and NT-3 increased synaptic efficacy at the *Xenopus* neuromuscular junction. Subsequently, these neurotrophins were shown to facilitate glutamatergic synaptic transmission, even in the hippocampus of the mammalian CNS [18-20]. There is now substantial evidence implicating BDNF in activity-dependent long-term synaptic plasticity [21,22]. The neurotrophin-binding Trk receptor activates many kinds of signaling pathways that promote neuronal survival and synaptic efficiency, although it is still unclear how the complex signaling pathways are

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systematically integrated to generate many biological functions.

ii. Activity-Dependent Expression of Neurotrophins

Among neurotrophins, BDNF shows the most ubiquitous expression in the developing and adult mammalian brain. BDNF expression levels are increased dramatically during the first few weeks of postnatal development. Expression of neurotrophins in neurons is linked to neuronal activity. BDNF and NGF mRNA levels are rapidly increased by seizure activity in the hippocampus and the cerebral cortex [23-25]. In contrast, blockade of visual input causes rapid down-regulation of BDNF mRNA in the rat visual cortex of dark-reared animals [26]. A similar phenomenon has been found in cultured neurons. The introduction of glutamate with high concentration potassium-induced depolarization increase levels of BDNF and NGF mRNA, while blockade of neuronal activity with γ-aminobutyric acid (GABA) decreases such levels [27,28].

iii. Processes of BDNF Secretion

BDNF is synthesized as a 32 kDa precursor protein (proBDNF) and proteolytically cleaved to generate the mature BDNF (13 kDa). The synthesis of the pro-BDNF occurs at the rough endoplasmic reticulum (ER). Following this, pro-BDNF is transported to the Golgi apparatus and concentrated in membrane stacks of the *trans*-Golgi network (TGN). Finally, BDNF-containing vesicles bud off the TGN to eventually transport to the releasing sites. Recent studies clarified some of the details of BDNF vesicular sorting. Specifically, the pro-region of BDNF has been implicated as a regulator of BDNF sorting to secretory vesicles [29]. Moreover, fusing the pro-region of BDNF to NT-4, which is rarely sorted into secretory vesicles, allowed NT-4 to sort more efficiently into specific vesicles [30]. These data support the importance of the BDNF pro-region as a potential target to help guide secretory granules. Furthermore, binding of BDNF to the lipid-raft-associated sorting receptor carboxypeptidase E (CPE) in the TGN is also important for sorting into secretory vesicles of the regulated pathway [31]. Sortilin, a trans-membrane protein, has also been implicated in the sorting of BDNF to secretory glanules. Sortilin is expressed in secretory granules and interacts specifically with the pro-region of BDNF. Interestingly, the truncated form of sortilin results in missorting of BDNF to the secretory vesicles [32].

It is still controversial as to where and how pro-neurotrophins are processed into mature neurotrophins in the CNS. Originally, it had been thought that pro-neurotrophins are prototypically cleaved by furin and pro-protein convertases (PCs) in the TGN or in secretory granules before secretion [33]. However, recent studies have indicated that a considerable amount of BDNF is secreted in the pro-form from neurons. Released pro-BDNF is subsequently processed to mature BDNF extracellularly by proteases such as plasmin or matrix metalloproteinases [34,35]. More recently, however, it was shown that pro-BDNF is rapidly converted into mature BDNF intracellularly and almost all BDNF was secreted as the mature form from hippocampal neurons [36].

iv. Constitutive and Regulated Secretion

Secretion of neurotrophins is classified into "constitutive" and "regulated" pathways, depending on whether the secretion occurs spontaneously or in response to neuronal activity, respectively. In hippocampal neurons, BDNF appears to be sorted primarily into the regulated pathway [37-39]. In the regulated pathway, BDNF-containing vesicles are transported into either presynaptic axon terminals or post-synaptic dendrites along microtubules for activity-dependent secretion [40-43]. Recently, Lessmann and colleagues conducted an elegant study that provided the long-awaited understanding of BDNF secretion. The activity-dependent postsynaptic secretion of neurotrophins critically depends on Ca^{2+} influx *via* ionotropic glutamate receptors or voltage-gated Ca^{2+} channels, Ca^{2+} release from internal stores, activation of Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), and intact protein kinase A (PKA) signaling. Trk signaling and activation of Na^{+} channels, on the other hand, are not required for BDNF secretion [44-46]. Furthermore, recent reports suggest that the Golgi apparatus exists in dendrites as well as the cell soma, and have gone so far as to identify a local BDNF secretory pathway in neuronal dendrites [47,48]. Future works may reveal more details concerning the secretory systems of neurotrophins at the subcellular level and that may be more complex and dynamic than we can presently imagine.

II. IMPAIRED SECRETION OF BDNF AND NEUROPSYCHIATRIC DISEASES

i. Huntington's Disease

Huntington's disease (HD) is a fatal, dominantly inherited, neurodegenerative disease that usually presents during midlife. It is characterized by relatively selective degeneration of striatal neurons which lead to psychiatric, cognitive and motor dysfunction. Polyglutamine expansion (polyQ) in the protein huntingtin (htt) is thought to be the principal mechanism for the neuronal toxicity in HD. Recently, evidence has indicated the possible link between HD and BDNF. Wild type htt plays a role as a transcription factor and facilitates expression of BDNF [49]. Furthermore, htt has been implicated in BDNF-containing vesicle transport. Mutant (PolyQ)-htt perturbs post-Golgi trafficking of BDNF in the regulated secretory pathway, though it does not influence the constitutive pathway [50,51]. Conversely, the exogenously transfected BDNF gene generated increased BDNF levels and TrkB signaling in the striatum, which resulted in improved symptoms in HD model mice [52]. These findings suggest that mutation of htt reduces levels of BDNF in the striatum by inhibiting gene expression and perturbing anterograde transport of BDNF-containing vesicles from cortex to striatum. Therefore, the development of therapies focused on the reduction of BDNF release should be important for future studies.

ii. Rett Syndrome

Rett syndrome (RTT) is an X-linked disorder characterized by arrested neurological development and subsequent cognitive decline. Methylation of DNA in vertebrates occurs preferentially on cytosine residues of dinucleotides in which the cytosine is followed by a guanine residue (CpGs). Meth-

ylated CpGs bind a variety of proteins. One of these proteins, methyl-CpG binding protein 2 (MeCP2), has been implicated in the long-term silencing of gene expression. Inactivating mutations in MeCP2 is caused in the majority of cases with Rett syndrome. Chen *et al.* showed that MeCP2 selectively binds to the BDNF promoter III and represses expression of BDNF [53]. Membrane depolarization triggers the calcium-dependent phosphorylation and release of MeCP2 from the BDNF promoter III, thereby facilitating transcription [54]. A conditional BDNF transgene increased BDNF expression in the MeCP2 mutant brain, which resulted in rescue of locomotor defects, recovery of electrophysiological deficits, and extension of lifespan in MeCP2 mutant animals [55]. Although MeCP2 null mice exhibited a slightly decreased content of BDNF in some brain areas, mutant neurons demonstrated equivalent secretion levels of BDNF compared to wild-type in response to high-frequency electrical stimulation [56]. Furthermore, BDNF expression in MeCP2 null neurons was significantly improved by chronic ampakine treatment, which was administered to facilitate AMPA receptor activation [57]. These results suggest that the expression of BDNF is still plastic in the MeCP2 null condition and manipulating the BDNF level or the BDNF signaling pathways may provide therapeutic opportunities for RTT patients.

iii. Autism

Autism is a severe neurodevelopmental disorder with a childhood onset, characterized by profound disturbances in socialization, language skills, communicative, and behavioral functions. BDNF is expressed abnormally in individuals with autism and, as a result, may be involved in the pathogenesis of autism [58,59]. Elevated levels of BDNF and NT4/5 measured by archived neonatal blood samples of autistic patients were reported [60]. Elevation of BDNF was also reported in a study of 18 Japanese children with autism compared with controls [61]. These findings suggest that excess BDNF during childhood may be involved in the neurobiological abnormalities observed in autism. The specific molecular mechanisms involving BDNF and autism remain unknown, though one report suggests that genetic changes in autistic individuals may account for altered neurotrophin levels [62]. Ca^{2+}-dependent activator protein for secretion 2 (CAPS2/CADPS2) is a secretory granule-associated protein that is abundant at the parallel fiber terminals of granule cells in the mouse cerebellum and is involved in the release of BDNF and NT-3. The human CAPS2/CADPS2 gene is located on chromosome 7q31.32 within a critical autism susceptibility locus 1 (AUTS1). CAPS2 knock-out mice demonstrate autistic-like behavioral phenotypes and deficient release of BDNF and NT-3. Moreover, phosphorylation of Trk receptors is decreased in the cerebellum, which may play a role in the pronounced impairment of cerebellar development and function, including neuronal survival, differentiation and migration of postmitotic granule cells, that these mice exhibit [63]. Although there have been few reports suggesting the relation between autism and BDNF secretion, further investigation may result in novel insights.

iv. Epilepsy

Epilepsy is a neurological disorder characterized by recurrent and unpredictable seizures. Various studies have shown that BDNF increases neuronal excitability and is up-regulated in areas implicated in epileptogenesis. Seizure activity increases expression of BDNF mRNA and protein, and recent studies have shown that interfering with BDNF signal transduction inhibits the development of the epileptic state *in vivo* [64]. Half of all drug-resistant individuals experience seizure control with dietary manipulation, such as isocaloric substitution of carbohydrates with fats and protein referred to as the 'ketogenic diet'. Daley *et al.* reported that an inhibitor of glycolysis is shown to have antiepileptic effects in the rat kindling model, which may be related to NADH-dependent regulation of BDNF expression [65]. This result may explain how the 'ketogenic diet' treatment works. Although it is unclear whether the up-regulation of BDNF is the cause or the consequence of epilepsy, the reduction of BDNF expression or BDNF signaling can be a useful tool for the treatment of epilepsy.

v. Psychiatric Disorders

Mood and anxiety disorders are the most common psychiatric diseases. BDNF has been implicated in these disorders, because decreased levels of BDNF in the hippocampus are correlated with stress-induced depressive behaviors [66]. Other studies also showed decreased plasma levels of BDNF in patients with major depression [67]. Many classes of antidepressants, including selective serotonin reuptake inhibitors, significantly increase BDNF mRNA expression in the hippocampus and prefrontal cortex [68,69]. The time course of such increase is consistent with the slow onset of therapeutic effects of antidepressants. More recently, striking evidence for the involvement of TrkB-dependent neurogenesis in the antidepressant effect has been reported. Mice lacking TrkB in the hippocampal neuron progenitor cells had impaired neurogenesis and proliferation induced by antidepressant treatment. These mice also demonstrated increased anxiety-like behavior and decreased sensitivity to antidepressants [70,71]. Taken together, BDNF may play a key role in the brains of recovering patients during antidepressant treatment [72,73].

Many reports have isolated the possible association between BDNF levels and schizophrenia in several brain regions [74]. However, results from these studies are contradictory in that some demonstrate decreased BDNF levels in the postmortem brain or serum, while others report that the BDNF level in patients was not significantly different from that in normal controls [75]. Moreover, samples used in each experiment differ in age, species (rodents, primates, human) and regions (i.e., hippocampus, frontal cortex, CSF and blood) [76]. Although there have been many studies examining the possible role of BDNF in schizophrenia, integrated knowledge concerning this has not been produced. Despite this, the neurobiological vulnerability paradigm remains an attractive concept, supporting that increased susceptibility may be a consequence of reduced expression of BDNF (neurotrophins) at a certain point of life [77].

In the region encoding BDNF's pro-region, a SNP was identified at amino acid 66 (Val66Met). Egan and colleagues reported that the met allele was associated with decreased episodic memory and abnormal hippocampal activation as assayed with fMRI in human subjects [29]. Furthermore, neurons transfected with met-BDNF-GFP showed lower

depolarization-induced secretion, while constitutive secretion was unchanged. Met-BDNF-GFP failed to localize to secretory granules or synapses [29]. Following this, a number of association studies of this polymorphism with psychiatric disorders have been done. Unexpectedly, the Met66 allele, which reduces BDNF release, has been suggested to be protective against developing bipolar disorder [78], although this association was not confirmed by large-scale studies [79,80]. The Met66 allele has also been implicated in other disorders like anorexia [81]. Future research is required to assess how the Val66Met is associated with particular psychiatric disorders.

CONCLUSIONS

The biological mechanisms of neurotrophins are critically important for neuronal functions that affect brain functions and behavior. Growing evidence has implicated BDNF in the pathophysiology of many neuropsychiatric diseases. Genetic variations leading to deranged expression or secretion due to altered transcription, vesicular sorting, vesicular trafficking and secretion of BDNF seem to play an important role in several neuropsychiatric diseases and related behavioral phenotypes. In order to develop treatment strategies for these diseases through targeting neurotrophins and their receptors, however, clarification of more detailed mechanisms is needed. Studies that reveal not only an increase/decrease in expression of neurotrophins, but also accurate spatiotemporal secretion profiles of neurotrophins are necessary.

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REFERENCES

[1] Lu B. Acute and long-term synaptic modulation by neurotrophins. Prog Brain Res 2004; 146: 137-50.

[2] Poo MM. Neurotrophins as synaptic modulators. Nat Rev Neurosci 2001; 2(1): 24-32.

[3] Barde YA. Trophic factors and neuronal survival. Neuron 1989; 2(6): 1525-34.

[4] Levi-Montalcini R, Hamburger V. A diffusible agent of mouse sarcoma, producing hyperplasia of sympathetic-ganglia and hyperneurotization of viscera in the chick embryo. J Exp Zool 1953; 123: 233-87.

[5] Cohen S, Levi-Montalcini R. A nerve growth-stimulating factor isolated from snake venom. Proc Natl Acad Sci USA 1956; 42: 571.

[6] Barde YA, Edgar D, Thoenen H. Purification of a new neurotrophic factor from mammalian brain. EMBO J 1982; 1: 549-53.

[7] Leibrock J, Lottspeich F, Hohn A, *et al.* Molecular cloning and expression of brain-derived neurotrophic factor. Nature 1989; 341: 149-52.

[8] Gotz R, Koster R, Winkler C, *et al.* Neurotrophin-6 is a new member of the nerve growth factor family. Nature 1994; 372: 266-69.

[9] Lai KO, Fu WY, Ip FCF, Ip NY. Cloning and expression of a novel neurotrophin, NT-7, from carp. Mol Cell Neurosci 1998; 11: 64-76.

[10] Lee R, Kermani P, Teng KK, Hempstead BL. Regulation of cell survival by secreted proneurotrophins. Science 2001; 294: 1945-48.

[11] Mazzoni IE, Saïd FA, Aloyz R, Miller FD, Kaplan D. Ras regulates sympathetic neuron survival by suppressing the p53-mediated cell death pathway. J Neurosci 1999; 19: 9716-27.

[12] Grewal SS, York RD, Stork PJ. Extracellular-signal-regulated kinase signalling in neurons. Curr Opin Neurobiol 1999; 9: 544-53.

[13] Vaillant AR, Mazzoni I, Tudan C, Boudreau M, Kaplan DR, Miller FD. Depolarization and neurotrophins converge on the phosphatidylinositol 3-kinase-Akt pathway to synergistically regulate neuronal survival. J Cell Biol 1999; 146: 955-66.

[14] Blum R, Konnerth A. Neurotrophin-mediated rapid signaling in the central nervous system: mechanisms and functions. Physiology 2005; 20: 70-8.

[15] Majdan M, Miller, FD. Neuronal life and death decisions functional antagonism between the Trk and p75 neurotrophin receptors. Int J Dev Neurosci 1999; 17: 153-61.

[16] Yoon SO, Casaccia-Bonnefil P, Carter B, Chao MV. Competitive signaling between TrkA and p75 nerve growth factor receptors determines cell survival. J Neurosci 1998; 18: 3273-81.

[17] Lohof AM, Ip NY, Poo MM. Potentiation of developing neuromuscular synapses by the neurotrophins NT-3 and BDNF. Nature 1993; 363: 350-53.

[18] Lessmann V, Gottmann K, Heumann R. BDNF and NT-4/5 enhance glutamatergic synaptic transmission in cultured hippocampal neurones. NeuroReport 1994; 6: 21-25.

[19] Kang H, Schuman EM. A requirement for local protein synthesis in neurotrophin-induced hippocampal synaptic plasticity. Science 1996; 273: 1402-06.

[20] Levine ES, Dreyfus CF, Black IB, Plummer MR. Brain-derived neurotrophic factor rapidly enhances synaptic transmission in hippocampal neurons *via* postsynaptic tyrosine kinase receptors. Proc Natl Acad Sci USA 1995; 92: 8074-77.

[21] Korte M, Carroll P, Wol E, Brem G, Thoenen H, Bonhoeffer T. Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. Proc Natl Acad Sci USA 1995; 92: 8856-60.

[22] Patterson SL, Abel T, Deuel TA, Martin KC, Rose JC, Kandel ER. Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. Neuron 1996; 16: 1137-45.

[23] Gall C M, Isackson PJ. Limbic seizures increase neuronal production of messenger RNA for nerve growth factor. Science 1989; 245: 758-61.

[24] Zafra F, Hengerer B, Leibrock J, Thoenen H, Lindholm D. Activity dependent regulation of BDNF and NGF mRNAs in the rat hippocampus is mediated by non-NMDA glutamate receptors. EMBO J 1990; 9: 3545-50.

[25] Ernfors P, Bengzon J, Kokaia Z, Persson H, Lindvall O. Increased levels of messenger RNAs for neurotrophic factors in the brain during kindling epileptogenesis. Neuron 1991; 7: 165-76.

[26] Castraen E, Zafra F, Thoenen H, Lindholm D. Light regulates expression of brain-derived neurotrophic factor mRNA in rat visual cortex. Proc Natl Acad Sci USA 1992; 89: 9444-48.

[27] Lindholm D, Castren E, Berzaghi M, Blochl A, Thoenen H. Activity-dependent and hormonal regulation of neurotrophin mRNA levels in the brain - implications for neuronal plasticity. J Neurobiol 1994; 25: 1362-72.

[28] Berninger B, Marty S, Zafra F, Berzaghi MP, Thoenen H. GABAergic stimulation switches from enhancing to repressing BDNF expression in rat hippocampal neurons during maturation *in vitro*. Development 1995; 121: 2327-35.

[29] Egan MF, Kojima M, Callicott JH, *et al.* The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell 2003; 112(2): 257-69.

[30] Brigadski T, Hartmann M, Lessmann V. Differential vesicular targeting and time course of synaptic secretion of the mammalian neurotrophins. J Neurosci 2005; 17; 25(33): 7601-14.

[31] Lou H, Kim SK, Zaitsev E, Snell CR, Lu B, Loh YP. Sorting and activity-dependent secretion of BDNF require interaction of a specific motif with the sorting receptor carboxypeptidase E. Neuron 2005; 45(2): 245-55.

[32] Chen ZY, Ieraci A, Teng H, *et al.* Sortilin controls intracellular sorting of brain-derived neurotrophic factor to the regulated secretory pathway. J Neurosci 2005; 25(26): 6156-66.

[33] Seidah NG, Benjannet S, Pareek S, *et al.* Cellular processing of the nerve growth factor precursor bythe mammalian pro-protein convertases. Biochem J 1996; 314: 951-60.

[34] Pang PT, Teng HK, Zaitsev E, *et al.* Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. Science 2004; 306(5695): 487-91.

[35] Lu B, Pang PT, Woo NH. The yin and yang of neurotrophin action. Nat Rev Neurosci 2005; 6(8): 603-14.

[36] Matsumoto T, Rauskolb S, Polack M, *et al.* Biosynthesis and processing of endogenous BDNF: CNS neurons store and secrete BDNF, not pro-BDNF. Nat Neurosci 2008; 11(2): 131-3.

[37] Farhadi HF, Mowla SJ, Petrecca K, Morris SJ, Seidah NG, Murphy, RA. Neurotrophin-3 sorts to the constitutive secretory pathway of hippocampal neurons and is diverted to the regulated secretory pathway by coexpression with brain-derived neurotrophic factor. J Neurosci 2000; 20: 4059-68.

[38] Goodman LJ, Valverde J, Lim F, *et al.* Regulated release and polarized localization of brain-derived neurotrophic factor in hippocampal neurons. Mol Cell Neurosci 1996; 7: 223-28.

[39] Mowla SJ, Farhadi HF, Pareek S, *et al.* Biosynthesis and post-translational processing of the precursor to brain-derived neurotrophic factor. J Biol Chem 2001; 276: 12660-66.

[40] Kojima M., Takei N, Numakawa T, *et al.* Biological characterization and optical imaging of brain-derived neurotrophic factor-green fluorescent protein suggest an activitydependent local release of brain-derived neurotrophic factor in neurites of cultured hippocampal neurons. J Neurosci Res 2001; 64: 1-10.

[41] Kohara K, Kitamura A, Morishima M, Tsumoto T. Activity-dependent transfer of brain-derived neurotrophic factor to postsynaptic neurons. Science 2001; 291(5512): 2419-23.

[42] Gartner A, Staiger V. Neurotrophin secretion from hippocampal neurons evoked by long-term-potentiation-inducing electrical stimulation patterns. Proc Natl Acad Sci USA 2002; 99: 6386-91.

[43] Adachi N, Kohara K, Tsumoto T. Difference in trafficking of brain-derived neurotrophic factor between axons and dendrites of cortical neurons, revealed by live-cell imaging. BMC Neurosci 2005; 6(1): 42.

[44] Hartmann M, Heumann R, Lessmann V. Synaptic secretion of BDNF after high-frequency stimulation of glutamatergic synapses. EMBO J 2001; 20(21): 5887-97.

[45] Kolarow R, Brigadski T, Lessmann V. Postsynaptic secretion of BDNF and NT-3 from hippocampal neurons depends on calcium calmodulin kinase II signaling and proceeds *via* delayed fusion pore opening. J Neurosci 2007; 27(39): 10350-64.

[46] Lessmann V, Gottmann K, Malcangio M. Neurotrophin secretion: current facts and future prospects. Prog Neurobiol 2003; 69(5): 341-74.

[47] Horton AC, Ehlers MD. Dual modes of endoplasmic reticulum-to-Golgi transport in dendrites revealed by live-cell imaging. J Neurosci 2003; 23(15): 6188-99.

[48] Horton AC, Rácz B, Monson EE, Lin AL, Weinberg RJ, Ehlers MD. Polarized secretory trafficking directs cargo for asymmetric dendrite growth and morphogenesis. Neuron 2005; 48(5): 757-71.

[49] Zuccato C, Cattaneo E. Role of brain-derived neurotrophic factor in Huntington's disease. Prog Neurobiol 2007; 81(5-6): 294-330.

[50] Gauthier LR, Charrin BC, Borrell-Pagès M, *et al.* Huntingtin controls neurotrophic support and survival of neurons by enhancing BDNF vesicular transport along microtubules. Cell 2004; 118(1): 127-38.

[51] del Toro D, Canals JM, Ginés S, Kojima M, Egea G, Alberch J. Mutant huntingtin impairs the post-Golgi trafficking of brain-derived neurotrophic factor but not its Val66Met polymorphism. J Neurosci 2006; 26(49): 12748-57.

[52] Gharami K, Xie Y, An JJ, Tonegawa S, Xu B. Brain-derived neurotrophic factor over-expression in the forebrain ameliorates Huntington's disease phenotypes in mice. J Neurochem 2008; 105(2): 369-79.

[53] Chen WG, Chang Q, Lin Y, *et al.* Derepression of BDNF transcription involves calcium-dependent phosphorylation of MeCP2. Science 2003; 302(5646): 885-9.

[54] Martinowich K, Hattori D, Wu H, *et al.* DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation. Science 2003; 302(5646): 890-3.

[55] Chang Q, Khare G, Dani V, Nelson S, Jaenisch R. The disease progression of Mecp2 mutant mice is affected by the level of BDNF expression. Neuron 2006; 49(3): 341-8.

[56] Wang H, Chan SA, Ogier M, *et al.* Dysregulation of brain-derived neurotrophic factor expression and neurosecretory function in Mecp2 null mice. J Neurosci 2006; 26(42): 10911-5.

[57] Ogier M, Wang H, Hong E, Wang Q, Greenberg ME, Katz DM. Brain-derived neurotrophic factor expression and respiratory function improve after ampakine treatment in a mouse model of Rett syndrome. J Neurosci 2007; 27(40): 10912-7.

[58] Tsai SJ. Is autism caused by early hyperactivity of brain-derived neurotrophic factor? Med Hypotheses 2005; 65(1): 79-82.

[59] Nelson PG, Kuddo T, Song EY, *et al.* Selected neurotrophins, neuropeptides, and cytokines: developmental trajectory and concentrations in neonatal blood of children with autism or Down syndrome. Int J Dev Neurosci 2006; (1): 73-80

[60] Nelson KB, Grether JK, Croen LA, *et al.* Neuropeptides and neurotrophins in neonatal blood of children with autism or mental retardation. Ann Neurol 2001; 49: 597-606.

[61] Miyazaki K, Narita N, Sakuta R, *et al.* Serum neurotrophin concentrations in autism and mental retardation: a pilot study. Brain Dev 2004; 26: 292-95.

[62] Sadakata T, Kakegawa W, Mizoguchi A, *et al.* Impaired cerebellar development and function in mice lacking CAPS2, a protein involved in neurotrophin release. J Neurosci 2007; 27(10): 2472-82.

[63] Sadakata T, Mizoguchi A, Sato Y, *et al.* The secretory granule-associated protein CAPS2 regulates neurotrophin release and cell survival. J Neurosci 2004; 24(1): 43-52.

[64] Binder DK, Croll SD, Gall CM, Scharfman HE. BDNF and epilepsy: too much of a good thing? Trends Neurosci 2001; (1): 47-53.

[65] Daley TJ, Pfender RM, Morrison JF, *et al.* 2-Deoxy-D-glucose reduces epilepsy progression by NRSF-CtBP-dependent metabolic regulation of chromatin structure. Nat Neurosci 2006; (11): 1382-7.

[66] Duman RS, Monteggia LM. A neurotrophic model for stress-related mood disorders. Biol Psychiatry 2006; 59: 1116-27.

[67] Karege F, Perret G, Bondolfi G, Schwald M, Bertschy G, Aubry JM. Decreased serum brain-derived neurotrophic factor levels in major depressed patients. Psychiatry Res 2002; 109: 143-48

[68] Nibuya M, Morinobu S, Duman RS. Regulation of BDNF and trkB mRNA in rat brain by chronic electroconvulsive seizure and antidepressant drug treatments. J Neurosci 1995; 15: 7539-47.

[69] Russo-Neustadt AA, Beard RC, Huang YM, Cotman CW. Physical activity and antidepressant treatment potentiate the expression of specific brain-derived neurotrophic factor transcripts in the rat hippocampus. Neuroscience 2000; 101: 305-12.

[70] Li Y, Luikart BW, Birnbaum S, *et al.* TrkB regulates hippocampal neurogenesis and governs sensitivity to antidepressive treatment. Neuron 2008; 59(3): 399-412.

[71] Bergami M, Rimondini R, Santi S, Blum R, Götz M, Canossa M. Deletion of TrkB in adult progenitors alters newborn neuron integration into hippocampal circuits and increases anxiety-like behavior. Proc Natl Acad Sci USA 2008; 105(40): 15570-5

[72] Martinowich K, Manji H, Lu B. New insights into BDNF function in depression and anxiety. Nat Neurosci 2007; (9):1089-93.

[73] Angelucci F, Brenè S, Mathé AA. BDNF in schizophrenia, depression and corresponding animal models. Mol Psychiatry 2005; (4): 345-52.

[74] William RP, Cynthia SW, Mayada A, Joel EK. Postmortem investigations of the pathophysiology of schizophrenia: the role of susceptibility genes. J Psychiatry Neurosci 2004; 29: 287-93.

[75] Ikeda Y, Yahata N, Ito I, *et al.* Low serum levels of brain-derived neurotrophic factor and epidermal growth factor in patients with chronic schizophrenia. Schizophr Res 2008; 101(1-3): 58-66.

[76] Buckley PF, Mahadik S, Pillai A, Terry A Jr. Neurotrophins and schizophrenia. Schizophr Res 2007; 94(1-3): 1-11.

[77] Castrén E. Neurotrophic effects of antidepressant drugs. Curr Opin Pharmacol 2004; (1): 58-64.
[78] Ribasés M, Gratacòs M, Armengol L, *et al.* Met66 in the brain-derived neurotrophic factor (BDNF) precursor is associated with anorexia nervosa restrictive type. Mol Psychiatry 2003; (8): 745-51.
[79] Kunugi H, Iijima Y, Tatsumi M, *et al.* No association between the Val66Met polymorphism of the brain-derived neurotrophic factor gene and bipolar disorder in a Japanese population: a multicenter study. Biol Psychiatry 2004; 56: 376-8.
[80] Green EK, Raybould R, Macgregor S, *et al.* Genetic variation of brain-derived neurotrophic factor (BDNF) in bipolar disorder: case-control study of over 3000 individuals from the UK. Br J Psychiatry 2006; 188: 21-5.
[81] Groves JO. Is it time to reassess the BDNF hypothesis of depression? Mol Psychiatry 2007; (12): 1079-88.

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Brief Research Communication

No Association Between Tagging SNPs of SNARE Complex Genes (STX1A, VAMP2 and SNAP25) and Schizophrenia in a Japanese Population

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Abnormalities in neural connections and the neurotransmitter system appear to be involved in the pathophysiology of schizophrenia. The soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex, which consists of Syntaxin1A, vesicle-associated membrane protein 2 (VAMP2) and synaptosomal-associated protein 25 kDa (SNAP25), plays an important role in the neurotransmitter system, and is therefore an attractive place to search for candidate genes for schizophrenia. We conducted a two-stage genetic association analysis of Syntaxin1A (*STX1A*), *VAMP2* and *SNAP25* genes with schizophrenia (first-set screening samples: 377 cases and 377 controls, second-set confirmation samples: 657 cases and 527 controls). Based on the linkage disequilibrium, 40 SNPs (*STX1A*, 8 SNPs; *VAMP2*, 3 SNPs; *SNAP25*, 29 SNPs) were selected as 'tagging SNPs'. Only nominally significant associations of an SNP (rs12626080) and haplotype (rs363014 and rs12626080) in *SNAP25* were detected in the first-set screening scan. To validate this significance, we carried out a replication analysis of these SNP and haplotype associations in second-set samples with a denser set of markers (including five additional SNPs). However, these associations could not be confirmed in the second-set analysis. These results suggest that the SNARE complex-related genes do not play a major role in susceptibility to schizophrenia in the Japanese population.

KEY WORDS: Schizophrenia; SNARE complex; Syntaxin; VAMP; SNAP25

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There is growing evidence that the presynapse is involved with the pathophysiology of schizophrenia. Within the presynaptic area, neurotransmitters are released by synaptic vesicle exocytosis, and the regulation of this release is critical for neural function. The machinery for this release consists of several groups of proteins that work together as a functional unit, the soluble *N*-ethylmaleimide sensitive factor attachments receptor (SNARE) complex [Montecucco et al., 2005].

The SNARE complex consists of Syntaxin1A, vesicle-associated membrane protein 2 (VAMP2) and synaptosomal-associated protein 25 kDa (SNAP25) [Marz and Hanson, 2002], and it has been reported that alterations in the components in the SNARE complex may underlie the pathophysiology of schizophrenia. First, postmortem studies measuring the level of SNARE complex protein or its mRNA revealed specific brain region alternations in schizophrenia [Gabriel et al., 1997; Thompson et al., 1998; Young et al., 1998; Karson et al., 1999; Sokolov et al., 2000; Hemby et al., 2002; Honer et al., 2002; Halim et al., 2003; Thompson et al., 2003]. Second, genetic association studies showed a significant association between SNPs in the Syntaxin1A gene (*STX1A*) and schizophrenic patients from Portugal and Toronto [Wong et al., 2004]. In addition, a very recent report showed that SNPs in *SNAP25* were associated with schizophrenia in Irish high-density families [Fanous et al., 2007].

In this study, we investigated whether genetic polymorphisms within *STX1A* (7p11.23: OMIM *186590), *VAMP2* (17p13.1: OMIM *185881) and *SNAP25* (20p12-p11.2: OMIM *600322) were associated with schizophrenia in a Japanese population.

A first-set screening analysis was conducted with 377 schizophrenic patients (196 males and 181 females; mean age ± standard deviation (SD) 42.4 ± 14.8 years) and 377 healthy controls (212 males and 172 females; 35.9 ± 14.7 years). In a

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confirmation analysis a different panel of samples was used, consisting of 657 patients with schizophrenia (350 male and 307 female; 50.1 ± 14.4 years) and 527 controls (303 male and 224 female; 40.8 ± 15.3 years).

The patients were diagnosed according to DSM-IV criteria with the consensus of at least two experienced psychiatrists on the basis of unstructured interviews and a review of medical records. All healthy control subjects were also psychiatrically screened based on unstructured interviews. None of the subjects was known to be related to each other, and all were ethnically Japanese.

Written informed consent was obtained from each subject. This study was approved by the ethics committees at Fujita Health University, Nagoya University Graduate School of Medicine, Osaka University Graduate School of Medicine and Teikyo University School of Medicine.

After consulting the HapMap database (release#16.c.1, June 2005, www.hapmap.org, population: Japanese Tokyo: minor allele frequencies (MAFs) of more than 0.05 for *STX1A* and *VAMP2*, and 0.1 for *SNAP25*), 39 SNPs (*STX1A*, 7 SNPs; *VAMP2*, 3 SNPs; *SNAP25*, 29 SNPs) were selected as 'tagging SNPs' based on the criterion of an r^2 threshold greater than 0.8 in 'pair-wise tagging only' mode using the 'Tagger' program (Paul de Bakker, http://www/broad.mit.edu/mpg/tagger). For *STX1A*, since a previous report showed the positive association of an SNP in intron 7 [Wong et al., 2004], we included this SNP with the aforementioned 'tagging SNPs' for the association analysis. Overall, 40 SNPs were examined in this study (Supplementary Figures 1–3).

For denser mapping in the confirmation analysis, we added five SNPs around nominally significant SNPs or haplotypes detected in the first-set screening scan (rs610457, rs363013, rs363015, rs6039792 and rs363050).

For genotyping of these SNPs, a TaqMan assay (Applied Biosystems, CA), PCR-RFLP assay, and direct sequencing techniques were used. Detailed information is available in Supplementary Table 1. Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by chi-square test (SAS/Genetics, release 8.2, SAS Japan Inc., Tokyo, Japan). Marker-trait association was evaluated by a likelihood ratio test (allele-wise and haplotype-wise analyses) and χ^2-test (genotype-wise analysis). For exhaustive screening, we tested all one-marker (by conventional allele-wise analysis), two-marker, and three-marker haplotypes (and seven-marker haplotypes for second-set confirmation analysis) using the COCAPHASE 2.403 program [Dudbridge, 2003].

The power and sample size calculations were performed with a statistical program (http://biostat.mc.vanderbilt.edu/twiki/bin/view/main/powersamplesize). This significance threshold for all statistical tests was 0.05.

All genotype frequencies of each group were in HWE (data not shown). The LD structures examined in our control samples were almost the same as the one shown in HapMap database (Supplementary Figure 1–3).

The SNP (rs12626080: SNAP25-M8: $P = 0.0236$, uncorrected) in *SNAP25* and the haplotype constructed by M7 (rs363014) and M8 in *SNAP25* showed a nominally significant association with schizophrenia in the first-set screening samples (global $P = 0.0215$, uncorrected), although no association was detected with any tagging SNP in *STX1A* and *VAMP2*, including the SNP reported to be associated with schizophrenia in Caucasian samples [Wong et al., 2003] (Tables I and II and Supplementary Table 2).

To validate this nominal significance, we carried out a replication analysis using an independent set of samples. In this analysis, five additional SNPs were further included for denser mapping around M7 and M8 (rs610457, rs363013, rs363015, rs6039792 and rs363050: Supplementary Figure 4). However, this second-set confirmation analysis showed no

TABLE I. First-Set Association Analysis of Tagging SNPs in STX1A and VAMP2

Genes	Marker IDs	Distance to next SNP (bp)	N[a]		MAF[b]		*P*-Values			
			SCZ	CON	SCZ	CON	Genotype	(1) Window[c]	(2) Window	(3) Window
STX1A (minus strand)	SNP1	rs867500	0	375	375	0.204	0.217	0.754	0.527			
	SNP2	Intron7 SNP	3592	373	372	0.260	0.261	0.767	0.978	0.947	0.946	
	SNP3	rs4363087	164	373	370	0.326	0.311	0.424	0.537	0.859	0.896	
	SNP4	rs3793243	3151	375	377	0.419	0.386	0.304	0.196	0.592	0.775	
	SNP5	rs875342	5751	375	370	0.228	0.238	0.211	0.653	0.553	0.698	
	SNP6	rs6951030	6143	373	372	0.0563	0.0565	0.826	0.594	0.781	0.924	
	SNP7	rs9654749	7145	376	374	0.483	0.469	0.460	0.602	0.593	0.872	
	SNP8	rs2030921	785	376	377	0.249	0.240	0.728	0.696	0.869		
VAMP2 (minus strand)	m1	rs2278637	0	372	369	0.430	0.436	0.348	0.724			
	m2	rs10610332	1981	377	377	0.399	0.403	0.750	0.975	0.732	0.802	
	m3	rs8067606	2800	375	371	0.425	0.431	0.490	0.765	0.694		

[a]N, number; SCZ, schizophrenia; CON, control.
[b]MAF, minor allele frequency.
[c]Identical as conventional allele-wise analysis.

TABLE II. First-Set Association Analysis of Tagging SNPs in SNAP25

Genes	Marker IDs		Distance to next SNP (bp)	N[a]		MAF[b]		P-Values			
				SCZ	CON	SCZ	CON	Genotype	(1) Window[c]	(2) Window	(3) Window
SNAP25	M1	rs6104567	0	377	370	0.263	0.242	0.298	0.341		
	M2	rs1889189	1,653	377	370	0.236	0.223	0.675	0.550	0.493	
	M3	rs3787303	11,662	377	376	0.312	0.300	0.306	0.630	0.830	0.825
	M4	rs2423487	4,347	374	368	0.171	0.171	0.146	0.978	0.874	0.721
	M5	rs363012	6,704	377	377	0.308	0.295	0.364	0.612	0.403	0.795
	M6	rs363039	697	375	368	0.432	0.397	0.128	0.173	0.552	0.364
	M7	rs363014	8,198	377	377	0.460	0.453	0.197	0.767	0.328	0.660
	M8	rs12626080	4,358	375	367	0.208	0.162	0.073	**0.0236**[d]	**0.0215**[d]	0.126
	M9	rs363052	2,374	375	369	0.163	0.159	0.177	0.866	0.0747	0.0882
	M10	rs363053	159	374	370	0.298	0.291	0.918	0.748	0.774	0.411
	M11	rs4813024	2,231	377	377	0.222	0.235	0.650	0.544	0.719	0.865
	M12	rs6074113	4,195	372	369	0.337	0.341	0.615	0.868	0.690	0.855
	M13	rs363022	383	374	369	0.394	0.413	0.950	0.457	0.509	0.643
	M14	rs362564	2,232	376	372	0.455	0.425	0.725	0.265	0.579	0.739
	M15	rs362547	513	372	376	0.222	0.184	0.380	0.0712	0.254	0.524
	M16	rs362567	952	377	376	0.144	0.129	0.259	0.379	0.0883	0.375
	M17	rs362570	773	375	368	0.351	0.331	0.572	0.414	0.615	0.134
	M18	rs362584	7,611	372	370	0.209	0.212	0.716	0.877	0.489	0.617
	M19	rs16991334	7,442	372	367	0.0970	0.0989	0.574	0.903	0.999	0.716
	M20	rs6039807	1,659	372	367	0.451	0.434	0.580	0.544	0.401	0.737
	M21	rs362995	13,463	377	377	0.269	0.248	0.764	0.361	0.601	0.891
	M22	rs363006	3,044	373	370	0.0134	0.0082	0.873	1	0.208	0.587
	M23	rs6108463	422	374	368	0.182	0.192	0.893	0.647	0.732	0.510
	M24	rs362988	865	374	370	0.379	0.401	0.325	0.391	0.679	0.689
	M25	rs6039820	657	377	376	0.400	0.373	0.253	0.286	0.526	0.788
	M26	rs6108464	1,923	377	376	0.401	0.405	0.892	889	0.612	0.807
	M27	rs3787283	468	376	375	0.460	0.465	0.614	0.850	0.942	0.548
	M28	rs3746544	2,666	372	367	0.260	0.249	0.746	0.634	0.642	0.862
	M29	rs6133852	3,876	377	377	0.237	0.206	0.176	0.156	0.437	0.676

[a]N, number; SCZ, schizophrenia; CON, control.
[b]MAF, minor allele frequency.
[c]Identical as conventional allele-wise analysis.
[d]Bold numbers represent significant *P*-values.

evidence of the significance of these markers (*P*-values for M7-M8 combination: 0.541: Supplementary Table 3). To increase the power, the genotypes of these five new SNPs in the first-set samples were determined and we then combined the samples (first-set and second-set samples), but again we could not detect an association in this explorative analysis (*P*-values for M7–M8 combination; 0.280: Table III and Supplementary Table 4).

This genetic two-stage case–control association study revealed no association between SNARE complex-related genes (*STX1A*, *VAMP2* and *SNAP25*) and schizophrenia in the Japanese population. Because postmortem studies showed a change in expression of SNARE complex genes (see Introduction), the most interesting variants of these genes are SNPs located in the promoter regions that might affect gene expression. To cover such regions, particularly the 5′ region of each gene, we applied the recently recommended 'gene-based' approach [Neale and Sham, 2004], in which it is important to include both the exon region and the flanking region. There is also emphasis on selecting genetic variants that adequately reflect the LD background in the targeted population (e.g., tagging SNPs). Our selection of tagging SNPs represented the all regions of these genes in the Japanese population, significantly reducing genotyping effort without much loss of power.

Moreover, we included confirmation analysis using an independent set of samples to check for Type I error, after significance was obtained in the screening samples. For *SNAP25*, an SNP and a two-marker haplotype were associated with schizophrenia in the first-set screening samples, but no significance could be seen in the larger second set, suggesting that the significance in the screening samples may have resulted from Type I error due to multiple testing or small sample size. We carried out power calculations and determined that our sample had sufficient power in the second-set analysis to detect association of 0.999 at $P < 0.05$, assuming an odds ratio of 1.69, which was shown in the first-set analysis of SNAP25-M8.

In addition, our sample size in the first-set screening analysis was large enough to deny Type II error in replicating the previous positive association of an SNP in *STX1A* intron 7 with schizophrenia in Caucasian samples [Wong et al., 2004]. The power was more than 0.997 at $P < 0.05$ when the odds ratio was set at 2.1, which is the estimated odds ratio of TDT in Wong's report [Wong et al., 2004]. One explanation for the different outcomes may be that *STX1A* susceptibility alleles were present in the Caucasian samples, but not in the Japanese population.

Although our sample size was large enough for replication of Wong's study, in general the odds ratios of common variants found to be associated with schizophrenia so far are less than 1.5. In this regard, a larger sample size might be required for conclusive results, since our sample size showed power surpassing 0.8 only when we set the odds ratio at more than 1.62.

With this statistical methodology, it is generally accepted that gene–gene interactions should be examined when a number of related genes are analyzed. We included explorative analysis to evaluate the interaction among these genes by multiple dimensionality reduction (MDR) [Hahn et al., 2003], but no interaction was detected (data not shown). In addition, we conducted MDR analysis for other genes related to SNARE complex genes, Complexin I and II (*CPLX I* and *CPLXII*), for which we previously found no association to schizophrenia [Kishi et al., 2006]. Again, no interaction could be detected in this analysis (data not shown).

There are numerous molecules related to the SNARE complex besides *CPLX* genes [Wang and Tang, 2006]. The most interesting molecule is dysbindin (*DTNBP1*: dustrobrevin-binding protein 1), for which there is evidence of an association

TABLE III. Confirmation Analysis Around the Nominally Significant SNPs Detected in First-Set Analysis

Marker IDs		Distance to next SNP (bp)	N[a]		MAF[b]		*P*-Values						
			SCZ	CON	SCZ	CON	Genotype	(1) Window[c]	(2) Window	(3) Window	(4) Window	(5) Window	(6) Window	(7) Window
	rs6104571	0	1,031 (656)	892 (527)	0.216 (0.196)	0.213 (0.217)	0.970 (0.469)	0.804 (0.200)						
	rs363013	1,661	1,031 (656)	892 (527)	0.011 (0.010)	0.011 (0.011)	0.902 (0.502)	0.872 (0.690)	00.992 (0.950)					
M7	rs363014	2,283	1,033 (656)	914 (537)	0.449 (0.444)	0.455 (0.422)	0.724 (0.155)	0.710 (0.280)	0.657 (0.523)	0.146 (0.648)	0.183 (0.704)			
	rs363015	5	1,031 (656)	892 (527)	0.060 (0.060)	0.062 (0.063)	0.340 (0.552)	0.733 (0.808)	0.789 (0.659)	0.759 (0.493)	0.194 (0.706)	0.136 (0.691)		
M8	rs12626080	4,353	1,032 (657)	890 (523)	0.177 (0.160)	0.166 (0.168)	0.617 (0.861)	0.362 (0.582)	0.192 (0.962)	0.195 (0.662)	0.142 (0.783)	0.142 (0.608)	0.107 (0.525)	
	rs6039792	1,040	1,031 (656)	892 (527)	0.212 (0.191)	0.209 (0.212)	0.953 (0.468)	0.766 (0.204)	0.119 (0.884)	0.144 (0.925)	0.142 (0.783)	0.107 (0.500)	0.107 (0.553)	0.076 (0.473)
	rs363050	165	1,031 (656)	892 (527)	0.225 (0.207)	0.209 (0.205)	0.511 (0.991)	0.246 (0.923)	0.056 (0.492)	0.136 (0.761)	0.136 (0.761)	0.107 (0.500)		

[a]N, number; SCZ, schizophrenia; CON, control.
[b]MAF, minor allele frequency.
[c]Identical as conventional allele-wise analysis.
[d]Numbers in parentheses indicate results from second-set samples.

with schizophrenia, since recent studies showed that dysbindin regulates the expression of *SNAP25* [Numakawa et al., 2004]. Therefore, it will be essential to evaluate the other candidate genes related to SNARE complex genes for conclusive results.

With regard to interpretation of the results from this study, several limitations should be mentioned. Firstly, we did not perform mutation screening of these genes. Secondly, our samples were un-matched for age and gender between cases and controls, and were not assessed with the use of a standard structured interview. Therefore, detailed association analysis with mutation search in well-phenotyped samples will be essential in future study.

To conclude, our results provide no evidence that SNARE complex genes play a major role in susceptibility for schizophrenia in the Japanese population. Our results also imply that caution is needed in drawing conclusions about positive associations from small-sample case–control studies. We strongly suggest that two-stage genetic association analysis be conducted when positive results are found in screening samples.

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REFERENCES

Dudbridge F. 2003. Pedigree disequilibrium tests for multilocus haplotypes. Genet Epidemiol 25(2):115–121.

Fanous A, van den Oord E, Zhao Z, Wormley B, Amdur R, O'Neill FA, Walsh D, Kendler K, Riley B. 2007. SNAP-25 is Associated with Schizophrenia in 270 Irish High Density Families. Biol Psychiatry 61(8S):191S.

Gabriel SM, Haroutunian V, Powchik P, Honer WG, Davidson M, Davies P, Davis KL. 1997. Increased concentrations of presynaptic proteins in the cingulate cortex of subjects with schizophrenia. Arch Gen Psychiatry 54(6):559–566.

Hahn LW, Ritchie MD, Moore JH. 2003. Multifactor dimensionality reduction software for detecting gene–gene and gene–environment interactions. Bioinformatics 19(3):376–382.

Halim ND, Weickert CS, McClintock BW, Hyde TM, Weinberger DR, Kleinman JE, Lipska BK. 2003. Presynaptic proteins in the prefrontal cortex of patients with schizophrenia and rats with abnormal prefrontal development. Mol Psychiatry 8(9):797–810.

Hemby SE, Ginsberg SD, Brunk B, Arnold SE, Trojanowski JQ, Eberwine JH. 2002. Gene expression profile for schizophrenia: Discrete neuron transcription patterns in the entorhinal cortex. Arch Gen Psychiatry 59(7):631–640.

Honer WG, Falkai P, Bayer TA, Xie J, Hu L, Li HY, Arango V, Mann JJ, Dwork AJ, Trimble WS. 2002. Abnormalities of SNARE mechanism proteins in anterior frontal cortex in severe mental illness. Cereb Cortex 12(4):349–356.

Karson CN, Mrak RE, Schluterman KO, Sturner WQ, Sheng JG, Griffin WS. 1999. Alterations in synaptic proteins and their encoding mRNAs in prefrontal cortex in schizophrenia: A possible neurochemical basis for 'hypofrontality'. Mol Psychiatry 4(1):39–45.

Kishi T, Ikeda M, Suzuki T, Kitajima T, Yamanouchi Y, Kinoshita Y, Ozaki N, Iwata N. 2006. No association of complexin1 and complexin2 genes with schizophrenia in a Japanese population. Schizophr Res 82(2–3):185–189.

Marz KE, Hanson PI. 2002. Sealed with a twist: Complexin and the synaptic SNARE complex. Trends Neurosci 25(8):381–383.

Montecucco C, Schiavo G, Pantano S. 2005. SNARE complexes and neuroexocytosis: How many, how close? Trends Biochem Sci 30(7): 367–372.

Neale BM, Sham PC. 2004. The future of association studies: Gene-based analysis and replication. Am J Hum Genet 75(3):353–362.

Numakawa T, Yagasaki Y, Ishimoto T, Okada T, Suzuki T, Iwata N, Ozaki N, Taguchi T, Tatsumi M, Kamijima K. et al. 2004. Evidence of novel neuronal functions of dysbindin, a susceptibility gene for schizophrenia. Hum Mol Genet 13(21):2699–2708.

Sokolov BP, Tcherepanov AA, Haroutunian V, Davis KL. 2000. Levels of mRNAs encoding synaptic vesicle and synaptic plasma membrane proteins in the temporal cortex of elderly schizophrenic patients. Biol Psychiatry 48(3):184–196.

Thompson PM, Egbufoama S, Vawter MP. 2003. SNAP-25 reduction in the hippocampus of patients with schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry 27(3):411–417.

Thompson PM, Sower AC, Perrone-Bizzozero NI. 1998. Altered levels of the synaptosomal associated protein SNAP-25 in schizophrenia. Biol Psychiatry 43(4):239–243.

Wang Y, Tang BL. 2006. SNAREs in neurons-beyond synaptic vesicle exocytosis (Review). Mol Membr Biol 23(5):377–384.

Wong AH, Macciardi F, Klempan T, Kawczynski W, Barr CL, Lakatoo S, Wong M, Buckle C, Trakalo J, Boffa E. et al. 2003. Identification of candidate genes for psychosis in rat models, and possible association between schizophrenia and the 14-3-3eta gene. Mol Psychiatry 8(2): 156–166.

Wong AH, Trakalo J, Likhodi O, Yusuf M, Macedo A, Azevedo MH, Klempan T, Pato MT, Honer WG, Pato CN. et al. 2004. Association between schizophrenia and the syntaxin 1A gene. Biol Psychiatry 56(1):24–29.

Young CE, Arima K, Xie J, Hu L, Beach TG, Falkai P, Honer WG. 1998. SNAP-25 deficit and hippocampal connectivity in schizophrenia. Cereb Cortex 8(3):261–268.

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Identification of *YWHAE*, a gene encoding 14-3-3epsilon, as a possible susceptibility gene for schizophrenia

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Schizophrenia is a complex mental disorder with a fairly high degree of heritability. Although the causes of schizophrenia remain unclear, it is now widely accepted that it is a neurodevelopmental and neurodegenerative disorder involving disconnectivity and disorder of the synapses. Disrupted-in-schizophrenia 1 (*DISC1*) is a promising candidate susceptibility gene involved in neurodevelopment, including maturation of the cerebral cortex. To identify other susceptibility genes for schizophrenia, we screened for *DISC1*-interacting molecules [NudE-like (*NUDEL*), Lissencephaly-1 (*LIS1*), 14-3-3epsilon (*YWHAE*), growth factor receptor bound protein 2 (*GRB2*) and Kinesin family 5A of Kinesen1 (*KIF5A*)], assessing a total of 25 tagging single-nucleotide polymorphisms (SNPs) in a Japanese population. We identified a *YWHAE* SNP (*rs28365859*) that showed a highly significant difference between case and control samples, with higher minor allele frequencies in controls ($P_{allele} = 1.01 \times 10^{-5}$ and $P_{genotype} = 4.08 \times 10^{-5}$ in 1429 cases and 1728 controls). Both messenger RNA transcription and protein expression of 14-3-3epsilon were also increased in the lymphocytes of healthy control subjects harboring heterozygous and homozygous minor alleles compared with homozygous major allele subjects. To further investigate a potential role for *YWHAE* in schizophrenia, we studied *Ywhae*$^{+/-}$ mice in which the level of 14-3-3epsilon protein is reduced to 50% of that in wild-type littermates. These mice displayed weak defects in working memory in the eight-arm radial maze and moderately enhanced anxiety-like behavior in the elevated plus-maze. Our results suggest that *YWHAE* is a possible susceptibility gene that functions protectively in schizophrenia.

INTRODUCTION

Recent neuroimaging studies show that structural brain abnormalities are an established feature of schizophrenia and are characterized by decreased total gray matter volume (1,2). These morphological correlates of schizophrenia range from a reduction in brain size to localized alterations in the morphology and molecular composition of specific neuronal, synaptic and glial populations in specific brain areas such as the hippocampus, dorsolateral prefrontal cortex and dorsal thalamus.

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