

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 $\beta 2$ -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 $\beta 2$ -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	Suicide; 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	Suicide; 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	Cirrhosis	90,000	C12	34	F	24	6.67	Cardiac disease
S13	32	F	38	6.6	Suicide; 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	Suicide; 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	Asthma
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	Suicide; 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	Suicide; 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.

^aLifetime neuroleptic 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 β 2-microglobulin gene (target molecule/ β 2-microglobulin 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

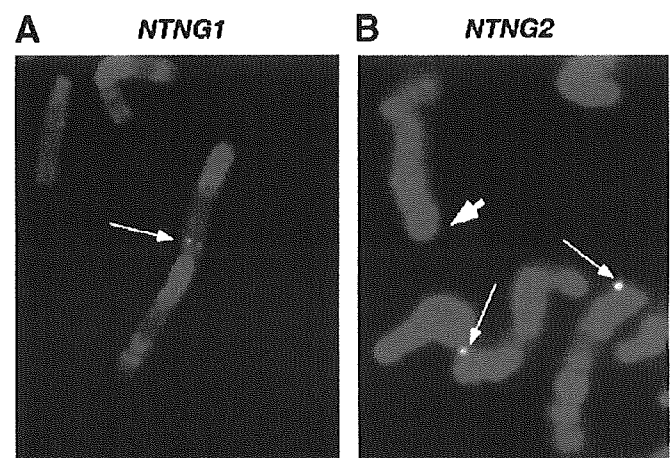


Figure 1. Fluorescence in situ hybridization of netrin-G1 (A) and netrin-G2 (B) sequences to normal metaphase human chromosomes. (A) The arrow denotes the positive signal at 1p13. (B) The thin arrows show the signal at 9q34, and the thick arrow shows weak signal at 12q24.3.

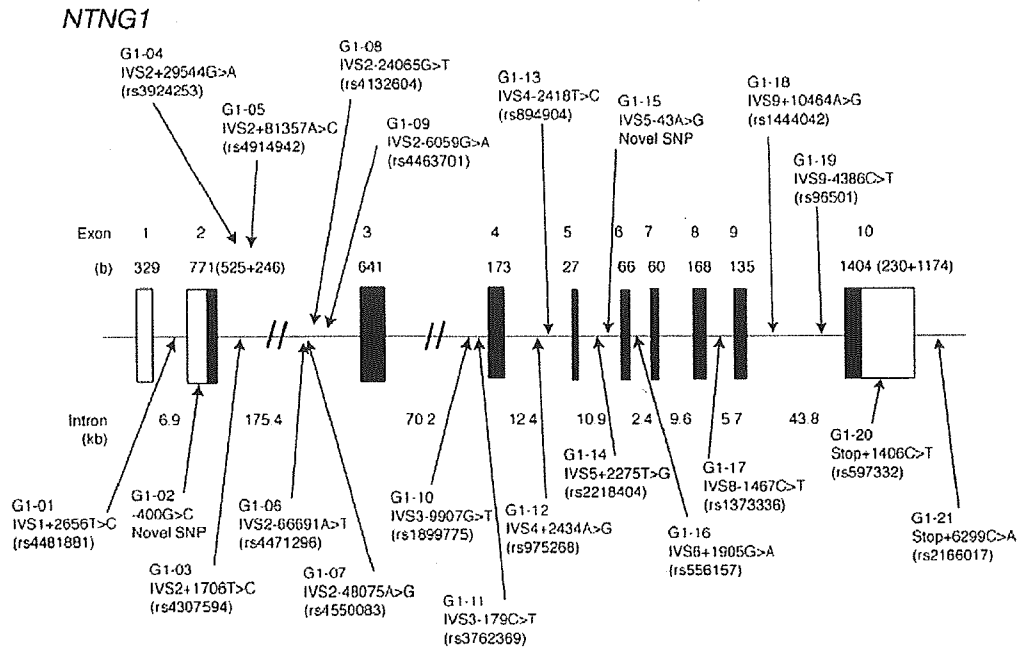


Figure 2. Genomic structure and location of polymorphic sites for human *NTNG1*. Exons are denoted by boxes, with untranslated regions in white and translated regions in black. The sizes of exons (bp) and introns (kb) are also shown. The rs number of each single nucleotide polymorphism (SNP) is the National 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' ,

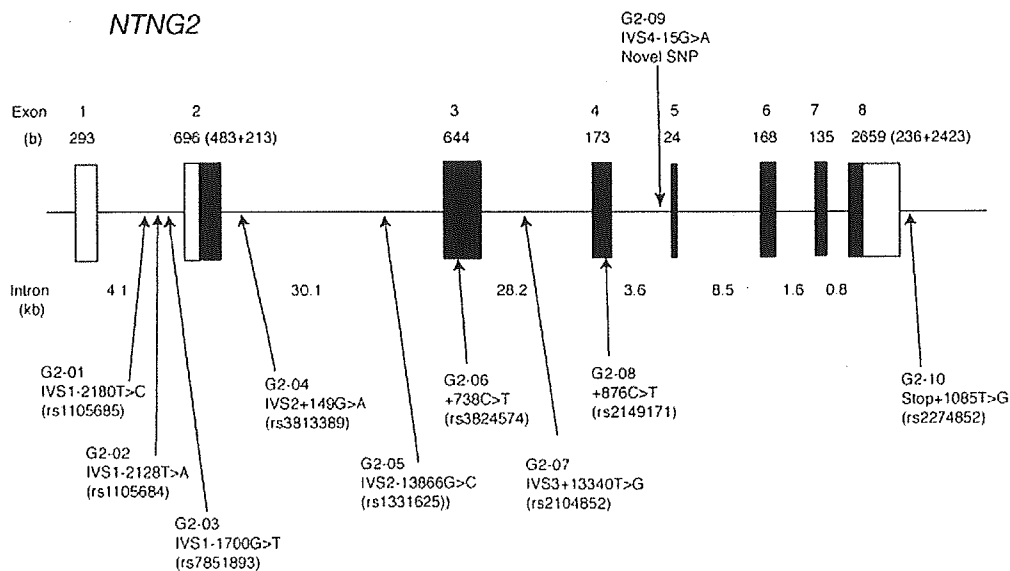


Figure 3. Genomic structure and locations of polymorphic sites for human *NTNG2*. Exons are denoted by boxes, with untranslated regions in white and translated regions in black. The sizes of exons (bp) and introns (kb) are also shown. The rs number of each single nucleotide polymorphism (SNP) is the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

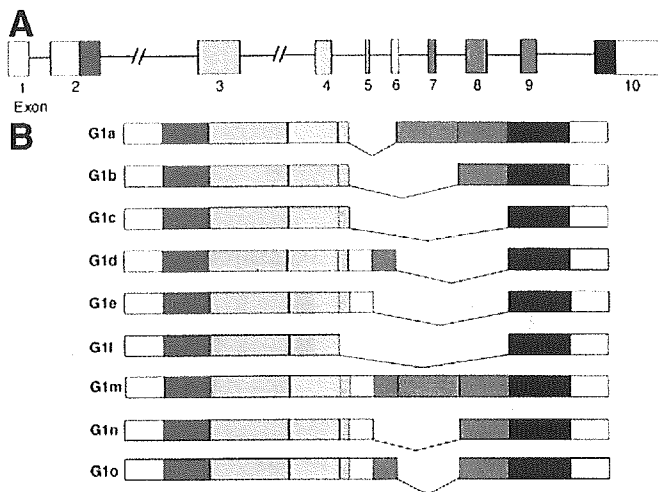


Figure 4. Splicing patterns of *NTNG1*. (A) All exons of *NTNG1*, with untranslated regions denoted in white and translated regions in color. (B) The nine different messenger ribonucleic acid isoforms.

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 ETD 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)

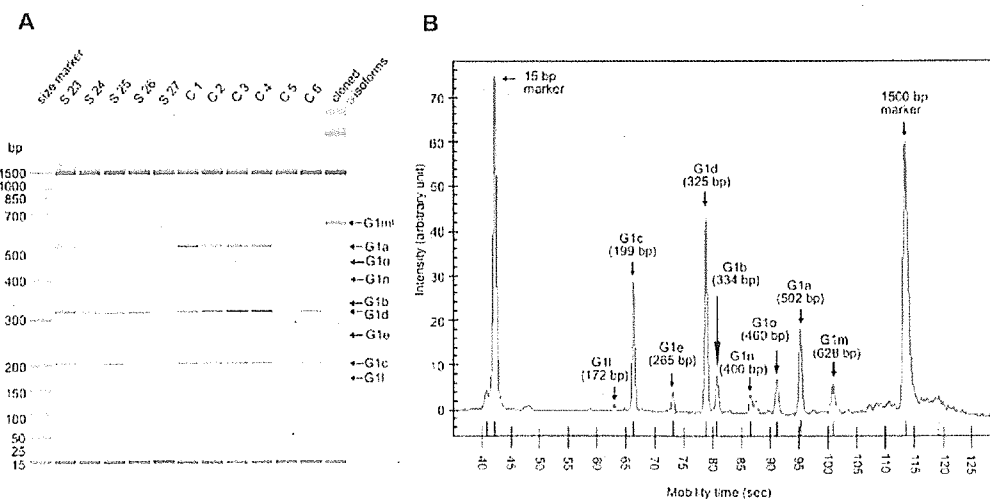


Figure 5. Demographic examples of semiquantitative analysis of *NTNG1* isoforms by the Agilent 2100 bioanalyzer using the DNA1000 LabChip kit. (A) Each analysis was represented by one lane in the gel-like image. The brain samples were Brodmann's Area 46 from the Stanley Foundation brain collection. Each 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
<i>NTNG1</i>					
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
<i>NTNG2</i>					
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.

^aSecond allele is a minor allele.

^bData on May, 2004; UCSC: University of California, Santa Cruz.

^cBased 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 semiquantitative 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

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

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

^aFor two-marker analysis, a sliding window of two markers was tested, with one-marker overlap.

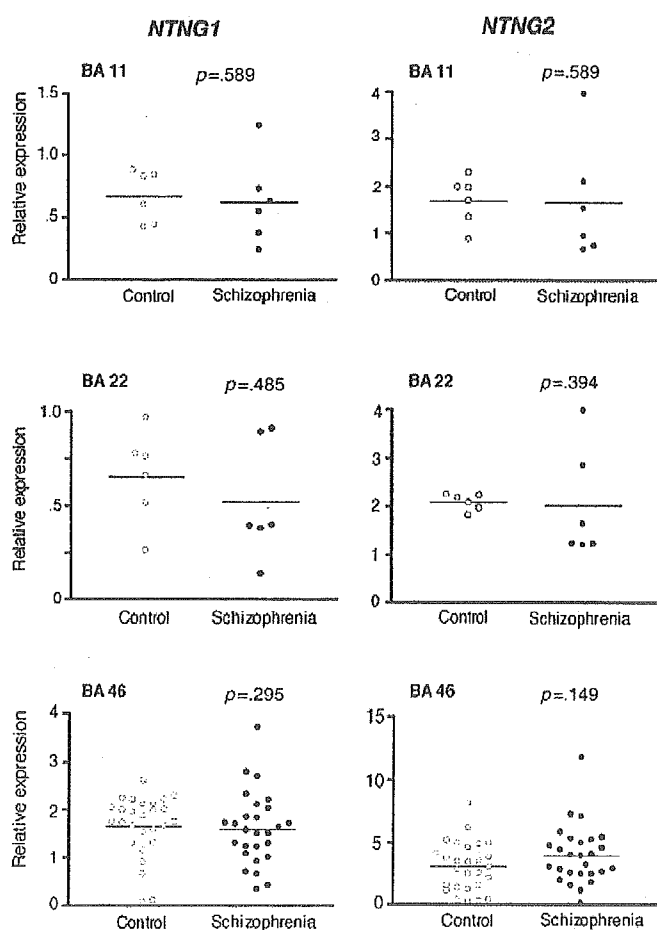
^bFor three-marker analysis, a sliding window of three markers was tested, with two-marker overlap.

^cThe 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)
<i>NTNG1</i> (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
<i>NTNG2</i> (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 (β -microglobulin) 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 *Ntn1* and *Ntn2* expression overlaps in limited brain areas (Nakashiba et al 2000, 2002). Mouse *Ntn1* 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 *Ntn2* expression occurs widely in the cerebral cortex and in the habenular nucleus and superior colliculus but not in regions that show *Ntn1* positivity. Northern analysis of human tissue also indicates that in the brain, *Ntn1* 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, *Ntn1* 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 *Ntn1*, animals displayed an augmented behavioral sensitization to repeated administration of a noncompetitive NMDA receptor antagonist, dizocilpine (MK-801), and mice devoid of *Ntn1* or *Ntn2* 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 Ntn1 and Ntn2 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

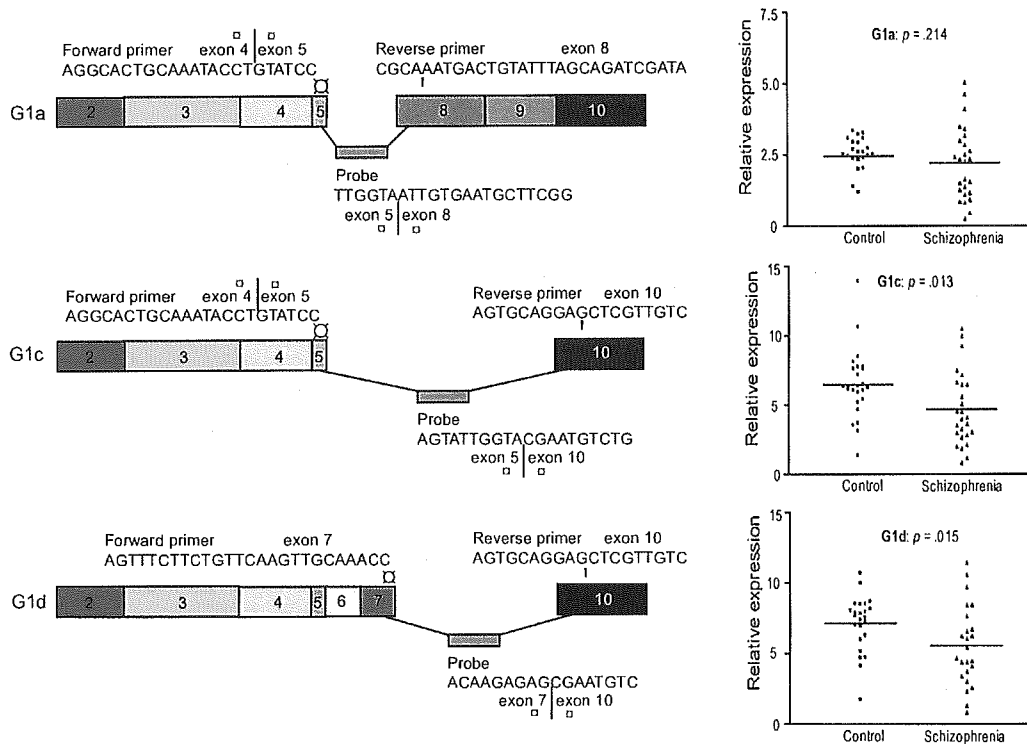


Figure 7. Isoform-specific expression analyses of *NTNG1*. (A) The design of polymerase chain reaction primers and TaqMan minor groove binding probes to analyze the three main isoforms G1a, G1c, and G1d. (B) Results of quantitative analysis of *NTNG1* isoforms. Each data point represents the relative expression level of a splice variant against that of the internal control (β 2-microglobulin) 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 dorso-lateral 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
<i>NTNG1</i>				
Exon 2	771	(F) 5'-TGCTTTATATTGCATCAGACCTC-3'	480	-603 (intron 1)
		(R) 5'-GACCTCAAAGCAGATCCCAAAA-3'		-167 (exon 2)
		(F) 5'-AGTATGTTAGGCTCCACCAA-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'-AAAACGCGAACCTGTC-3'		+176118 (exon 3)
		(F) 5'-ACGAACATGGCAGCCCTATCAG-3'	529	+175966 (exon 3)
		(R) 5'-AATGCCTTCAGAACCTACT-3'		+178445 (intron 3)
		(F) 5'-GGCCTGCAAATCTATCTCTACTA-3'	511	+246439 (intron 3)
Exon 4	173	(R) 5'-GATGACTGAATTTTACTGAT-3'		+246858 (intron 4)
		(F) 5'-TGCACCTGTATTTTGTGTGTGTC-3'	283	+258962 (intron 4)
Exon 5	27	(R) 5'-CCTATTACATCAGAAATGGACACT-3'		+259198 (intron 5)
		(F) 5'-AATTGCTTGCTCTGTG-3'	347	+269832 (intron 5)
Exon 6	66	(R) 5'-TTCAAAGACATAGCATTAT-3'		+270142 (intron 6)
		(F) 5'-CTAATTTAGGGCTACTTTTCA-3'	254	+272404 (intron 6)
Exon 7	60	(R) 5'-TACACTTCACAGATATTCAGATT-3'		+272813 (intron 7)
		(F) 5'-ATGCCATTCCACCGTCTTT-3'	406	+282063 (intron 7)
Exon 8	168	(R) 5'-AGGATATTTTCTACATTGAG-3'		+282431 (intron 8)
		(F) 5'-TCATTAATGGACATCTTT-3'	352	+287968 (intron 8)
Exon 9	135	(R) 5'-GGATCTTTTCTGCTCTGA-3'		+288282 (intron 9)
		(F) 5'-GGCTGAAAACATGATGTACCAGATG-3'	453	+331947 (intron 9)
Exon 10	1404	(R) 5'-AGGCCTCTTAGTGTACACTGTC-		+332351 (exon 10)
<i>NTNG2</i>				
Exon 2	698	(F) 5'-GTTTGCAAAGCTTCAGTGCTCG-3'	432	-133 (intron 1)
		(R) 5'-CAAGGATCTCCTCCAGACCTCCT-3'		+257 (intron 2)
Exon 3	644	(F) 5'-ACAGAGCAGGTTTCTCGGTTCCG-3'	494	+31048 (intron 2)
		(R) 5'-GAGTACTCCTCGGTGCAGAGCA-3'		+31499 (exon 3)
		(F) 5'-CTGGCAGCCCTACCACTTCTA-3'	469	+31421 (exon 3)
		(R) 5'-ACCAGGTAACATCCCAGGTATC-3'		+31848 (intron 3)
		(F) 5'-GGAATCAAGGAGTTTCTGGCCT-3'	418	+59870 (intron 3)
Exon 4	173	(R) 5'-ATCCACAGCTCCTGGGATTGA-3'		+60248 (intron 4)
		(F) 5'-CACCCCGTCCCCACAC-3'	320	+63577 (intron 4)
Exon 5	24	(R) 5'-GGCCCAAGTCCCAGAGG-3'		+63864 (intron 5)
		(F) 5'-CCTGGGGTGAGTCCTCC-3'	273	+72205 (intron 5)
Exon 6	168	(R) 5'-TGCCGTGTCGGTGCCTCTC-3'		+72435 (intron 8)
		(F) 5'-AACCGCTGCAGCTACATTGA-3'	247	+72316 (exon 6)
		(R) 5'-ACAGTCTCCCAGGTGATTCT-3'		+72523 (intron 6)
Exon 7	135	(F) 5'-AGTCTTCTCCAGGCCTGGCCA-3'	444	+73900 (intron 6)
		(R) 5'-TCTTGCTAGGCCAGTCCACC-3'		+74301 (intron 7)
Exon 8	2659	(F) 5'-AGCCTCTACATCCCCGGCCCA-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.



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

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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. © 2004 Elsevier Ireland Ltd. All rights reserved.

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 $\epsilon 1$ 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-

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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'-GAAGGAAGCATGTGGGA-AATGCAG-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.celera.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

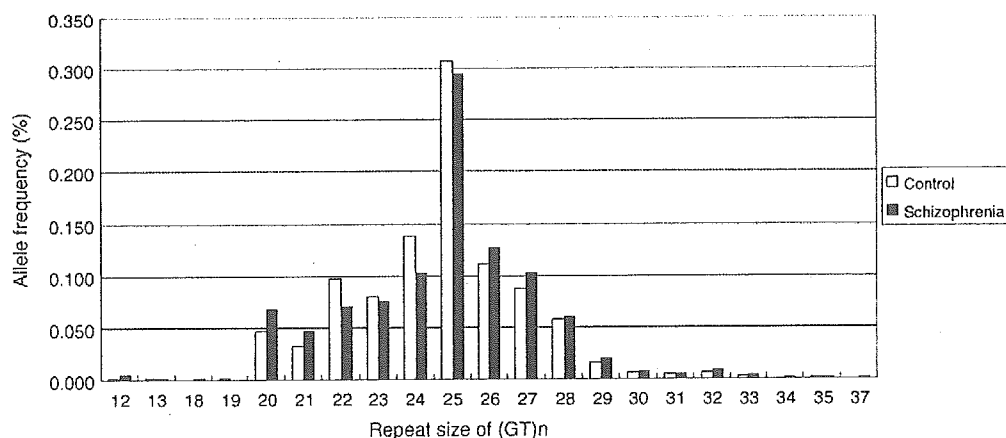


Fig. 1. Allele frequency distribution of the *GRIN2A* (GT)_n repeat in schizophrenic patients and controls. Allele size is expressed as the number of GT repeats.

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
<i>P</i> value	0.022	0.0042	0.0041	0.000011
Empirical <i>P</i> 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, $\text{Pr}(K = 1, 2, 3, 4, 5)$, 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 $\text{Pr}(K = 1) > 0.99$.

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 multi-site 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.

Acknowledgements

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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

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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.

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The receptor forms a cation channel, the opening of which is modulated by an allosteric 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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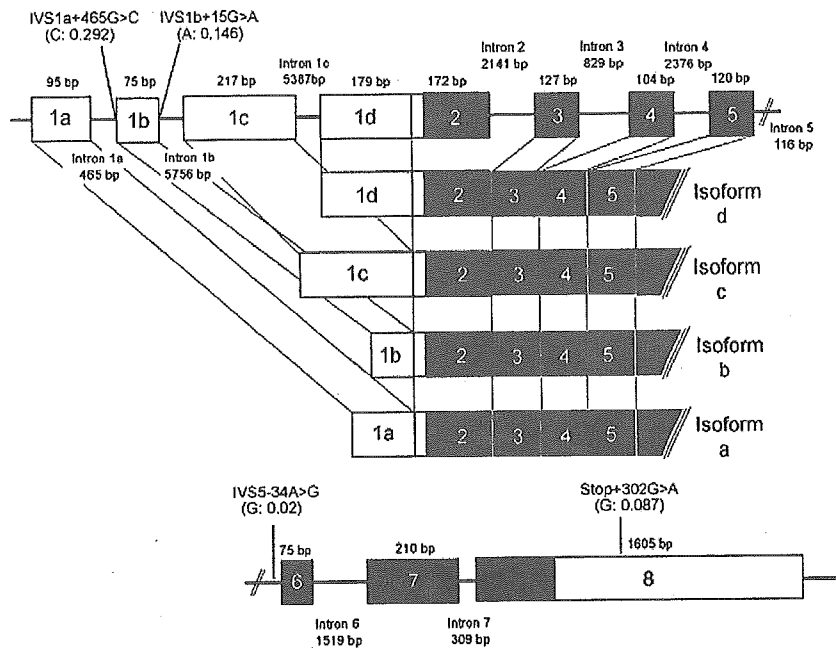


Figure 1. Genomic structure, transcript isoforms, and location of polymorphic sites for human *SRR*. Exons are denoted by boxes, with untranslated regions in white and translated regions in black. Note that the alternative use of 5' end untranslated exons of 1a, 1b, 1c, and 1d generates four mRNA isoforms,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

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***SRR* isoform**

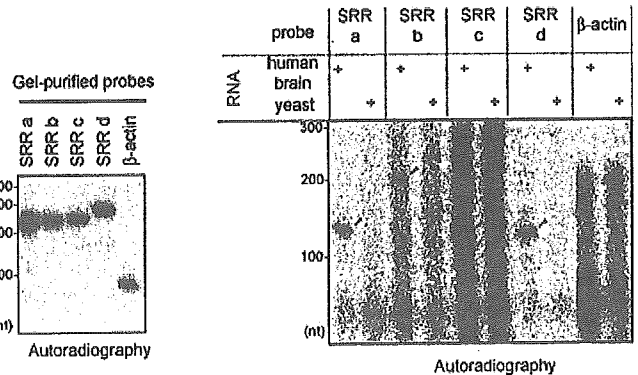
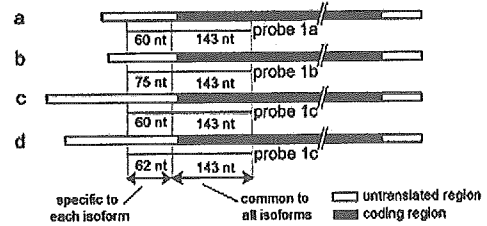


Figure 2. RNase protection assay of *SRR* transcripts. Upper panel shows *SRR* mRNA isoforms and positions of specific probes (probes 1a, 1b, 1c, and 1d). Filled boxes show the coding region of mRNA and open boxes indicate 5'- or 3'-untranslated regions. Note that all four probes consist of an isoform-specific 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'-TCTTGCCCTCCACCTCTTTC-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) -12174	Taq and Master Amp K
5' Upstream of Exon 1b	(F) 5'-AGAAAGAGGGTGGAGGCAGG-3'	741	(Exon 1a, 556 bp Upstream of Exon 1b)	Taq and Master Amp K
Exon 1b	(R) 5'-CTGTCAACCCGAGTCCCAGA-3' (F) 5'-TGGCCGCGCTGGGAGGAAAA-3' (R) 5'-ACCCGAGTCCCAGACTAGCAGT-3'	261	-11434 (Intron 1b) -11700 (Intron 1a) -11440 (Intron 1b)	Platinum TaqPCRx and Enhancer Solution 1X
5' Upstream of Exon 1c	(F) 5'-TGGAAATCATCTCCCAGCATTCTCC-3'	450	(Intron 1b, 430 bp Upstream of Exon 1c)	Taq and Master Amp K
Exon 1c	(R) 5'-GCCAGGGTATGAGGTCACTGAAGA-3' (F) 5'-TCCTTTCCTCCCTCCTTAGG-3' (R) 5'-TGGAGCTTCAGGAACTACTGG-3'	476	-5768 (Exon 1c) -5995 (Intron 1b) -5509 (Intron 1c)	Taq and Master Amp K
5' Upstream of Exon 1d	(F) 5'-GAAAGGTGGAGCTGGGCAGC-3'	422	(Intron 1c, 425 bp Upstream of Exon 1d)	Taq and Master Amp K
Exon 1d	(R) 5'-CTGGTGAAGCTTTGAGGGAAG-3' (F) 5'-TGCCCAACAGTGCCAAGAGATG-3'	528	-187 (Exon 1d) -279 (Intron 1c)	Taq
Exon 2	(R) 5'-CATTGGGTGGTCAGGAGAATC-3' (F) 5'-ACAGGCCCCAGGTCTATTCTG-3'	362	+249 (Intron 2) -104 (Intron 1)	Taq
Exon 3	(R) 5'-TGCCTATCTCATTGGGTGGTCAG-3' (F) 5'-GGTGACAGATTGGATGTGCATG-3'	326	+258 (Intron 2) +2202 (Intron 2)	Taq
Exon 4	(R) 5'-CTCCACAGCTATCATCACTC-3' (F) 5'-ATTTCTGACCTTGATCCGCCT-3'	311	+2527 (Intron 3) +3133 (Intron 3)	Taq
Exon 5	(R) 5'-GACCAAATGGAACCTGTTGGGGA-3' (F) 5'-ATTCTCTCCACCTACAGGTA-3'	351	+3443 (Intron 4) +5678 (Intron 4)	Taq
Exon 6	(R) 5'-CAATTGCCCTGGAAGTGCTGAA-3' (F) 5'-GTGTGCAGAATGTTGAGCACGT-3'	309	+6028 (Exon 6) +5840 (Exon 5)	Taq
Exon 7	(R) 5'-GTGTTGGGATTACAGGTGTGAG-3' (F) 5'-TTACCTAGTCAGGTTCCCGTG-3'	424	+6148 (Intron 6) +7468 (Intron 6)	Taq
Exon 8	(R) 5'-TATGTCAGCCTGTCACTCCACT-3' (F) 5'-AAGCAGGCTGAAAGGCCAGCTT-3'	539	+7891 (Intron 7) +8010 (Intron 7)	Taq
Exon 8	(R) 5'-CGAGTCTTCCCAAATGGACTT-3' (F) 5'-AGTGGACTGACAGGCTGACATA-3'	469	+8548 (Exon 8) +8269 (Exon 8)	Taq
Exon 8	(R) 5'-GATTCTAAGAGGATGTGCTGTGG-3' (F) 5'-CCATGGGTACCTAGAAAGACATC-3'	501	+8737 (Exon 8) +8527 (Exon 8)	Taq
Exon 8	(R) 5'-AGCCTCAGATTGGGCTAGGGCA-3' (F) 5'-GATGGCCTGTAGCAATGAGGCT-3'	592	+9027 (Exon 8) +8805 (Exon 8)	Taq
Exon 8	(R) 5'-TGCGCCAATCACTTTTCTCTCT-3'	560	+9396 (Exon 8) +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'-TTCGCGTTCCTGCAGATAAGATTTG (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-

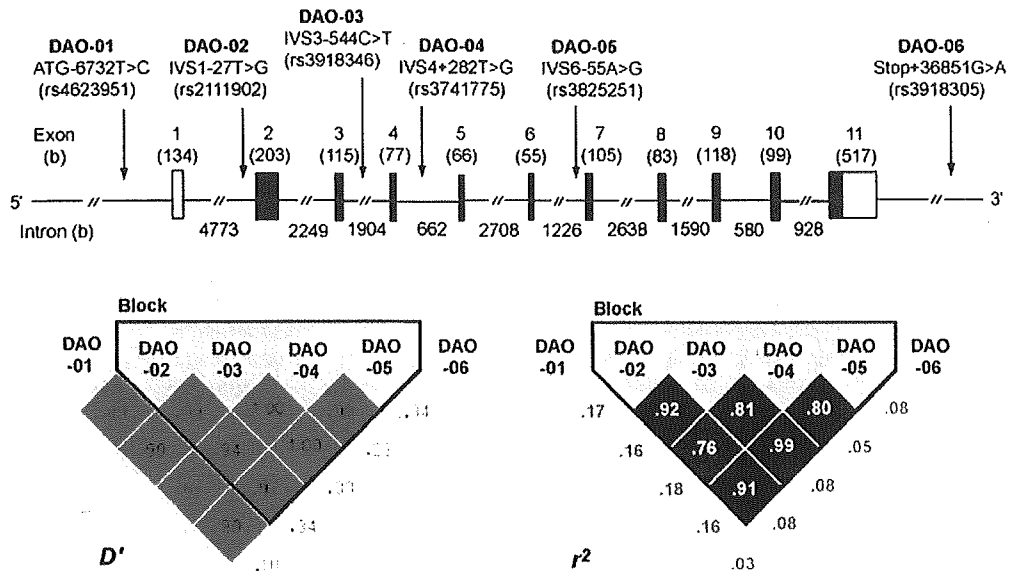


Figure 3. Genomic structure, location of polymorphic sites and haplotype block structure for human *DAO*. In the top panel, exons are denoted by boxes, with untranslated regions in white and translated regions in black. The sizes of exons (b) and introns (b) are also shown. The “rs” number of each single nucleotide polymorphism (SNP) is the National Center for Biotechnology Information SNP cluster ID from the dbSNP database (<http://www.ncbi.nlm.nih.gov/SNP/>). In the lower panel, the haplotype block structure of *DAO* is shown, with linkage disequilibrium parameters D' (left) and r^2 (right). The D' and r^2 values were calculated from the sample set B.

GCGCGTGCAGAGGTG and Ex2R; for isoform c, 5'-TTC-CATATGGAACCAACTCGCCT and Ex2R; for isoform d, 5'-CTC-TTCAATAAACATACTGTCTC and Ex2R. Note that these four fragments comprise a common 143 nt sequence and each isoform-specific 60–75 nt sequence. Similarly, a region common to the four isoforms, as well as β -actin, were amplified using the primer sets, 5'-GGCGTCAGAAGCTTGGTTCTTCT (forward)/5'-TGTCCCTTGCCAGCTATCACT (reverse), and 5'-GCGGACTATGAC TTAGTTGCGT (forward)/5'-TAAAGCCATGCCAATCTCATCTTG (reverse), respectively. These PCR amplicons were cloned into the pCR2.1 vector (Invitrogen, Carlsbad, California), and sequence integrity was confirmed. Antisense [32 P]-UTP-labeled riboprobes were synthesized using these constructs and the MAXIscript kit (Ambion, Austin, Texas). An RNase protection assay was performed using Human Brain Total RNA (BD Biosciences Clontech) or yeast total RNA, and the RPAIII kit (Ambion), according to the manufacturer's instructions.

Mutation Screening of *SRR*

Genomic DNA was isolated from blood samples using standard methods. The complete coding region, exon/intron boundaries, and the 5' upstream regions of *SRR* were screened for polymorphisms by direct sequencing of PCR products from 30 unrelated schizophrenia samples. The primers and DNA polymerases used for amplification are listed in Table 1. The PCR was performed with an initial denaturation at 94°C for 1 min, followed by 35 cycles at 94°C for 15 sec, 55°–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. Direct sequencing of PCR products was performed using the BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, Foster City, California) and the ABI PRISM 3730 Genetic Analyzer (Applied Biosystems). Polymorphisms were detected using the SEQUENCHER program (Gene Codes Corporation, Ann Arbor, Michigan).

Selection of Polymorphisms for *DAO*

For genetic analysis of *DAO*, we first chose three single nucleotide polymorphisms (SNPs) previously reported to be associated with schizophrenia (DAO-02, DAO-03, and DAO-04 in Figure 3; Chumakov et al 2002; Liu et al 2004; Schumacher et al 2004). To examine the 5' and 3'-regions of the gene more thoroughly, we selected SNPs from databases including the Celera Discovery System (Celera; <http://www.celera-discovery-system.com/>) and the Entrez SNP on NCBI (dbSNP; <http://www.ncbi.nlm.nih.gov/SNP/>). Three additional SNPs—DAO-01, DAO-05, and DAO-06 (Figure 3)—were analyzed after an examination for informativeness using 30 randomly chosen schizophrenic samples (minor allele frequency > .01). We found one missense polymorphism (992G>T, Gly331Val, rs4262766) in the database, but in our screening panel, this polymorphism was always Gly331.

SNP Genotyping

The SNPs were typed in all samples using the TaqMan system (Applied Biosystems). Probes and primers were designed using Assays-by-Design SNP genotyping (Applied Biosystems). The PCR reactions were performed in an ABI 9700 thermocycler and fluorescence was determined using an ABI 7900 sequence detector single point measurement and SDS v2.0 software (Applied Biosystems). Each marker was checked for allele-inheritance inconsistency within a pedigree of the sample set C using PEDCHECK software (O'Connell and Weeks 1998), and no inconsistencies were found, proving the accuracy of the genotyping.

Determination of Total Serine, D-Serine, and L-Serine

Measurement of total, D-, and L-serine levels was carried out according to established methods using a column-switching high-performance liquid chromatography (HPLC) system (Shi-

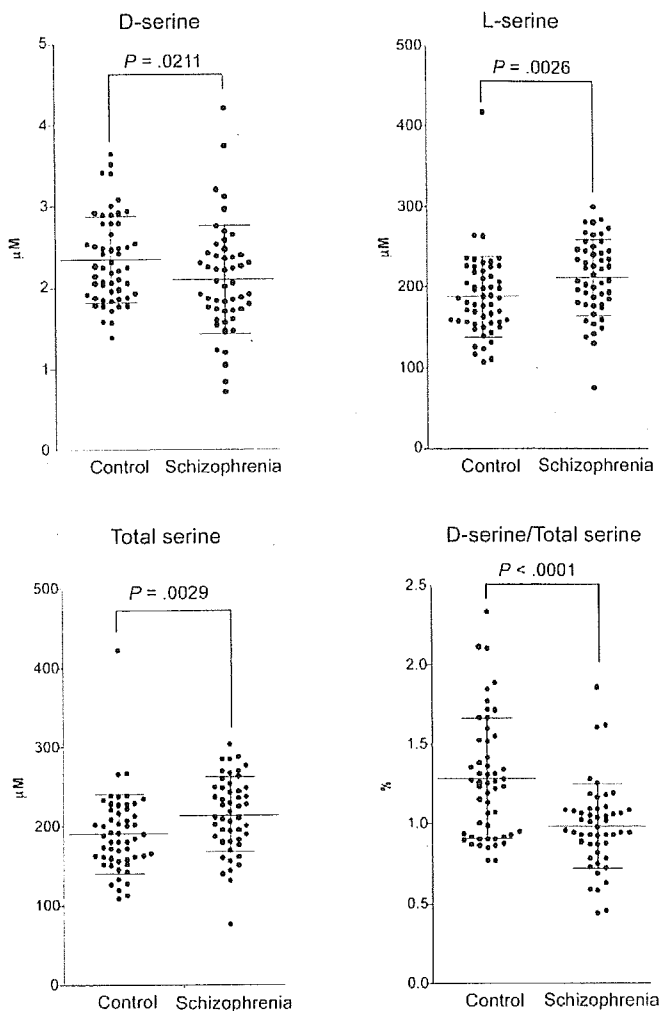


Figure 4. Significant differences in serum levels of serine isomers between healthy control subjects and schizophrenia patients. Examined samples included 52 control subjects and 50 patients with schizophrenia. Horizontal bars show mean \pm SD.

madzu Corporation, Kyoto, Japan). A 20- μ L aliquot of the human serum was added to 20 μ L of .1 M borate buffer (pH 8.0) and 60 μ L of 50 mmol/mL 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F; Tokyo Kasei Kogyo, Tokyo, Japan) in CH_3CN . The reaction mixture was then heated at 60°C for 1 min and immediately supplemented with 100 μ L of $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ (90/10) containing .1% trifluoroacetic acid (TFA) to stop the reaction. A 10- μ L aliquot of the resultant solution was injected into the HPLC system. A reversed-phase ODS column (TSKgel ODS-80T_s [Tosoh Corporation, Tokyo, Japan] as Column 1) was used for the separation and quantification of total (D- and L-) serine, and the gradient elution of the mobile phase was maintained at a constant flow rate of .8 mL/min. Mobile phase 1a consisted of $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ (90/10) containing .1% TFA, and phases 1b and 1c, of $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ (10/90) containing .1% TFA and CH_3CN , respectively. The time program for gradient elution was as follows: 0–25 min 1a : 1b : 1c = 92 : 8 : 0; 25–35 min 1a : 1b : 1c = 0 : 100 : 0; and 35–45 min, 1a : 1b : 1c = 0 : 0 : 100. The chiral column (Column 2) used for the separation and quantification of D- and L-serine with NBD-F comprised two Sumichiral OA-2500 columns (S) (Sumika Chemical Analysis Service, Osaka, Japan),

which were connected in tandem. The mobile phase was 15 mmol/mL citric acid in MeOH. The flow rate was isocratically pumped at .8 mL/min. The column temperature of all columns was maintained at 35°C. Fluorescence detection was performed at 530 nm with an excitation wavelength at 470 nm.

Statistical Analyses

Allelic and genotypic frequencies of markers between patients and control subjects in the case-control study were assessed using Fisher's Exact Test. Haplotype frequencies, normalized linkage disequilibrium (LD) coefficient D' and squared correlation coefficient r^2 in the sample set B were calculated using the expectation-maximization algorithm implemented in COCAPHASE software (Dudbridge 2003) (<http://www.hgmp.mrc.ac.uk/~fdudbrid/software/>). To examine haplotype block structures in the genomic region of *DAO*, we used the Haploview program (Barrett et al 2005; <http://www.broad.mit.edu/mpg/haploview/>).

All members of the 124 families in sample set C were analyzed using the pedigree disequilibrium test (PDT) program, v3.12 (<http://www.chg.duke.edu/software/pdt.html>; Martin et al 2000). The complete 80 trio set in sample set C was also analyzed using 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 using the MCETDT program, version 1.3 (<http://www.mds.qmw.ac.uk/statgen/dcurtis/software.html>; Zhao et al 1999). TRANSMIT software (Clayton 1999; <http://watson.hgen.pitt.edu/docs/transmit.html>) was run as a global test of haplotype transmission for the set of complete 80 trios. Genetic Power Calculator (Purcell et al 2003; <http://statgen.iop.kcl.ac.uk/gpc/>) was used to compute statistical power.

The differences in serum levels of D-serine, and ratio (%) of D-serine to total (D- and L-) serine between the two groups and among multiple groups were examined using the two-tailed Mann-Whitney U test and the Kruskal-Wallis test.

Results

cDNA and Genomic Structures and Polymorphisms of *SRR*

The cDNA sequence of the protein coding region for human *SRR* and its genomic structure have been reported (De Miranda et al 2000) and are thought to contain no 5' untranslated exons. Our current 5'-RACE analysis and comparison of cDNA and genomic sequences revealed the existence of four novel 5' untranslated exons, herein referred to as exons 1a–1d, and showed that the alternative use of these 5' end exons gives rise to four different transcripts in the brain; we designate these mRNA variants isoforms a, b, c, and d (Figure 1). These results suggest that at least four promoters, each flanking the 5' portion of exons 1a, 1b, 1c, or 1d, drive and control the expression of *SRR*. We deposited this genomic information into GenBank under the Accession Numbers AY743705, AY743706, AY743707, and AY743708. The reported cDNA sequence (De Miranda et al 2000) started from our exon 1d but contained unknown sequences that could not be found in the human genome sequence database. The University of California at Santa Cruz (UCSC) July 2003 draft assembly of the human genome (<http://genome.ucsc.edu/>) displays our isoform b structure as a genomic organization of *SRR*. Interestingly, the exon 1d sequences are used as intronic sequences of isoforms a, b, and c (Figure 1). We previously reported this type of rare genomic organization in a different gene (Kikuchi et al 2003a, 2003b; Yoshikawa et al 1998).

Table 2. Results of Case–Control and Family-Based Association Studies Between *SRR* and Schizophrenia

Sample Set A	Schizophrenia (n = 50)	Control (n = 52)	Case–Control <i>p</i> Value	
			Allele	Genotype
Minor Allele Frequency				
IVS1a+465G>C	.220	.241	.743	.038
IVS1b+15G>A	.190	.087	.041	.026
Sample Set B	Schizophrenia (n = 570)	Control (n = 570)	Case–Control <i>p</i> Value	
Minor Allele Frequency			Allele	Genotype
IVS1a+465G>C (SNP1)	.273	.264	.635	.321
IVS1b+15G>A (SNP2)	.105	.096	.529	.802
LD between the two SNPs				
<i>D'</i>	1.000	1.000		
<i>r</i> ²	.044	.038		
Haplotype Frequency (SNP1–SNP2)			Global <i>p</i> value	
G-A	.105	.097		
G-G	.622	.640	.643	
C-G	.272	.263		
Sample Set C (Family Panel)	<i>p</i> for PDT-SUM	<i>p</i> for PDT-AVE	<i>p</i> for ETDT	Global <i>p</i> for TRANSMIT
IVS1a+465G>C	.592	.439	.786	.890
IVS1b+15G>A	.250	.310	.837	

PDT, pedigree disequilibrium test; PDT-SUM places greater weight on larger families; PDT-AVE places equal weight on all families.

To evaluate the abundance of each transcript in human brain, we performed an RNase protection assay (RPA). We designed four probes specific to each isoform, which shared 143 nt downstream and harbored upstream a 60–75 nt region specific to each isoform (Figure 2). When probe 1a, 1c, or 1d was used, the 143 nt protection fragment was clearly detected, whereas the expected > 200 nt fragment could not be detected (Figure 2), suggesting that isoforms a, c, and d are the minority in brain tissue, and that isoform b, containing the common 143 nt region, forms the majority of *SRR* in the brain. This idea is supported by two lines of evidence: 1) we detected a weak but apparent signal around 218 (= 143 + 75) nt and no signal around 143 nt (Figure 2), and 2) when the 5' region of the *SRR* transcript was amplified by 5'-RACE, clones with fragments corresponding to isoform 1b were predominant but those corresponding to the other isoforms were very scarce (data not shown). The lower signal intensity of the 218 nt band detected with probe b, relative to the signal of the 143 nt fragment detected with probes 1a, 1c, or 1d, could be due to the high G/C content in the cDNA sequence of probe b (60/75 = 80% G/C). Increased G/C content can produce complex secondary structures in both probe and target sequences, leading to decreased signal-to-noise ratios. The results were not improved by altering the probe sequence.

The *SRR* spans a genomic region of more than 50 kb. Mutation screening of all exons including the newly identified ones in this study, splice boundaries and 5' flanking regions of exons 1a–1d identified four polymorphisms, two SNPs (IVS1a+465G>C, rs408067, IMS-JST095603; IVS1b+15G>A, rs3744270, IMS-JST095602) surrounding exon 1b, and two novel SNPs, one in intron 5 (IVS5-34A>G) and the other in the 3'-untranslated region (Stop+302G>A; Figure 1). The IVS1a+465G>C is located next to the 5' end nucleotide of exon 1b that was defined by the present 5'-RACE analysis. The SNP databases display an exonic SNP (Stop+821T>C, rs2273983, IMS-JST069708), but in our sample it was not polymorphic (only the T allele was detected). Because only IVS1a+465G>C and IVS1b+15G>A were genetically informative (minor allele frequencies $\geq .10$), we examined these two SNPs in the subsequent genetic study.

Serum D-Serine Levels in Schizophrenia and Control Subjects

In a prior study, we examined 42 schizophrenia patients and 42 healthy control subjects for their serum levels of serine isomers (Hashimoto et al 2003). Among them, DNA was available from only 19 patients and 8 control subjects. To determine both serum levels of serine and genotypes in the same individuals, we newly recruited 31 schizophrenia patients and 46 normal control subjects and established sample set A (50 schizophrenia and 52 control subjects). Using sample set A, we were able to replicate the previous findings: significant decreases in the concentration of serum D-serine ($p = .0211$) and the percentage of D-serine in total serine ($p < .0001$), and significant increases in total serine ($p = .0029$) and L-serine ($p = .0026$) concentrations in the schizophrenia group compared with the control group (Figure 4). When we analyzed these measures using only the newly recruited samples, we observed similar trends in the comparison of schizophrenia and control groups, with the following *p* values: $p = .7221$ for serum D-serine concentration, $p = .0145$ for percentage of D-serine in total serine, $p = .0072$ for total serine level, and $p = .0075$ for L-serine concentration.

As in the prior study (Hashimoto et al 2003), we detected no correlation between serum total serine (Pearson correlation coefficient $r = -.183$, $p = .202$), L-serine ($r = -.184$, $p = .020$), and D-serine ($r = -.077$, $p = .597$) levels, and age of onset of any of the patients (the detailed characteristics of individual subjects are partly described elsewhere [Hashimoto et al 2003], and the remainder are available on request). Likewise, we confirmed no significant correlation between the duration of illness and any of the serum levels of total serine ($r = .093$, $P = .521$), L-serine ($r = .091$, $P = .531$), and D-serine ($r = .205$, $P = .154$). Gender, age, medication use, and smoking had no effect on any of the three serine-related measures, in the healthy control subjects or patients from the present sample panel A, which is consistent with our previous report (Hashimoto et al 2003). Current thinking suggests that medication by typical and atypical antipsychotic drugs is unlikely to influence serum levels of D-serine (Tsai et al 1999).