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A Family-Based Association Study and Gene Expression Analyses of Netrin-G1 and -G2 Genes in Schizophrenia

Mika Aoki-Suzuki, Kazuo Yamada, Joanne Meerabux, Yoshimi Iwayama-Shigeno, Hisako Ohba, Kazuya Iwamoto, Hitomi Takao, Tomoko Toyota, Yumiko Suto, Noriaki Nakatani, Brian Dean, Sachiko Nishimura, Kenjiro Seki, Tadafumi Kato, Shigeyoshi Itohara, Toru Nishikawa, and Takeo Yoshikawa

Background: *The netrin-G1* (NTNG1) *and -G2* (NTNG2) *genes, recently cloned from mouse, play a role in the formation and/or maintenance of glutamatergic neural circuitry. Accumulating evidence strongly suggests that disturbances of neuronal development and the N-methyl-D-aspartate receptor–mediated signaling system might represent a potential pathophysiology in schizophrenia. We therefore set out to examine the genetic contribution of human* NTNG1 *and* NTNG2 *to schizophrenia.*

Methods: *Twenty-one single nucleotide polymorphisms (SNPs) from* NTNG1 *and 10 SNPs from* NTNG2 *were analyzed in 124 schizophrenic pedigrees. All genotypes were determined with the TaqMan assay. The expression levels of* NTNG1 *and* NTNG2 *were examined in the frontal (Brodmann's Area [BA]11 and BA46) and temporal (BA22) cortices from schizophrenic and control postmortem brains. The isoform-specific expression of* NTNG1 *splice variants was assessed in these samples.*

Results: *Specific haplotypes encompassing alternatively spliced exons of* NTNG1 *were associated with schizophrenia, and concordantly, messenger ribonucleic acid isoform expression was significantly different between schizophrenic and control brains. An association between* NTNG2 *and schizophrenia was also observed with SNPs and haplotypes that clustered in the 5′ region of the gene.*

Conclusions: *The* NTNG1 *and* NTNG2 *genes might be relevant to the pathophysiology of schizophrenia.*

Key Words: Neurodevelopment, laminet-1, laminet-2, postmortem brain, real-time quantitative polymerase chain reaction, alternative splicing

Schizophrenia is a severe brain disorder that usually produces a lifetime of disability, with emotional and cognitive distress for the affected individuals (Lewis and Lieberman 2000). Although schizophrenia has a worldwide prevalence of approximately 1%, the specific factors that underlie its predisposition remain elusive. A number of studies have focused on identifying genetic and environmental components that separately, or in combination, contribute to the manifestation of the disease (Lewis and Levitt 2002).

A strongly supported theory is that schizophrenia evolves from a fixed brain lesion occurring early in neurodevelopment that responds abnormally to later maturational stimuli (Chua and Murray 1996; Marenco and Weinberger 2000; Weinberger 1987). Developmental neurobiology has made great strides in detailing the molecular machinery that leads to the assembly of brain systems (Jessell 2000; Tessier-Lavigne and Goodman 1996). Several gene families that are responsible for encoding cues for guiding axonal growth cones and cell migration have been identified. These genes include members of the immunoglobulin superfamily, ephrins, semaphorins, slits, and netrins (Chisholm and Tessier-Lavigne 1999; Tessier-Lavigne and Goodman 1996). For the cellular and signaling bases for schizophrenia pathophysiology, disturbances in *N*-methyl-D-aspartate (NMDA) and dopamine neurotransmission have also been strongly implicated.

The netrins are a family of diffusible axon guidance molecules related to laminin that are conserved from *Caenorhabditis elegans* to vertebrates (Ishii et al 1992; Serafini et al 1994). A netrin-related molecule, netrin-G1 (Ntng1) (also called laminet-1), has been identified in mice and shown to be distinct from the classical netrins in several aspects (Nakashiba et al 2000; Yin et al 2002). Unlike classical netrins, Ntng1 is predominantly tethered to the membrane through a carboxyl-terminal glycosyl phosphatidyl-inositol anchor. It generates several splice variants, none of which bind deleted in colorectal carcinoma (DCC) or uncoordinated 5 (UNC5), the best characterized netrin receptors. Nakashiba et al (2002) and Yin et al (2002) have recently identified a close paralogue, netrin-G2 (Ntng2) (also called laminet-2). Both *Ntng1* and *Ntng2* are elaborated within the vertebrate lineage. Comparative analysis of mouse *Ntng1* and *Ntng2* revealed complementary expression in the brain, implying nonredundant roles (Nakashiba et al 2002; Yin et al 2002). Our ongoing studies with mutant mice devoid of these proteins suggest that Ntng1 and Ntng2 are necessary in correct NMDA receptor functioning and that mutant mice show behavioral phenotypes related to schizophrenia (Nishimura et al 2004).

To define the possible roles of *NTNG1* and *NTNG2* molecules in schizophrenia, we mapped the genomic layout of human *NTNG1* and *NTNG2*, confirmed their chromosomal localization, and detected multiple splice variants of *NTNG1* transcripts. On the basis of these findings, we performed a

From the Laboratories for Molecular Psychiatry (MAS, KY, JM, YIS, HO, HT, TT, NN, TY), Molecular Dynamics of Mental Disorders (KI, TK), and Behavioral Genetics (SN, KS, SI), RIKEN Brain Science Institute, Wako; Section of Psychiatry and Behavioral Sciences (MAS, TN), Tokyo Medical and Dental University Graduate School, Tokyo; Department of Pediatric Cardiology (YS), Tokyo Women's Medical University, Tokyo, Japan; and The Rebecca L. Cooper Research Laboratories (BD), Mental Health Research Institute of Victoria, Parkville, Victoria, Australia.

Address reprint requests to Takeo Yoshikawa, M.D., Ph.D., RIKEN Brain Science Institute, Laboratory for Molecular Psychiatry, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan; E-mail: takeo@brain.riken.go.jp.

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genetic and expression study of these two closely related genes in schizophrenia.

Methods and Materials

Subjects

The schizophrenia pedigrees were all derived from a geographic area located in central Japan. The probands, both in- and outpatients, were followed up by hospital doctors for at least 6 months. The samples consisted of 124 families with 376 members, of whom 163 were affected. This included 1) 80 independent and complete trios (schizophrenic offspring and their parents); 2) 15 probands with one parent; 3) 13 probands with affected siblings; and 4) 30 probands with discordant siblings (Yamada et al 2004). There was some overlap in the samples from 1), 3), and 4). Consensual diagnosis was made according to DSM-IV criteria by at least two experienced psychiatrists, on the basis of direct interviews, available medical records, and information from hospital staff and relatives. None of the patients had additional Axis I disorders as defined by DSM-IV, and none of the present family members suffered from neurodegenerative disorders, including Parkinson's and Alzheimer's diseases.

The present study was approved by the ethics committee of RIKEN Brain Science Institute. All patients and family members gave informed and written consent to participate in the study.

Determination of the Exon/Intron Structures of NTNG1 and NTNG2

The complete genomic structure of *NTNG1* was not available from the databases and was determined as described (Meerabux et al, unpublished data): briefly, mouse netrin-G1a complementary deoxyribonucleic acid (cDNA) sequence NM_030699 and G1d sequence AB038664, as well as human *NTNG1* clones BC030220 and AB023193, were aligned with human genomic bacterial artificial chromosome (BAC) clones RP11-270C12, RP11-396N10, and RP11-436H6 (GenBank identification [ID] numbers AC114491, AL590427, and AL513187, respectively; http://www.ncbi.nlm.nih.gov/Entrez/index.html) using the National Center for Biotechnology Information (NCBI) BLAST 2 sequences algorithm (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). For *NTNG2* genomic organization, we consulted the Ensembl Human Genome Browser (http://www.ensembl.org/Homo_sapiens/), which provided annotation on the longest cDNA clone available in the databases (Vega Gene ID: OTTHUMG00000020835).

Fluorescent In Situ Hybridization (FISH)

For fluorescent in situ hybridization (FISH) analysis, *NTNG1* cDNA probe was prepared by polymerase chain reaction (PCR) amplification of Marathon-Ready Human adult brain cDNA (Clontech, Palo Alto, California) with a forward primer (5′-AGGGGGCGACTTGCAGGAGGC, 3′ end at nucleotide (nt) -570: A of the ATG initiation codon is counted as +1) and a reverse primer (5′-GCAGTGCCTTTGGGGATGGGG, 3′ end at nt 1032 in BC030220). The procedure was repeated for *NTNG2* (forward primer: 5′-CTGAGAGGTTCTGCTCCCATG, 3′ end at nt 3; reverse primer: GGGTAGCAGCCCTGGCGCCAG, 3′ end at nt 1313 in AB058760). The PCR products were separated on an agarose gel, and the DNA was excised, purified, and sequenced to check the fidelity of the probes. The purified DNA samples were fluorescently labeled under standard conditions and used as probes against normal metaphase chromosomes.

Detection of Ntng1 Transcript Isoforms

On the basis of information regarding the mouse *Ntng1* splice variation, which is generated by alternative splicing of downstream exons (Nakashiba et al 2000), we designed a PCR assay to detect human splice variants from Marathon-Ready Human adult brain cDNA (Clontech), using a forward primer designed to exon 4 and a reverse primer in exon 10 (Meerabux et al, unpublished data). The PCR products were cloned into pCR2.1 (Invitrogen, Carlsbad, California) and sequenced with the DYEnamic ET terminator cycle sequencing kit (Amersham Pharmacia Biotech, Piscataway, New Jersey). The exonic composition of each fragment was determined by aligning each sequence result against the known *NTNG1* exon sequences with SEQUENCHER software (Gene Codes, Ann Arbor, Michigan).

We also performed semiquantitative analysis of *NTNG1* messenger ribonucleic acid (mRNA) splice variants by separating the PCR fragments with an Agilent 2100 bioanalyzer with the DNA1000 LabChip kit (Agilent Technologies, Palo Alto, California). The bioanalyzer software automatically calculates migration time (sec), area, size (bp), concentration (ng/μL), and molarity (nmol/L) of each separated band and displays the results in real time. The identities of the detected peaks were determined by comparison with the cloned isoforms run in a separate well on the same chip.

Mutation Screening

Genomic DNA was isolated from blood samples according to standard methods. The complete coding region and exon/intron boundaries of *NTNG1* and *NTNG2* were screened for polymorphisms by direct sequencing of PCR products, from 40 unrelated schizophrenia samples. Primers used for amplification are listed in Appendix 1 (available online). Polymerase chain reaction was performed with an initial denaturation at 94°C for 1 min, followed by 35 cycles at 94°C for 15 sec, 50°–70°C (optimized for each primer pair) for 15 sec, 72°C for 45 sec, and a final extension at 72°C for 2 min, with TaKaRa Taq polymerase (Takara Bio, Shiga, Japan). Detailed information on amplification conditions is available upon request. Direct sequencing of PCR products was performed with the BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, Foster City, California) and the ABI PRISM 3700 Genetic Analyzer (Applied Biosystems). Polymorphisms were detected with SEQUENCHER. The Japanese Single Nucleotide Polymorphisms (http://snp.ims.u-tokyo.ac.jp/index.html), The SNP Consortium (http://snp.cshl.org/news/), and the NCBI (http://www.ncbi.nlm.nih.gov/) databases were searched for additional single nucleotide polymorphisms (SNPs).

SNP Genotyping

Single nucleotide polymorphisms were typed in all 124 families with the TaqMan system (Applied Biosystems). Polymerase chain reactions were performed in an ABI 9700 thermocycler, and fluorescence was determined with an ABI 7900 sequence detector single point measurement and SDS v2.2 software (Applied Biosystems). Each marker was checked for allele–inheritance inconsistency within a pedigree with PEDCHECK software (O'Connell and Weeks 1998), and conflicts or flagged alleles were resolved by regenotyping.

Statistical Analyses

The initial panel of 124 families was analyzed with the pedigree disequilibrium test (PDT) program, v3.12 (http://www.chg.duke.edu/software/pdt.html) (Martin et al 2000, 2001). The statistically more conservative follow-up study was carried out

Table 1. Demographic and Treatment Data for the Australian Schizophrenic and Control Brains (BA11 and BA22)

Subject ID	Age (y)	Gender	PMI (h)	pH	Cause of Death	FRAD	FAPDD
Schizophrenic Patients							
1	79	F	26	6.27	Hypothermia	Fluphenazine decanoate	89
2	57	M	24	6.06	Coronary artery atheroma	Fluphenazine decanoate	45
3	65	M	41	6.57	Ischemic heart disease	Fluphenazine decanoate	45
4	47	F	50	6.31	Pneumonia	Resperidone	1200
5	65	F	50	6.35	Rupture of abdominal aortic aneurysm	Fluphenazine decanoate + haloperidol	339
6	69	M	48	6.44	Suicide: Carbon monoxide poisoning	Haloperidol	350
Mean ± SD	63.67 ± 10.86		39.83 ± 11.97	6.33 ± .17			
Control Subjects							
1	77	F	17	6.32	Hypertension heart disease		
2	57	M	27	6.43	Ischemic heart disease		
3	68	M	41	6.06	Aortic stenosis		
4	39	F	65	6.38	Mitral valve prolapse		
5	62	F	40	6.45	Ischemic heart disease		
6	68	M	69	6.59	Coronary artery atheroma		
Mean ± SD	61.83 ± 13.04		43.17 ± 20.52	6.37 ± .18			

BA, Brodmann's Area; ID, identification number; PMI, postmortem interval; FRAD, final recorded antipsychotic drug; FAPDD, final recorded antipsychotic drug dose (mg chlorpromazine equivalents/day); M, male; F, female.

with the complete 80-trio set and the extended transmission disequilibrium test (ETDT) algorithm, v2.2 (Sham and Curtis 1995). Empirical significance levels of the ETDT results were simulated from 10,000 Monte Carlo permutations with the MCETDT program, v1.3 (http://www.mds.qmw.ac.uk/statgen/dcurtis/software.html) (Zhao et al 1999). The TRANSMIT program, v2.5.4 (Clayton 1999; Clayton and Jones 1999) was used for haplotype-based transmission disequilibrium testing. Uncorrected p values for individual haplotypes (haplotypic p) and corrected p values for multi-allele testing (global p) were calculated with the TRANSMIT program.

Genomic linkage disequilibrium (LD) patterns retained in the Japanese population were determined by pairwise LD examination of markers within *NTNG1* and *NTNG2*, in 186 unrelated individuals from our schizophrenic pedigree panel. The standardized disequilibrium coefficient (D') (Lewontin 1988) and the squared correlation coefficient (r^2) were calculated with COCAPHASE software (http://www.hgmp.mrc.ac.uk/~fdudbrid/software/unphased/) (Dudbridge 2003).

Collection of Human Central Nervous System Tissues

Two sources of postmortem brain samples were used in this study. In one sampling, Brodmann's Area (BA)11 (lateral orbitofrontal cortex) and BA22 (superior temporal cortex) were collected by the Victorian Institute of Forensic Medicine, affiliated with the State Coroner's Office in Australia. At autopsy, brains were removed, and 1-cm coronal slices from the left hemispheres were rapidly frozen to −80°C. The postmortem diagnoses were confirmed according to DSM-IV criteria by a psychiatrist and a senior psychologist using the Diagnostic Instrument for Brain Studies (Keks et al 1999). Ethical approval for the study was given by the North-Western Healthcare Network Human Ethics Committee. The gender distribution, mean age, mean postmortem interval, and pH for the tissues were not significantly different between schizophrenia (n = 6) and control (n = 6) groups (Table 1). The control subjects had no known history of psychiatric illness.

The second set of samples consisted of RNA extracted from BA46 (dorsolateral prefrontal cortex [DLPFC]), obtained from the Stanley Foundation brain collection. Detailed information on the subjects and the extraction method are described on the web site, http://www.stanleyresearch.org/programs/brain_collection.asp. Both schizophrenia and control groups consisted of 27 subjects. Diagnoses were made according to DSM-IV. A summary of the demographic information on these subjects is shown in Table 2. There were no significant demographic differences between schizophrenic and control brains (Torrey et al 2000). This study was performed unblinded.

RNA Preparation and cDNA Synthesis

Total RNA was extracted from the brain sample with an acid guanidium thiocyanate/phenol chloroform extraction method (ISOGEN; NIPPON Gene, Toyama, Japan). In the Australian samples, single-stranded cDNA was synthesized with SuperScript III RT (Invitrogen, Carlsbad, California) and oligo(dT) and random hexamers. For the Stanley Foundation brain collection samples, the extracted RNA was purified by RNAeasy column (Qiagen, Hilden, Germany). Single-stranded cDNA was synthesized by SuperScript II RT (Invitrogen) and oligo(dT) primers (Invitrogen) and converted to double-stranded cDNA by T4 DNA polymerase (Invitrogen).

Real-Time Quantitative PCR

The mRNA levels were determined by real-time quantitative PCR, with TaqMan universal PCR mastermix, transcript-specific minor groove binding (MGB) probes (Assays-on-Demand, Applied Biosystems), and an ABI 7900 sequence detection system, according to the manufacturer's instructions. The MGB probe for total *NTNG1* (detects all isoforms) was derived from exons 3 and 4 and that for *NTNG2* from exons 1 and 2. The probe specific to each *NTNG1* isoform is described in the corresponding sections.

The β2-microglobulin gene was chosen as a control from the TaqMan Human Endogenous Control Plate (Applied Biosystems) after evaluation of 11 available internal control probes. The PCR assay was performed simultaneously with test and standard samples and no template controls in the same plate. A standard curve plotting the cycle of threshold values against input quantity (log scale) was constructed for both the β2-microglobulin gene

Table 2. Demographic and Treatment Data for the Stanley Schizophrenic and Control Brains (BA46)

Sample ID	Age (y)	Gender	PMI (h)	pH	Cause of Death	Lifetime Antipsychotics[a]	Sample ID	Age (y)	Sex	PMI (h)	pH	Cause of Death
Schizophrenic Patients (n = 27)							Control Subjects (n = 27)					
S1	45	F	52	8.51	Sucide: jumped	20,000	C1	49	M	48	8.5	Cardiac disease
S2	40	M	34	6.18	Pneumonia	75,000	C2	53	M	9	6.4	Cardiac disease
S3	51	M	43	6.63	Cardiac disease	130,000	C3	37	M	13	6.5	Cardiac disease
S4	19	M	28	6.73	Overdose	2,500	C4	51	M	31	6.7	Cardiac disease
S5	53	F	13	8.49	Cardiac disease	15,000	C5	53	M	28	6	Cardiac disease
S6	37	M	30	6.8	Cardiac disease	20,000	C6	38	F	33	6	Cardiac disease
S7	52	M	10	8.1	Cardiac disease	100,000	C7	38	F	26	6.7	Cardiac disease
S8	24	M	15	8.2	Sucide: overdose	12,000	C8	60	M	47	6.6	Cardiac disease
S9	39	M	80	6.6	Motor vehicle accident	120,000	C9	35	M	62	6.7	Myocarditis
S10	33	M	29	6.5	Cardiac disease	20,000	C10	34	M	22	6.48	Cardiac disease
S11	50	M	9	6.2	Cardiac disease	34,000	C11	45	M	29	6.94	Cardiac disease
S12	43	M	18	6.3	Cirrhosia	90,000	C12	34	F	24	6.67	Cardiac disease
S13	32	F	38	6.6	Sucide: jumped	10,000	C13	42	M	37	6.91	Cardiac disease
S14	35	M	47	6.4	Cardiac disease	200,000	C14	44	F	10	6.2	Cardiac disease
S15	44	M	32	6.67	Cardiac disease	20,000	C15	57	M	26	6.4	Cancer
S16	47	M	13	6.3	Acute pancreatitis	300,000	C16	45	M	18	8.81	Cardiac disease
S17	45	M	35	6.66	Cardiac disease	50	C17	49	M	23	6.93	Cardiac disease
S18	36	F	27	8.49	Sucide: hanging	600	C18	49	F	45	6.72	Cardiac disease
S19	53	M	38	8.17	Cardiac disease	120,000	C19	33	F	29	6.52	Asthama
S20	54	F	42	6.65	Pneumonia	400,000	C20	48	M	31	6.60	Cardiac disease
S21	44	F	26	8.58	Pulmonary thrombosis	50,000	C21	50	M	49	6.75	Cardiac disease
S22	39	M	28	6.6	Sucide: hanging	48,000	C22	32	M	13	8.57	Cardiac disease
S23	38	M	35	6.68	Overdose	15,000	C23	47	M	11	8.8	Cardiac disease
S24	43	M	65	6.67	Sucide: hanging	70,000	C24	48	M	31	6.67	Cardiac disease
S25	42	M	19	6.48	Cardiac disease	18,000	C25	48	M	24	6.91	Cardiac disease
S26	46	M	30	6.72	Pneumonia	200,000	C26	39	F	58	8.48	Cardiac disease
S27	59	F	38	6.93	Cardiac disease	30,000	C27	47	M	38	8.57	Cardiac disease
Mean ±	42.3 ±		32.1 ±	8.53 ±		78,524 ±		44.8 ±		29.7 ±	8.81 ±	
SD	9.1		16.3	.23		97,532		7.6		13.3	.28	

BA, Brodmann's Area; PMI, postmortem interval; F, female; M, male.
[a]Lifetime neuroteptic dose in fluphenazine milligram equivalent dose.

and the target molecules (*NTNG1* and *NTNG2*) for each PCR assay. All real-time quantitative PCR data was captured with SDS v2.2 (Applied Biosystems). The ratio of the relative concentration of the target molecule to β2-microgloblin gene (target molecule/β2-microgloblin gene) was calculated. We used the Mann-Whitney *U* test (two-tailed) to detect significant changes in gene expression levels for each gene.

Results

Genomic Organizations and Chromosomal Localizations of NTNG1 and NTNG2

The FISH analysis showed that human *NTNG1* and *NTNG2* map to chromosomes 1p13.3 and 9q34, respectively (Figure 1), in keeping with their database assignment, although the *NTNG2* probe showed weak signal at 12q24.3 in 20% of the cells. Genomic analysis demonstrated that the *NTNG1* spans 341 kilobases (kb) and is composed of 10 exons (Figure 2), whereas human *NTNG2* is encoded by eight exons and spans an interval of 82 kb (Figure 3). Both genes are located in schizophrenia linkage regions (Lewis et al 2003; Kaufman et al 1998).

Isoform Variation of NTNG1 Transcripts

Detection of human *NTNG1* mRNA is generally limited to brain and kidney by Northern analysis (Lin et al 2003). An initial assay of *NTNG1* splice products in human adult brain detected at least nine alternatively spliced transcripts, involving exons 5, 6, 7, 8, and 9 (Figure 4). The relative abundance of each isoform in postmortem brain samples is shown in Figure 5A. The main isoforms in the brain were G1a, G1c, and G1d (Figure 5B).

SNP Identification and Genetic Analyses

Our mutation screening and database search detected a total of 21 SNPs in *NTNG1* (2 novel SNPs in our mutation screening) (Figure

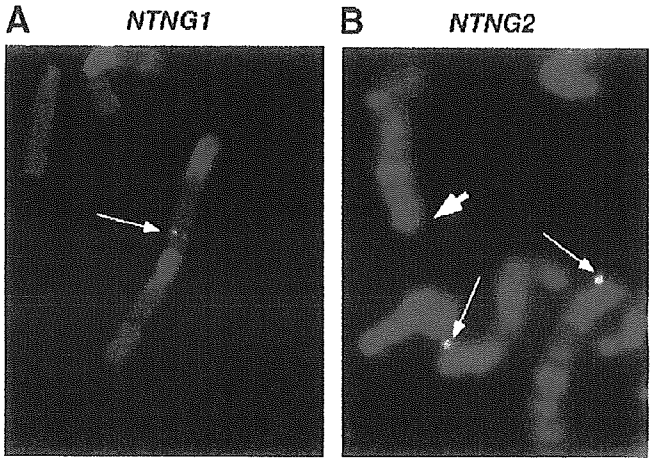

Figure 1. Fluorescence in situ hybridization of netrin-G1 **(A)** and netrin-G2 **(B)** sequences to normal metaphase human chromosomes. **(A)** The arrow denotes the positive signal at 1p13. **(B)** The thin arrows show the signal at 9q34, and the thick arrow shows weak signal at 12q24.

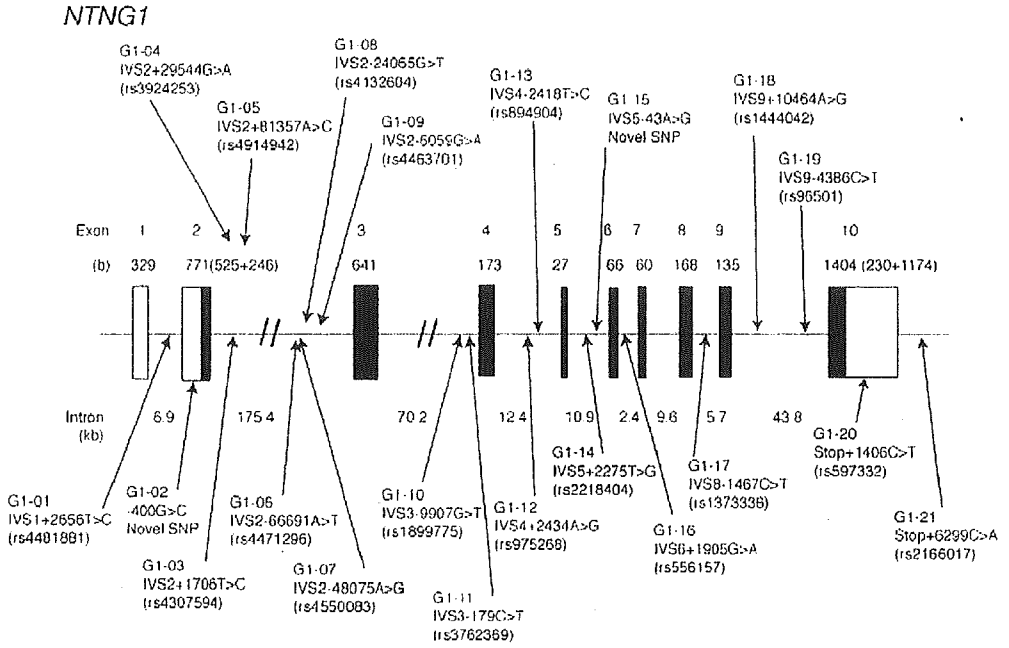

Figure 2. Genomic structure and location of polymorphic sites for human *NTNG1*. Exons are denoted by boxes, with untranslated regions in white and translated regions in black. The sizes of exons (bp) and introns (kb) are also shown. The rs number of each single nucleotide polymorphism (SNP) is the National Center for Biotechnology Information SNP cluster identification number from the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/).

2) and 10 SNPs in *NTNG2* (6 SNPs in our screening) (Figure 3). SNPs -400G>C (G1-02) and IVS5-43A>G (G1-15) in *NTNG1* and IVS4-15G>A (G2-09) in *NTNG2* were novel. No TaqMan typing probe could be designed for G2-09 in *NTNG2*. All other SNPs were unambiguously typed, with the exceptions of IVS4+2434A>G (G1-12) and Stop+1406C/T (G1-20) in *NTNG1*. The detailed information on these SNPs including heterozygosity is shown in Table 3. For subsequent genetic analyses, we chose 12 SNPs in *NTNG1* and 8 SNPs in *NTNG2*, on the basis of their relative abundance, with minor allele frequencies greater than .04 (Table 3).

Two different pairwise LD statistics, D' (normalized D) and r^2 (squared correlation coefficient) values, both of which measure LD values between 0 and 1, were computed between markers within the two genes, in 186 unrelated individuals from the present pedigree panel. Abecasis et al (2001) suggested a D' value greater than .33 as the minimum useful amount of LD, whereas Nakajima et al (2002) designated $r^2 > .1$ as the criterion for useful LD. In *NTNG1*, LD blocks with moderate strength exist in the intervals of G1-01 through to G1-03 and G1-11 through to G1-18 (Table 4). In *NTNG2*, intermediate LD measured by D',

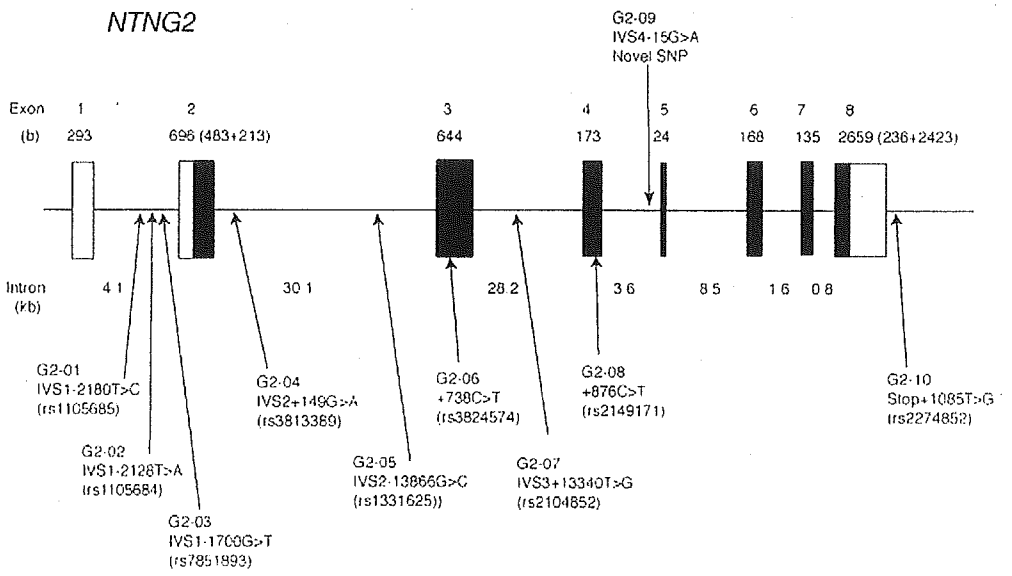

Figure 3. Genomic structure and locations of polymorphic sites for human *NTNG2*. Exons are denoted by boxes, with untranslated regions in white and translated regions in black. The sizes of exons (bp) and introns (kb) are also shown. The rs number of each single nucleotide polymorphism (SNP) is the National Center for Biotechnology Information SNP cluster identification number.

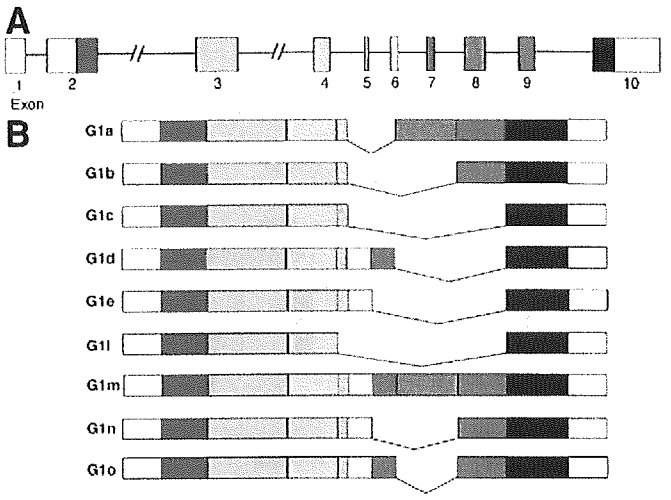

Figure 4. Splicing patterns of *NTNG1*. **(A)** All exons of *NTNG1*, with untranslated regions denoted in white and translated regions in color. **(B)** The nine different messenger ribonucleic acid isoforms.

was observed among the markers in the 5′ region of the gene (G2-01–G2-06) (Table 5).

Table 6 shows the results of family-based association tests for the entire pedigree panel. The PDT program computes two statistical measures, PDT-sum and PDT-ave. Briefly, PDT-sum gives more weight to larger families, whereas PDT-ave places equal weight on all families. The suitability of both statistical methods depends on family structure and genetic models (Martin et al 2001). In *NTNG1*, the SNP G1-19 showed significant association with schizophrenia (nominal p = .02) by PDT-sum. In *NTNG2*, the SNP G2-02 displayed significant association with schizophrenia with both PDT-sum (p = .008) and PDT-ave (p = .014). The SNPs G2-03 (p = .022) and G2-06 (p = .045) detected nominally significant associations by PDT-sum. After corrections for multiple SNP testing, the empirical p values for SNP transmission of *NTNG1* were .703 (PDT-ave) and .416 (PDT-sum), and those for *NTNG2* were .144 (PDT-ave) and .060 (PDT-sum). To test the SNP associations in a more stringent manner, we performed ETDT analysis on the 80 complete triad families. This detected a significant association between the G2-02 of *NTNG2* and schizophrenia (p = .045) (Table 6).

The transmission of individual haplotypes (haplotypic p) was assessed with the TRANSMIT program (Table 6). Significant distortions of haplotype transmission were observed for multiple haplotype blocks in *NTNG1* and *NTNG2* based on haplotypic p values, but the global p values were significant only with the (G1-14)–(G1-17)–(G1-18) haplotype of *NTNG1* (p = .049) and with the G2-02~G2-03 haplotype of *NTNG2* (p = .049). After Bonferroni correction for multiple testing, these transmission deviations were not significant. Further inspection of individual haplotypes revealed that the G-C-A haplotype (G1-14~G1-17~G1-18) of *NTNG1* was significantly less frequently transmitted (p = .002) and the A-T haplotype (G2-02~G2-03) of *NTNG2* was excessively transmitted (p = .016) to patients (Table 7), suggesting a protective role for the former and a risk factor for the latter.

Expression Analyses of NTNG1 and NTNG2 in Postmortem Brains

We quantified *NTNG1* and *NTNG2* mRNA expression levels in the two frontal regions BA11 (lateral orbitofrontal cortex) and BA46 (DLPFC) and one temporal region BA22 (superior temporal cortex) from schizophrenic and control postmortem brains, using MGB reactions. We analyzed six BA11 and six BA22 tissues each from schizophrenic patients and control subjects and 27 BA46 samples each from patients and control subjects. For *NTNG1*, transcript expressions were not significantly different between schizophrenic patients and control subjects in the three brain regions (Figure 6); nor were there significant differences in *NTNG2* expression levels between schizophrenic patients and normal subjects in the brain regions examined (Figure 6).

Expression Analysis of NTNG1 Isoforms

The *NTNG1* expression assay designed to detect the common exons 3 and 4 showed no significant changes between schizophrenic and control brains (Figure 6); however, because the haplotype spanning the alternatively spliced exons in *NTNG1* and composed of genetic variants (G1-14, G1-17, and G-18)

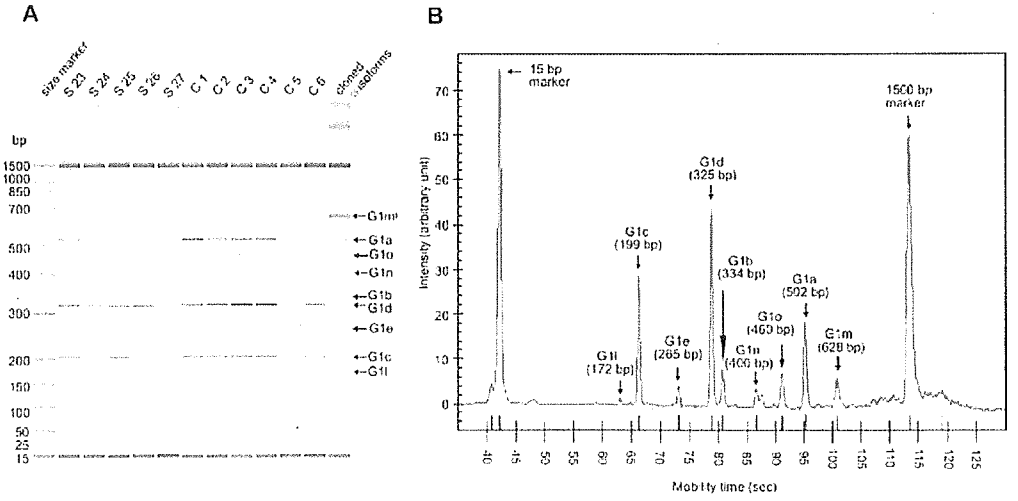

Figure 5. Demographic examples of semiquantitative analysis of *NTNG1* isoforms by the Agilent 2100 bioanalyzer using the DNA1000 LabChip kit. **(A)** Each analysis was represented by one lane in the gel-like image. The brain samples were Brodmann's Area 46 from the Stanley Foundation brain collection. Each sample number corresponds to the "Sample ID" shown in Table 2. The 15-bp and 1500-bp size markers were included in all samples for calibration of size and total amount. **(B)** The analysis of the S25 sample is presented as an electropherogram.

Table 3. Allele Frequencies for SNP Markers Located Within *NTNG1* and *NTNG2*

Marker No.	Polymorphism[a]	Genome Location by UCSC (bp)[b]	Distance from Neighboring SNP (bp)	Minor Allele Frequency[c]	Heterozygosity[c]
NTNG1		Chromosome 1p13.3			
G1-01	IVS1+2656T/C	107398460		.339	.448
G1-02	−400G/C	107402858	4398	.095	.171
G1-03	IVS2+1706T/C	107405209	2351	.289	.411
G1-04	IVS2+29544G/A	107433047	27838	.209	.330
G1-05	IVS2+81357A/C	107484861	51814	.048	.091
G1-06	IVS2−66691A/T	107512251	27390	0	0
G1-07	IVS2−48075A/G	107530867	18616	0	0
G1-08	IVS2−24065G/T	107554881	24014	.462	.497
G1-09	IVS2−6059G/A	107572887	18006	0	0
G1-10	IVS3−9907G/T	107639911	67024	0	0
G1-11	IVS3−179C/T	107649639	9728	.238	.363
G1-12	IVS4+2434A/G	107652424	2785	ND	
G1-13	IVS4−2418T/C	107659928	7504	.238	.363
G1-14	IVS5+2275T/G	107664647	4719	.238	.363
G1-15	IVS5−43A/G	107873201	8554	.035	.068
G1-16	IVS6+1905G/A	107675214	2013	.016	.032
G1-17	IVS8−1467C/T	107689862	14648	.347	.453
G1-18	IVS9+10464A/G	107701927	12085	.323	.437
G1-19	IVS9−4386C/T	107730889	28962	.194	.312
G1-20	Stop+1406C/T	107736910	6021	ND	
G1-21	Stop+6299C/A	107741803	4893	0	0
NTNG2		Chromosome 9q34			
G2-01	IVS1−2180T/C	132069110		.054	.102
G2-02	IVS1−2128T/A	132069162	52	.173	.286
G2-03	IVS−1700G/T	132069590	428	.284	.407
G2-04	IVS2+149G/A	132072134	2544	.071	.132
G2-05	IVS2−13866G/C	132089041	16907	.116	.205
G2-06	738C/T	132103431	14390	.390	.476
G2-07	IVS3+13340T/G	132116890	13459	0	0
G2-08	876C/T	132131808	14918	.465	.498
G2-09	IVS4−15G>A	132135498	3690	ND	
G2-10	Stop+1085T/G	132148138	12640	.446	.494

SNP, single nucleotide polymorphism; ND, genotypes were not unambiguously determined.
[a]Second allele is a minor allele.
[b]Data on May, 2004; UCSC: University of California, Santa Cruz.
[c]Based on the genotype data from 186 unrelated subjects.

showed a preferential transmission to schizophrenic probands (global $p = .049$) (Table 6), we examined the composition of mRNA isoforms in BA46 of postmortem brains in a semiquantitative manner, using the Agilent bioanalyzer system (Figure 5A). Because semiquantitave analysis suggested differential expressions of the three main isoforms, G1a, G1c, and G1d, between schizophrenic and control brains (data not shown), we measured these transcripts in a more stringent manner, by design-

Table 4. Pairwise Marker-to-Marker LD Statistics of *NTNG1*

SNP Marker	G1-01	G1-02	G1-03	G1-04	G1-05	G1-08	G1-11	G1-13	G1-14	G1-17	G1-18	G1-19
G1-01		.939[a]	.887[a]	.386[a]	.180	.046	.052	.052	.052	.039	.200	.084
G1-02	.189[a]		1.000[a]	.069	.873[a]	.671[a]	.036	.036	.036	.214	.114	.013
G1-03	.628[a]	.260[a]		.150	.193	.231	.055	.055	.055	.104	.074	.153
G1-04	.020	.002	.002		.067	.670[a]	.006	.006	.008	.084	.020	.084
G1-05	.003	.004	.005	.001		1.000[a]	.144	.144	.144	.429[a]	.013	.217
G1-08	.001	.041	.019	.101	.042		.319[a]	.319[a]	.319[a]	.057	.238	.083
G1-11	.000	.000	.000	.000	.003	.027		1.000[a]	1.000[a]	1.000[a]	.901[a]	.143
G1-13	.000	.000	.000	.000	.003	.027	1.000[a]		1.000[a]	1.000[a]	.901[a]	.143
G1-14	.000	.000	.000	.000	.003	.027	1.000[a]	1.000[a]		1.000[a]	.901[a]	.143
G1-17	.001	.002	.008	.001	.005	.002	.166[a]	.166[a]	.166[a]		.960[a]	.253
G1-18	.010	.001	.001	.000	.000	.031	.120[a]	.120[a]	.120[a]	.233[a]		.148
G1-19	.001	.000	.002	.006	.010	.002	.002	.002	.002	.008	.011	

For each pair of markers, the standardized D' is shown above the diagonal, and r^2 is shown below the diagonal. LD, linkage disequilibrium; SNP, single nucleotide polymorphism.
[a]D' values of >.3 and r^2 values of >.1.

Table 5. Pairwise Marker-to-Marker LD Statistics of *NTNG2*

SNP Marker	G2-01	G2-02	G2-03	G2-04	G2-05	G2-06	G2-08	G2-09
G2-01		1.000[a]	1.000[a]	.025	1.000[a]	1.000[a]	.661[a]	.092
G2-02	.012		1.000[a]	1.000[a]	.049	.794[a]	.119	.154
G2-03	.146[a]	.506[a]		.787[a]	.448[a]	.443[a]	.116	.164
G2-04	.000	.017	.121[a]		.046	.416[a]	.280	.418[a]
G2-05	.008	.002	.010	.000		.643[a]	.080	.215
G2-06	.036	.199[a]	.122[a]	.020	.085		.136	.004
G2-08	.029	.003	.006	.005	.001	.014		.176
G2-10	.001	.004	.008	.011	.005	.000	.022	

For each pair of markers, the standardized D' is shown above the diagonal, and r^2 is shown below the diagonal. LD, linkage disequilibrium; SNP, single nucleotide polymorphism.

[a] D' values of >.3 and r^2 values of >.1.

Table 6. Transmission Analyses of Each SNP and Haplotype for *NTNG1* and *NTNG2* by PDT and ETDT

	PDT		ETDT	TRANSMIT							
Genes and Polymorphisms	SUM PDT	AVE PDT	Allele	Specific Haplotype p (2 SNPs)[a]	Global p[c]			Specific haplotype p (3 SNPs)[b]	Global p[c]		
NTNG1											
G1-01	.337	.168	.172	.162							
G1-02	.199	.156	.222	.371	.099			.177			
G1-03	.284	.247	.117		.216	.039[d]		.492	.061 .396		
G1-04	1.000	.509	.504			.177	.181			.026[d] .303	
G1-05	.695	1.000	.667	.124			.402				.095 .517
G1-08	.758	.992	.392	.256	.335			.207 .563			
G1-11	.833	.626	.891		.795	.809			.361 .809		
G1-13	.753	.542	1.000			.809	.809			.809 .809	
G1-14	.753	.542	1.000	.045[d]			.809				.045[d] .112
G1-17	.120	.172	.199	.112	.068			.002[a] .049[d]			
G1-18	.855	.647	.692		.289	.154			.094 .261		
G1-19	.020[d]	.088	.095			.284					
NTNG2											
G2-01	.655	.571	.761	.046[d]							
G2-02	.008[d]	.014[d]	.045[d]	.119	.018[d]			.016[d] .098			
G2-03	.022[d]	.061	.264		.049[d]	.062			.016[d] .057		
G2-04	.748	.571	1.000	.056		.090				.043[d] .098	
G2-05	.121	.118	.076	.143	.019[d]						.041[d] .068
G2-06	.045[d]	.055	.199		.088	.099		.035[d] .362			
G2-08	.421	.749	.535	.235		.373			.114 .707		
G2-10	.571	.724	.802	.664							

SNP, single nucleotide polymorphism; PDT, pedigree disequilibrium test; ETDT, extended transmission DT.

[a] For two-marker analysis, a sliding window of two markers was tested, with one-marker overlap.

[b] For three-marker analysis, a sliding window of three markers was tested, with two-marker overlap.

[c] The global p values represent the overall significance when the observed versus expected frequencies of all of the haplotypes are considered together.

[d] $p < .05$.

Table 7. Transmission of Individual *NTNG1* and *NTNG2* Haplotypes that Showed Global Significance in Transmission Analyses

Gene and Haplotype	Observed Transmissions	Expected Transmissions	Var (O-E)	χ^2	*p* Value (1 df)
NTNG1 (G1-14)-(G1-17)-(G-18)					
G-C-A	7.0	15.3	7.0	9.741	.002[a]
T-C-A	36.0	35.0	13.0	.077	.781
G-T-A	63.0	56.7	18.3	2.145	.143
G-C-G	51.0	50.5	16.7	.016	.900
T-C-G	1.0	1.0	.5	.000	.989
G-T-G	2.0	1.5	.8	.325	.569
NTNG2 (G2-02)-(G2-03)					
T-G	107.0	111.9	15.9	1.518	.218
A-G	0	.5	.3	1.028	.311
T-T	18.0	20.6	6.8	.981	.322
A-T	35.0	27.0	11.0	5.817	.016[a]

[a] $p < .05$.

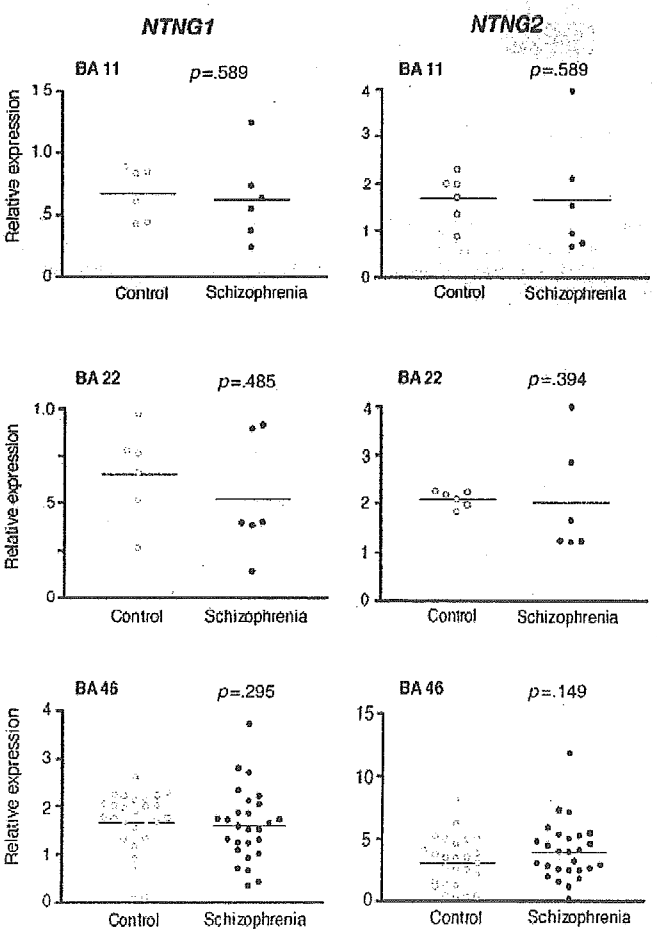

Figure 6. Messenger ribonucleic acid levels for *NTNG1* and *NTNG2* in the indicated brain regions (Brodmann's Area [BA]11, BA22, and BA46) of control and schizophrenic subjects. Each data point represents the relative expression level against that of the internal control (β2-microgloblin) for each sample. Horizontal bars indicate the mean value. BA11 and BA22 samples were from the Victorian Institute of Forensic Medicine in Australia (n = 6 for each group) and BA 46 were obtained from the Stanley Foundation brain collection (n = 27 for each group). The p values were calculated by Mann-Whitney U test (two-tailed).

ing isoform-specific PCR primers and TaqMan MGB probes (Figure 7). This quantitative analysis showed that the expression levels of G1c (p = .013) and G1d (p = .015) were significantly decreased in schizophrenic compared with control brains (Figure 7).

Discussion

In contrast to the classical netrin genes, netrin-G1 and -G2 are specific for vertebrates. In situ hybridization studies in mice show that *Ntng1* and *Ntng2* expression overlaps in limited brain areas (Nakashiba et al 2000, 2002). Mouse *Ntng1* is expressed with greatest abundance in the dorsal thalamic nuclei and the inferior colliculus, followed by perforant pathways of the entorhinal cortex and the olfactory pathway in the piriform cortex. Strikingly, mouse *Ntng2* expression occurs widely in the cerebral cortex and in the habenular nucleus and superior colliculus but not in regions that show *Ntng1* positivity. Northern analysis of human tissue also indicates that in the brain, *Ntng1* is expressed maximally in thalamic regions (Lin et al 2003). This spatial dissociation is suggestive of a specific role for each gene in these areas, and the strong expression in the thalamus and cortical areas hint that the role of netrin-Gs could include maintaining neuronal plasticity associated with sensory and/or cognitive functioning (Nakashiba et al 2002).

Abnormalities in measures of attention and cognition have been observed in patients with schizophrenia. These deficits might be linked to impairments in sensorimotor gating, whereby intrusive, overwhelming stimuli are improperly filtered (Braff and Geyer 1990). Interestingly, *Ntng1* knockout mice exhibit a reduced level of prepulse inhibition (Nishimura et al 2004), a behavioral paradigm that examines sensory filtering function (Geyer et al 2001). Furthermore, in the same study of mice lacking *Ntng1*, animals displayed an augmented behavioral sensitization to repeated administration of a noncompetitive NMDA receptor antagonist, dizocilpine (MK-801), and mice devoid of *Ntng1* or *Ntng2* showed reduced NMDA receptor–mediated postsynaptic responses in electrophysiologic analysis of brain slices (Nishimura et al 2004 and unpublished data). These data demonstrate that the proteins Ntng1 and Ntng2 are imperative for NMDA receptor function, lending further support to the disturbance of NMDA neurotransmission hypothesis for schizophrenia.

Our association analysis of the *NTNG1* gene detected a nominal association of the most 3′ variant G1-19 with schizophrenia. More importantly, the haplotype block constructed

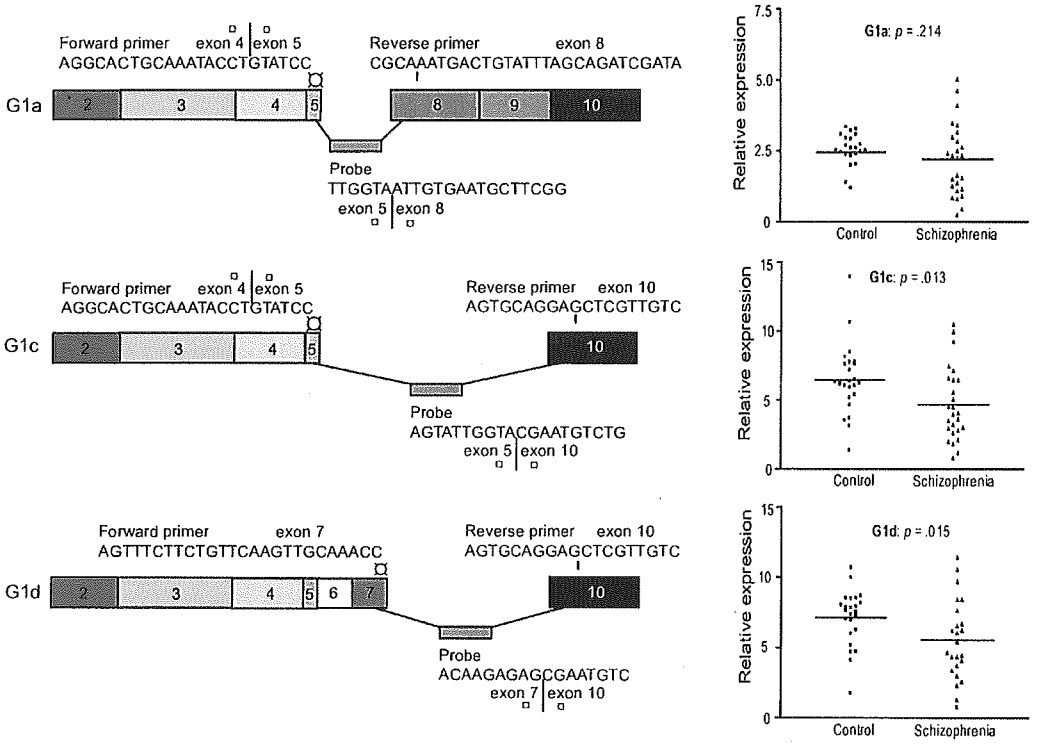

Figure 7. Isoform-specific expression analyses of *NTNG1*. **(A)** The design of polymerase chain reaction primers and TaqMan minor groove binding probes to analyze the three main isoforms G1a, G1c, and G1d. **(B)** Results of quantitative analysis of *NTNG1* isoforms. Each data point represents the relative expression level of a splice variant against that of the internal control (β2-microgloblin) for each brain sample. The data for failed amplifications are omitted. Horizontal bars indicate the mean value. The brain samples were Brodmann's Area 46 from the Stanley Foundation brain collection. The *p* values were calculated by the Mann-Whitney *U* test.

by G1-14~G1-17~G1-18, located in the 3′ region of the gene showed a globally significant excess of transmission to the disease. These results suggest that a potential disease-causing variant(s) might reside in the 3′ region of the gene. The mouse genomic structure of *Ntng1* is very similar to that of the human orthologue, as is the generation of multiple mRNA isoforms by alternative splicing (Nakashiba et al 2000). In humans, splicing involves the exons 5, 6, 7, 8, and 9. These exons are within the same LD block and overlap with the associated haplotype block. Therefore it is tempting to speculate that aberrant transcript processing might underpin this genetic association. In support of this speculation, splice variant netrin-G1c and -G1d distributions were significantly different between the DLPFC (BA46) of schizophrenic patients and control subjects. Although netrin-G1c and -G1d bind equally well to the NTNG1 ligand (NGL-1), the first molecule known to interact extracellularly with NTNG1 (Lin et al 2003; Nishimura et al, unpublished data), these isoforms might have distinct affinities for other unknown interacting molecules.

Both individual SNP and haplotype transmissions suggest a possible association between *NTNG2* and disease. The association signals were clustered within the same LD block located in the 5′ region of the gene. These results imply that the risk variant(s) might influence the regulation of gene expression. Our quantification assay, however, did not detect significantly different expression of *NTNG2* mRNA in BA46 of schizophrenic and control brains. A larger number of brain samples need to be examined before firm conclusions regarding differential gene expression between groups (schizophrenic vs. control) and regarding haplotype-dependent expressional levels of both *NTNG1* and *NTNG2* in each group can be drawn. Having stated this, it is practically and technically difficult to determine the exact haplotypes of genetically independent brain samples in the case of *NTNG1* (the SNPs G1-14, G1-17, and G1-18 of *NTNG1* span approximately 40 kb in the genome).

The anatomic significance of the frontal cortex in the pathogenesis of schizophrenia has long been recognized (Wong 2003). The DLPFC circuit involves the DLPFC in the cortex, the dorsolateral caudate nucleus of the striatum, the lateral dorsal medial nucleus of the globus pallidus, the posterolateral nucleus of the substantia nigra, and the ventral anterior, medial dorsalis, pars parvocellularis subnuclei of the dorsal medial nucleus of the thalamus. Defects in any of these structures could produce alterations in intrinsic and extrinsic functional connectivity that might be related to the symptomology of schizophrenia (Bunney et al 2000). In this context, the present findings of a potential disturbance of at least *NTNG1* gene regulation at the transcriptional level might suggest a molecular contribution by netrin-G gene(s) to the disrupted higher-order brain functions in schizophrenia.

In conclusion, our data suggest a possible involvement of human *NTNG1* and *NTNG2* in the vulnerability to schizophrenia; however, to draw a robust conclusion regarding the disease-promoting role of these vertebrate-specific genes, further replication studies are warranted in independent samples. In addition, it would be interesting to include the gene for transmembrane protein NGL-1, which has recently been identified as a specific binding partner of NTNG1 (Lin et al 2003), in future genetic studies to determine the role of the netrin-G system in schizophrenia.

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This article is dedicated to Dr. Masayuki Fukasawa (1973–2004).

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Appendix 1. PCR Primers Used to Search for Nucleotide Variants in *NTNG1* and *NTNG2*

Region	Exon Length (bp)	Primers	Product Size (bp)	3′ End of Primer
NTNG1				
Exon 2	771	(F) 5′-TGCTTTATATTGCATCAGACCTC-3′	480	−603 (intron 1)
		(R) 5′-GACCTCAAAGCAGATCCCAAAA-3′		−167 (exon 2)
		(F) 5′-AGTATGTTAGGCTTCCACCAA-3′	568	−218 (exon 2)
		(R) 5′-GTCTTCACACTCACCACATC-3′		+309 (intron 2)
Exon 3	641	(F) 5′-TAGGGCAAATAAAAATGA-3′	584	+175568 (intron 2)
		(R) 5′-AAAAACGCGAACCTGTC-3′		+176118 (exon 3)
		(F) 5′-ACGAACATGGCAGCCCTATCAG-3′	529	+175966 (exon 3)
		(R) 5′-AATGCCTTCAGAACCTACT-3′		+178445 (intron 3)
Exon 4	173	(F) 5′-GGCCTGCAAATCTATCTCTTACTA-3′	511	+246439 (intron 3)
		(R) 5′-GATGACTGAATTTTACTGAT-3′		+246858 (intron 4)
Exon 5	27	(F) 5′-TGCACCTGTATTTTGTGTGTGTGC-3′	283	+258962 (intron 4)
		(R) 5′-CCTATTACATCAGAAATGGACACT-3′		+259198 (intron 5)
Exon 6	66	(F) 5′-AATTGCTTGCTCTTGTT-3′	347	+269832 (intron 5)
		(R) 5′-TTTCAAAGACATAGCATTCAT-3′		+270142 (intron 6)
Exon 7	60	(F) 5′-CTTAATTTAGGGCTACTTTTCA-3′	254	+272404 (intron 6)
		(R) 5′-TACACTTCACAGATATTCCAGATT-3′		+272813 (intron 7)
Exon 8	168	(F) 5′-ATGCCATTCCACCGTCTTT-3′	406	+282063 (intron 7)
		(R) 5′-AGGATATTTTCTACATTGAG-3′		+282431 (intron 8)
Exon 9	135	(F) 5′-TCATTAATGGACATCTTT-3′	352	+287968 (intron 8)
		(R) 5′-GGATCTTTTTCTGCTCTGA-3′		+288282 (intron 9)
Exon 10	1404	(F) 5′-GGCTGAAAACATGATGTACCAGATG-	453	+331947 (intron 9)
		(R) 5′-AGGCCTTCTTAGTTTGTACACTGTC-		+332351 (exon 10)
NTNG2				
Exon 2	698	(F) 5′-GTTTGCAAAGCTTCAGTGCTCG-3′	432	−133 (intron 1)
		(R) 5′-CAAGGATCTCCTCCAGACCTCCT-3′		+257 (intron 2)
Exon 3	644	(F) 5′-ACAGAGCAGGTTTCTCGGTTCG-3′	494	+31048 (intron 2)
		(R) 5′-GAGTACTCCTCGGTGCAGAGCA-3′		+31499 (exon 3)
		(F) 5′-CTGGCAGCCCTACCAGTTCTA-3′	469	+31421 (exon 3)
		(R) 5′-ACCAGGTAACATCCCAGGTATC-3′		+31848 (intron 3)
Exon 4	173	(F) 5′-GGAATCAAGGAGTTTCTGGCCT-3′	418	+59870 (intron 3)
		(R) 5′-ATCCACAGCTCCTGGGATTGA-3′		+60248 (intron 4)
Exon 5	24	(F) 5′-CACCCCCGTCCCCACAC-3′	320	+63577 (intron 4)
		(R) 5′-GGCCCAAGTCCCAGAGG-3′		+63864 (intron 5)
Exon 6	168	(F) 5′-CCTGGGGTGAGTCCTTCC-3′	273	+72205 (intron 5)
		(R) 5′-TGCCGTGTCCGTGCCCTCTC-3′		+72435 (intron 8)
		(F) 5′-AACCGCTGCAGCTACATTGA-3′	247	+72316 (exon 6)
		(R) 5′-ACAGTCTCCCCAGGTGATTCT-3′		+72523 (intron 6)
Exon 7	135	(F) 5′-AGTCTTCTCTCCAGGCCTGGCCA-3′	444	+73900 (intron 6)
		(R) 5′-TCTTGCTAGGCCCAGTCCACC-3′		+74301 (intron 7)
Exon 8	2659	(F) 5′-AGCCTCCTACATCCCCGGCCCA-3′	489	+75766 (intron 7)
		(R) 5′-GCCCTGCTGAGTAGCACCTGGGA-3′		+757167 (exon 8)

Nucleotide positions are counted from A of the start codons on the genomic stretches of *NTNG1* (GenBank accession No. NM_014917) and *NTNG2* (NM_032536). F, forward; R, reverse.

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Extended analyses support the association of a functional $(GT)_n$ polymorphism in the *GRIN2A* promoter with Japanese schizophrenia

Yoshimi Iwayama-Shigeno[a], Kazuo Yamada[a], Masanari Itokawa[a,b], Tomoko Toyota[a], Joanne M.A. Meerabux[a], Yoshio Minabe[c], Norio Mori[c], Toshio Inada[d], Takeo Yoshikawa[a,*]

[a] *Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute, Saitama, Japan*
[b] *Department of Schizophrenia Research, Tokyo Institute of Psychiatry, Tokyo, Japan*
[c] *Department of Psychiatry and Neurology, Hamamatsu University School of Medicine, Hamamatsu, Japan*
[d] *Department of Psychiatry and Psychobiology, Nagoya University Graduate School of Medicine, Nagoya, Japan*

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Abstract

Dysfunction of the *N*-methyl-D-aspartate (NMDA) type glutamate receptor has been proposed as a mechanism in the etiology of schizophrenia. Recently, we identified a variable $(GT)_n$ repeat in the promoter region of the NMDA NR2A subunit gene (*GRIN2A*), and showed its association with schizophrenia in a case-control study, together with a correlation between the length of the repeat and severity of chronic outcome. In this study, we extended our analyses, by increasing the number of case-control samples to a total of 672 schizophrenics and 686 controls, and excluded potential sample stratification effects. We confirmed the significant allelic association between the repeat polymorphism and disease ($P=0.011$), and as in the previous study, we observed an over-representation of longer alleles in schizophrenia. These results suggest a probable genetic effect for the *GRIN2A* promoter $(GT)_n$ variation on the predisposition to schizophrenia in Japanese cohorts.

Keywords: NMDA receptor; NR2A subunit; Chromosome 16; Bipolar disorder; Linkage

Schizophrenia is a chronic and disabling brain disease of unknown etiology. Multiple genes with weak to moderate effects are thought to play a role in pathogenesis. A number of candidate genes for the disease have been proposed, of which, genes for the *N*-methyl-D-aspartate (NMDA) receptor are of special interest [19,22]. The NMDA receptor contains a non-competitive binding site for psychotomimetics, such as phencyclidine, MK-801 and ketamine. These antagonists trigger psychotic behavior, including both schizophrenia-like positive (delusions and hallucinations) and negative (affective blunting and specific cognitive defects) symptoms in normal subjects [10]. Conversely, the NMDA receptor co-agonists, such as glycine, D-serine and D-cycloserine, are reported to improve the negative symptoms of schizophrenia to varying degrees [6,11].

At least seven genes encoding NMDA receptor subunits have been identified in humans, NR1 (*GRIN1*), NR2A to D (*GRIN2A* to *D*) and NR3A and B (*GRIN3A* and *B*) [2]. Functionally distinct NMDA receptor subtypes are formed by heteromeric assembly of NR1 and NR2 subunits [16]. Expression of NR2A occurs predominately in the neocortices [13] and begins around puberty [20], corresponding to the period of schizophrenia onset. Mice lacking the ε1 subunit gene, orthologous to human *GRIN2A*, exhibited phenotypes resembling both the positive and negative symptoms of schizophrenia [12]. In a previous study, we identified a variable $(GT)_n$ repeat in the 5′-regulatory region of the *GRIN2A*, and showed that the repeat sequence repressed transcriptional activity in a length-dependent manner, such that the longer the repeat, the greater the repression of promoter activity [8]. Case-control genetic analysis detected marginally significant association of the repeat polymorphism with schizophrenia in Japanese cohorts ($P=0.05$), and showed that longer alle-

* Corresponding author. Tel.: +81 48 467 5968; fax: +81 48 467 7462.
E-mail address: takeo@brain.riken.jp (T. Yoshikawa).

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les were overrepresented in schizophrenics [8]. In this study, we seek to confirm this association between the *GRIN2A* promoter $(GT)_n$ polymorphism and schizophrenia by analyzing extended case-control samples while excluding potential population stratification.

All subjects examined in this study were derived from a geographic area located in the mid region of Japan. In this case-control study, subjects were increased from 375 schizophrenics and 378 controls, to 672 unrelated schizophrenics (mean age 45.4 ± 10.6 years) and 686 mentally healthy control subjects (mean age 44.8 ± 11.0 years), almost doubling the size of samples. Consensual diagnosis was made by at least two experienced psychiatrists according to criteria from the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV) [1]. The present study was approved by the Ethical Committee of RIKEN. Written informed consent was obtained from all participants after an explanation of the study.

DNA was extracted from whole blood according to a standard protocol. Amplification of the $(GT)_n$ repeat region was performed using the following primers: a FAM-labeled upstream primer, 5′-GAAGGAAGCATGTGGGAAATGCAG-3′ (the 3′ end is 88 bp upstream of the 5′ end of the GT repeat; see GenBank accession no. AF443855) and a non-labeled downstream primer, 5′-gtttcttGCTGGGTACAGTTATCCCCCT-3′ (the 3′ end is 19 bp downstream of the 3′ end of the GT repeat). The underlined tail sequence was added because Taq DNA polymerase catalyzes the non-templated addition of adenosine to the 3′ end of PCR products affecting the degree of product adenylation. By placing the sequence GTTTCTT at the 5′ end of reverse primers, nearly 100% adenylation of the 3′ end of the forward strand is achieved, facilitating accurate genotyping [7,8]. PCR was performed with an initial denaturation at 95 °C for 12 min, followed by 35 cycles of 94 °C for 15 s, 58 °C for 15 s and 72 °C for 30 s, and a final extension at 72 °C for 30 min, using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). PCR products were analyzed using an ABI 3700 sequencer equipped with GeneScan software (Applied Biosystems).

We assessed the population structure of case-control samples by genotyping 21 single nucleotide polymorphisms (SNPs). The markers were selected from a database of Japanese Single Nucleotide Polymorphisms (JSNP: http://snp.ims.u-tokyo.ac.jp/), Celera Discovery System (Celera: http://www.celeradiscoverysystem.com/) and Entrez SNP on NCBI (dbSNP: http://www.ncbi.nlm.nih.gov/SNP/) (these SNP information are available upon request). SNPs were scored using the Assays-on-Demand™ SNP genotyping system (Applied Biosystems, Foster City, CA), based on TaqMan assay methods [15]. Genotypes were determined using an ABI7900 sequence detection instrument (Applied Biosystems) and the SDS v2.0 software package (Applied Biosystems).

Fig. 1 shows the allelic distributions of the $(GT)_n$ in schizophrenia and control groups. In concordance with the previous finding, the allele distribution histogram of schizophrenic patients was shifted to the right, with longer alleles over-represented in schizophrenics. Because of the length-dependent suppressive effect of the $(GT)_n$ repeat on gene transcription [8] and the observed allele distribution patterns (Fig. 1), we divided the tandem repeat alleles into two classes according to length: short (25 or fewer repeats) and long (more than 25 repeats). The distributions of these two categories were significantly different between schizophrenia and control groups (Fisher's exact test, two-sided, $P = 0.014$, odds ratio = 1.23, 95% CI = 1.04–1.45). When we set the boundary of "short" alleles at 24 and "long" alleles at 25 repeats (cf. mean repeat sizes were 24.71 in schizophrenics and 24.66 in controls), the P value was 0.094. We assessed the overall differences of allelic distributions between the two groups using the CLUMP program (http://www.mds.qmw.ac.uk/statgen/dcurtis/software.html) [18]. We performed 10,000 Monte Carlo permutations to simulate the empirical significance levels of the statistics produced by the program, and this analysis gave significant

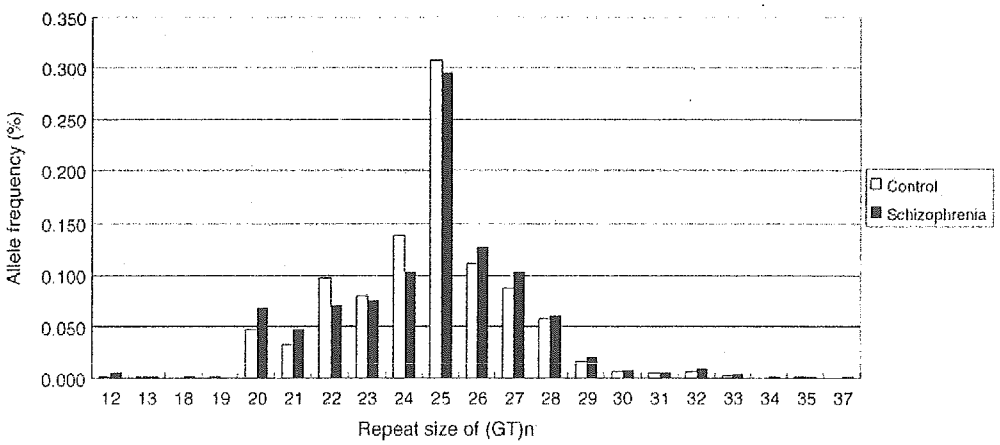

Fig. 1. Allele frequency distribution of the *GRIN2A* $(GT)_n$ repeat in schizophrenic patients and controls. Allele size is expressed as the number of GT repeats.

Table 1
Comparison of $(GT)_n$ allele distributions by the CLUMP program

	T1	T2	T3	T4
Chi-squared	34.75	28.86	8.25	19.32
d.f.	20	12	1	1
P value	0.022	0.0042	0.0041	0.000011
Empirical *P* value	0.011	0.011	0.045	0.011

P values are uncorrected for multi-allele testing. Empirical *P* values were simulated from 10,000 Monte Carlo permutations.

results in all T1–T4 statistics (empirical $P<0.05$) (Table 1). There was no deviation of genotypes from Hardy–Weinberg equilibrium, which was examined using Arlequin software (http://anthropologie.unige.ch/arlequin/methods.html) [17]. When we tested for single allelic association of common alleles [frequency > 3%: $(GT)_{20}$ to $(GT)_{28}$], Fisher's exact test gave the following *P* values: 0.029 for $(GT)_{20}$, 0.074 for $(GT)_{21}$, 0.009 for $(GT)_{22}$, 0.612 for $(GT)_{23}$, 0.004 for $(GT)_{24}$, 0.499 for $(GT)_{25}$, 0.187 for $(GT)_{26}$, 0.186 for $(GT)_{27}$ and 0.869 for $(GT)_{28}$.

We used the computer program STRUCTURE [14] to group individuals, in an attempt to identify genetically similar diploid subpopulations. Employing this Markov chain Monte Carlo method, 1,000,000 replications were used for the burn-in period of the chain and for parameter estimation. The number of populations present in the sample (*K*) is unknown, therefore, we ran the analysis at $K=1$, 2, 3, 4 and 5. The best estimate of *K* was found by calculating the posterior probabilities, $Pr(K=1, 2, 3, 4, 5)$, as described by Pritchard et al. [14]. The Japanese case-control samples in this study showed no evidence of a cryptic population structure, as demonstrated by $Pr(K=1)>0.99$.

In this study, we have replicated the previous association finding under more stringent conditions, by analyzing a larger panel of age-/sex-matched case-control samples and excluded the potential confounding factor of population stratification. Our recent genome-wide linkage disequilibrium scan of schizophrenia in Japanese pedigrees has revealed evidence of significant association with the marker *D16S423*, located at chromosomal region 16p13.1 and 3.8 Mb upstream from the *GRIN2A* $(GT)_n$ polymorphism [23]. Moreover, the Japanese Schizophrenia Sib-pair Linkage Group, a multisite collaborative, has identified 16p13.13 as a susceptibility region in schizophrenia (*D16S748*, located 1.9 Mb downstream of the $(GT)_n$ polymorphism) in a first-stage genome-wide scan [9]. Previously reported linkage data suggested that 16p13 might encode a susceptibility gene for bipolar disorder [4]. Edenberg et al. [5] have reported evidence of linkage to mood disorders with markers *D16S2618*, *D16S2619* and *D16S749* located at 16p12–13 in the National Institute of Mental Health (NIMH) initiative pedigrees. These markers map within 6.6 Mb upstream and 9.6 Mb downstream of the $(GT)_n$ repeat. We have recently examined the *GRIN2A* in the same NIMH panel and found evidence of a modest association [7]. Therefore, *GRIN2A* may be a potential susceptibility gene on 16p for both schizophrenia and affective disorders, consistent with the notion of a shared pathway in the predisposition to schizophrenia and bipolar disorder [3,21]. Further phenotypically defined examination of the *GRIN2A* repeat polymorphism is warranted.

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Identification of Multiple Serine Racemase (*SRR*) mRNA Isoforms and Genetic Analyses of *SRR* and *DAO* in Schizophrenia and D-Serine Levels

Kazuo Yamada, Tetsuo Ohnishi, Kenji Hashimoto, Hisako Ohba, Yoshimi Iwayama-Shigeno, Manabu Toyoshima, Akira Okuno, Hitomi Takao, Tomoko Toyota, Yoshio Minabe, Kazuhiko Nakamura, Eiji Shimizu, Masanari Itokawa, Norio Mori, Masaomi Iyo, and Takeo Yoshikawa

Background: *We previously reported a reduction in serum levels of D-serine, an endogenous co-agonist of the N-methyl-D-aspartate (NMDA) receptor, in schizophrenia, supporting the hypofunction hypothesis of NMDA neurotransmission in schizophrenia. In this study, we examined the genetic roles of serine racemase (SRR), an enzyme catalyzing the formation of D-serine from L-serine, and D-amino-acid oxidase (DAO) in the susceptibility to schizophrenia and the regulation of serum D-serine levels.*

Methods: *We determined the complete cDNA and genomic structures of SRR and performed mutation screening. Single nucleotide polymorphisms (SNPs) in SRR and DAO were tested for their association with schizophrenia in both case–control and family-based designs and for correlation with serum levels of D-serine.*

Results: *Genomic analyses revealed that human brain SRR transcripts consist of four isoforms with one major species, which were derived from alternative use of various 5' end exons. Genetic association analyses showed no significant association between SRR/DAO and schizophrenia. We replicated the decreased serum D-serine levels in schizophrenia in the sample set, but D-serine levels did not correlate with SRR/DAO genotypes.*

Conclusions: *The SRR/DAO are not likely to be major genetic determinants in the development of schizophrenia or control of serum D-serine levels.*

Key Words: Alternative splicing, genotype–phenotype correlation, glycine site, mutation screening, N-methyl-D-aspartate receptor, polymorphism.

The precise etiology of schizophrenia remains largely unknown, and the genetic determinants of the disease are complex, making identification of definitive susceptibility genes a formidable task (Gottesman 1991; Kendler 2005). Converging evidence, including genetic studies, has, however, supported the hypofunction hypothesis of glutamatergic neurotransmission via the N-methyl-D-aspartate (NMDA)-type glutamate receptor in schizophrenic brains. This hypothesis originally stemmed from clinical observations that phencyclidine and its congener anesthetic ketamine, both acting as noncompetitive antagonists of the receptor, evoke a schizophrenia-like psychosis including positive and negative symptoms in healthy control subjects and that phencyclidine exacerbates schizophrenic symptoms in patients (Javitt and Zukin 1991; Krystal et al 1999). The cognate NMDA receptor is heteromeric, consisting of the indispensable NR1 subunit and one of six possible subunits, NR2A, NR2B, NR2C, NR2D, NR3A, or NR3B (Andersson et al 2001; Dingledine et al 1999). Genetic variations in the receptor molecules themselves (Itokawa et al 2003; Miyatake et al 2003), and other molecules that may indirectly influence NMDA receptor function (for review, see Harrison and Owen 2003) have been reported to confer a risk for schizophrenia.

From the Laboratory for Molecular Psychiatry (KY, TO, HO, YI-S, MT, AO, HT, TT, TY), RIKEN Brain Science Institute, Wako, Saitama; Department of Psychiatry (KH, ES, MIy), Chiba University Graduate School of Medicine, Chiba; Department of Psychiatry and Neurology (YM, KN, NM), Hamamatsu University School of Medicine, Hamamatsu; Department of Schizophrenia Research (MIt), Tokyo Institute of Psychiatry, Tokyo, Japan.

Address reprint request to Takeo Yoshikawa, M.D., Ph.D., Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan; E-mail: takeo@brain.riken.go.jp.

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The receptor forms a cation channel, the opening of which is modulated by an arosteric glycine-binding site (Danysz and Parsons 1998; Leeson and Iversen 1994). The endogenous ligands for this strychnine-insensitive glycine site are deemed to include D-serine (Snyder and Ferris 2000). Importantly, therapeutic trials with D-serine have been shown to improve the positive and negative symptoms, and cognitive deficit, of patients with schizophrenia (Tsai et al 1998). These results suggest that endogenous D-serine could play an imperative role in the pathophysiology of schizophrenia (Goff and Coyle 2001). D-serine is synthesized by a glial serine racemase (SRR), a novel pyridoxal-5'-phosphate (vitamin B6)-dependent enzyme converting L-serine to D-serine in the mammalian brain (Schell 2004; Snyder and Ferris 2000; Wolosker et al 1999a, 1999b). Degradation of D-serine is mediated by D-amino acid oxidase (DAO), but this enzyme is not present in forebrain areas that are highly enriched for D-serine (Hashimoto et al, in press; Nagata 1992; Schell 2004). It is also known that glycine is converted to L-serine by the pyridoxal-5'-phosphate-dependent enzyme, serine hydroxymethyltransferase (Bauwe and Kolukisaoglu 2003). We recently reported that serum levels of D-serine and the ratio of D-serine to total serine were indeed significantly decreased in schizophrenia patients, suggesting that the activity of SRR may be reduced in schizophrenia (Hashimoto et al 2003). In this study, we first clarified the genomic architecture of the human *SRR* gene and then examined the genetic role of this gene in the susceptibility to schizophrenia and regulation of serum D-serine levels. We also studied the genetic contribution of the DAO, which catalyzes the oxidative deamination of D-amino acids, with the exception of D-aspartate and D-glutamate (which are oxidized by D-aspartate oxidase; Sacchi et al 2002), to the previously mentioned phenotypes.

Methods and Materials

Subjects

Fifty patients with schizophrenia (mean age 36.9 ± 14.2 years) and 52 healthy control subjects (mean age 30.3 ± 8.0

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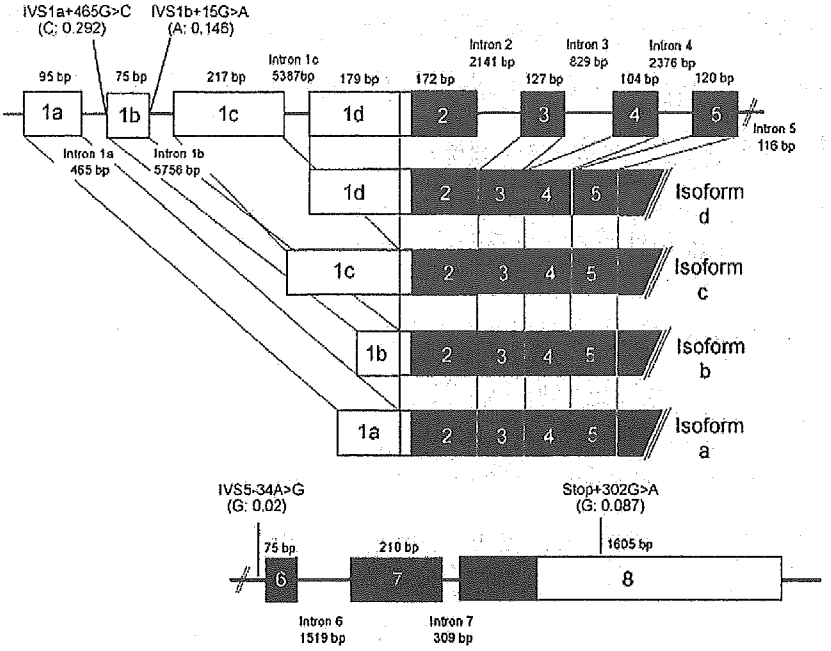

Figure 1. Genomic structure, transcript isoforms, and location of polymorphic sites for human *SRR*. Exons are denoted by boxes, with untranslated regions in white and translated regions in black. Note that the alternative use of 5′ end untranslated exons of 1a, 1b, 1c, and 1d generates four mRNA isoforms, of which isoform b is the major transcript in the brain.

years; hereafter referred to as sample set A) were used for evaluation of both serum levels of D and L serines and genotypes. Among these subjects, 31 schizophrenia patients (62%) and 44 normal control subjects (85%) were newly recruited in this study; the remainder were the same as those analyzed previously (Hashimoto et al 2003).

For a large-scale case–control genetic association study, an independent sample panel (referred to as sample set B) was used, which comprised 570 unrelated schizophrenia patients (285 men, 285 women; mean age 47.0 ± 11.4 years) and 570 age- and gender-matched control subjects who showed no history of mental illness in a brief psychiatric interview (285 men, 285 women; mean age 46.7 ± 11.1 years).

The third independent sample panel (referred to as sample set C), which was used for a family-based association test, consisted of 124 families with 376 members, of whom 163 were affected. This included 80 independent and complete trios (schizophrenic offspring and their parents), 15 probands with one parent, 13 probands with affected siblings, and 30 probands with discordant siblings.

All subjects resided in central Japan. A consensual diagnosis was made according to DSM-IV by at least two experienced psychiatrists on the basis of direct interviews, available medical records, and information from hospital staff and relatives. None of the patients had additional Axis I disorders as defined by DSM-IV.

The study was approved by the ethics committees of RIKEN and Chiba University Graduate School of Medicine. All control subjects and patients and family members gave informed written consent to participate in the study after provision and explanation of study protocols and purposes.

5′-RACE (Rapid Amplification of cDNA Ends) and Determination of the Genomic Structure for SRR

A partial cDNA sequence for *SRR* was obtained from GenBank (accession No. NM_021947) and the full length transcript was isolated by 5′-RACE using a brain-derived Marathon cDNA kit (BD Biosciences Clontech, Palo Alto, California), according to

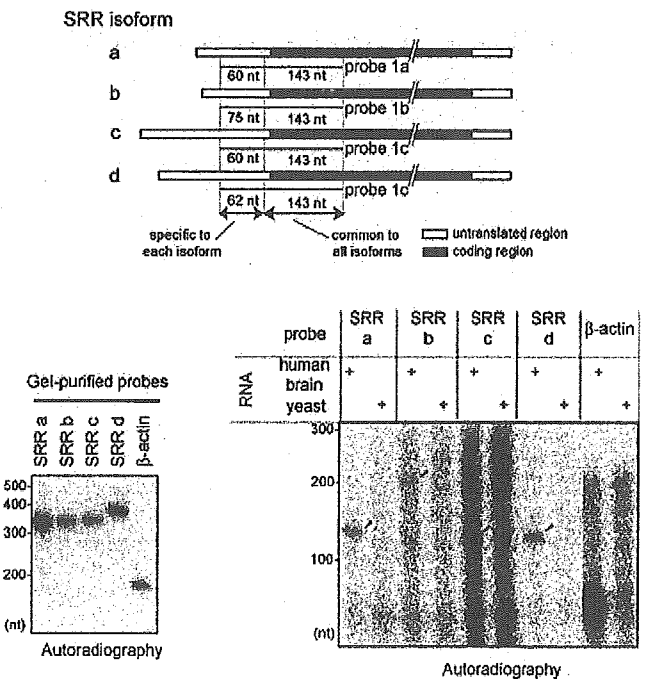

Figure 2. RNase protection assay of *SRR* transcripts. Upper panel shows *SRR* mRNA isoforms and positions of specific probes (probes 1a, 1b, 1c, and 1d). Filled boxes show the coding region of mRNA and open boxes indicate 5′- or 3′-untranslated regions. Note that all four probes consist of an isoform-specific region (60–75 nt) and common region (143 nt). Each probe is flanked by 71 nt at the 5′ end and 41 nt at 3′ end with vector derived sequences. The lower left panel is an autoradiogram, showing the integrity of radio-labeled riboprobes. Probes for *SRR* isoforms a–d and β-actin were gel-purified after synthesis and re-electrophoresed in a denaturing gel to evaluate the integrity of the probes. The lower right panel shows the results of the RNase protection assay. Each probe was hybridized to human total RNA or yeast total RNA (negative control), and then an RNase protection assay was performed. Arrowheads in the autoradiogram show the positions of specific signals.

Table 1. PCR Primers Used to Search for Nucleotide Variants in the SRR Gene

Region	Primers	Product Size (bp)	5′ End of Primer	Polymerase and Buffer
5′ Upstream of Exon 1a	(F) 5′-CTATTGCCTAACGCAGGAGAGGT-3′ (R) 5′-TCCTGCCTCCACCCTCTTTC-3′	440	−12593 (415 bp Upstream of Exon 1a) −12154 (Exon 1a)	Taq and Master Amp K
Exon 1a	(F) 5′-GGCGGCCTGGAGAGCGATA-3′ (R) 5′-TTCCCCCAGCAGTAGGCG-3′	267	−12301 (Upstream of Exon 1a) −12035 (Intron 1a)	Taq and Master Amp K
5′ Upstream of Exon 1b	(F) 5′-AGAAAGAGGGTGGAGGCAGG-3′ (R) 5′-CTGTCAACCCGAGTCCCAGA-3′	741	−12174 (Exon 1a, 556 bp Upstream of Exon 1b) −11434 (Intron 1b)	Taq and Master Amp K
Exon 1b	(F) 5′-TGGCCGCGCTGGGAGGAAAA-3′ (R) 5′-ACCCGAGTCCCAGACTAGCAGT-3′	261	−11700 (Intron 1a) −11440 (Intron 1b)	Platinum TaqPCRx and Enhancer Solution 1X
5′ Upstream of Exon 1c	(F) 5′-TGGAATCATCTCCCCAGCATTCTCC-3′ (R) 5′-GCCAGGGTATGAGGTCACTGAAGA-3′	450	−6217 (Intron 1b, 430 bp Upstream of Exon 1c) −5768 (Exon 1c)	Taq and Master Amp K
Exon 1c	(F) 5′-TCCTTTCCTCCCTCCTTAGG-3′ (R) 5′-TGGAGCTTCAGGAACTACTGG-3′	476	−5995 (Intron 1b) −5509 (Intron 1c)	Taq and Master Amp K
5′ Upstream of Exon 1d	(F) 5′-GAAAGGTGGAGCTGGGCACG-3′ (R) 5′-CTGGTGAAGCTTTGAGGGAAG-3′	422	−608 (Intron 1c, 425 bp Upstream of Exon 1d) −187 (Exon 1d)	Taq and Master Amp K
Exon 1d	(F) 5′-TGCCAACAGTGCCAAGAGATG-3′ (R) 5′-CATTGGGTGGTCAGGAGAATC-3′	528	−279 (Intron 1c) +249 (Intron 2)	Taq
Exon 2	(F) 5′-ACAGGCCCCAGGTCTATTCTG-3′ (R) 5′-TGCCTATCTCATTGGGTGGTCAG-3′	362	−104 (Intron 1) +258 (Intron 2)	Taq
Exon 3	(F) 5′-GGTGACAGATTGGATGTGCATG-3′ (R) 5′-CTTCCCACAGCTATCATCACTC-3′	326	+2202 (Intron 2) +2527 (Intron 3)	Taq
Exon 4	(F) 5′-ATTTCCTGACCTTGTGATCCGCCT-3′ (R) 5′-GCAGCCAGTAAGGTAGAAAGAGCC-3′	311	+3133 (Intron 3) +3443 (Intron 4)	Taq
Exon 5	(F) 5′-GACCAAATGGAACTTGTTGGGGA-3′ (R) 5′-ATTCCTCCTCCACCTACAGGTA-3′	351	+5678 (Intron 4) +6028 (Exon 6)	Taq
Exon 6	(F) 5′-CAATTGCCCTGGAAGTGCTGAA-3′ (R) 5′-GTGTGCAGAATGTTGAGCACGT-3′	309	+5840 (Exon 5) +6148 (Intron 6)	Taq
Exon 7	(F) 5′-GTGTTGGGATTACAGGTGTGAG-3′ (R) 5′-TTACCTAGTCAGGTTCCCGTG-3′	424	+7468 (Intron 6) +7891 (Intron 7)	Taq
Exon 8	(F) 5′-CTGGACACGTATTCTCATCTG-3′ (R) 5′-TATGTCAGCCTGTCAGTCCACT-3′	539	+8010 (Intron 7) +8548 (Exon 8)	Taq
Exon 8	(F) 5′-AAGCAGGCTGAAAGGCCAGCTT-3′ (R) 5′-CGAGTCTTCCCCAAATGGACTT-3′	469	+8269 (Exon 8) +8737 (Exon 8)	Taq
Exon 8	(F) 5′-AGTGGACTGACAGGCTGACATA-3′ (R) 5′-GATTCTAAGAGGATGTGCTGTGG-3′	501	+8527 (Exon 8) +9027 (Exon 8)	Taq
Exon 8	(F) 5′-CCATGGGTACCTAGAAAGACATC-3′ (R) 5′-AGCCTCAGATTGGGCTAGGGCA-3′	592	+8805 (Exon 8) +9396 (Exon 8)	Taq
Exon 8	(F) 5′-GATGGCCTGTAGCAATGAGGCT-3′ (R) 5′-TGCGCCAATCACTTTTCCTCCT-3′	560	+9240 (Exon 8) +9799 (Downstream of Exon 8)	Taq

F, forward; R, reverse.

Nucleotide positions are counted from A of the start codon on the genomic stretch of the *SRR* gene.

Taq polymerase was purchased from Takara (Tokyo, Japan). Master Amp buffer was from Epicentre (Madison, Wisconsin), and Platinum TaqPCRx/Enhancer Solution were from Invitrogen (Carlsbad, California).

the manufacturer's instructions. Polymerase chain reaction (PCR) amplifications were carried out using the Expand Long Template PCR System (Buffer 1; Roche, Indianapolis, Indiana), and the following two gene-specific primers located in exon 3 (Figure 1) and the AP1/AP2 primers supplied by the manufacturer: for the first PCR: 5′-AGCTTCTGACGGCATTGAGAGCACCACG (5′ end at nt +199 on the cDNA sequence; A of the initiation codon was designated as +1), and for the nested PCR: 5′-TTCGCGTTCCT-GCAGATAAGATTTG (5′ end at nt +287 on the cDNA sequence). The genomic structure was predicted by comparing the cDNA sequence with the *Homo sapiens* chromosome 17 genomic contig (accession No. NT_010718). This structure was confirmed by sequencing each exon and flanking intron portion of the genome (see Mutation Screening later in the article).

RNase Protection Assay (RPA) of SRR

Four DNA fragments corresponding to isoforms a, b, c, and d were amplified from Marathon human brain cDNA (BD Biosciences Clontech), using the following primer sets (see Figures 1 and 2): for isoform a, 5′-GGCCAGGCTCTCCCGGAGCT (forward) and 5′-CACATTTGAAGAAAAGATTGCG (named Ex2R, a reverse primer located in exon 2); for isoform b, 5′-GCT-