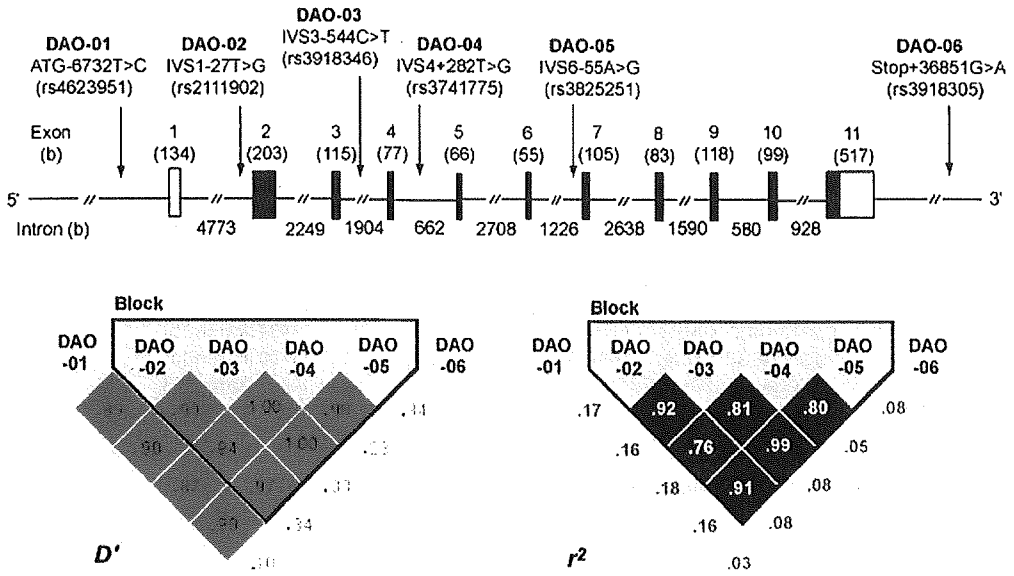

Figure 3. Genomic structure, location of polymorphic sites and haplotype block structure for human *DAO*. In the top panel, exons are denoted by boxes, with untranslated regions in white and translated regions in black. The sizes of exons (b) and introns (b) are also shown. The "rs" number of each single 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.

GCGCGTGCGCAGAGGTG and Ex2R; for isoform c, 5'-TTCCATATGGAACCAACTCGCCT and Ex2R; for isoform d, 5'-CTCTTCAATAAACATACTGTCTC 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'-GGCGTCAGAAGCTTGGTTCCT (forward)/5'-TGTCCCTTGTCCAGCTATCACT (reverse), and 5'-GCGGACTATGACTTAGTTGCGT (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.celeradiscoverysystem.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-

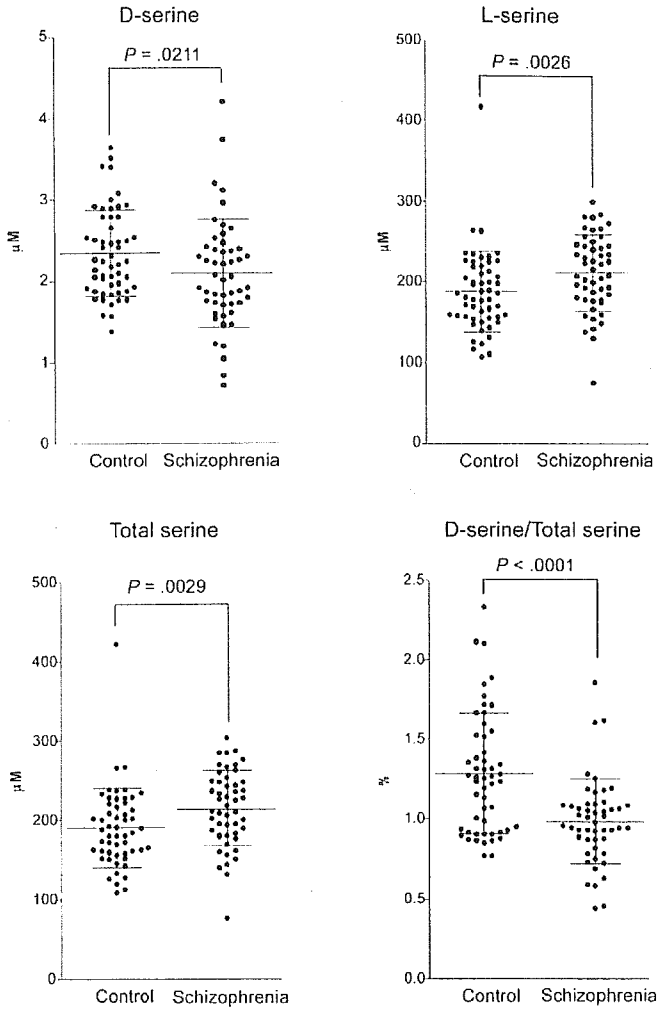

Figure 4. Significant differences in serum levels of serine isomers between healthy control subjects and schizophrenia patients. Examined samples included 52 control subjects and 50 patients with schizophrenia. Horizontal bars show mean ± SD.

madzu Corporation, Kyoto, Japan). A 20-μL aliquot of the human serum was added to 20 μL of .1 M borate buffer (pH 8.0) and 60 μL 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 μL of H_2O/CH_3CN (90/10) containing .1% trifluoroacetic acid (TFA) to stop the reaction. A 10-μL aliquot of the resultant solution was injected into the HPLC system. A reversed-phase ODS column (TSKgel ODS-80Ts [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 H_2O/CH_3CN (90/10) containing .1% TFA, and phases 1b and 1c, of H_2O/CH_3CN (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 p Value	
Minor Allele Frequency			Allele	Genotype
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 p 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				
D'	1.000	1.000		
r^2	.044	.038		
Haplotype Frequency (SNP1–SNP2)			Global p value	
G-A	.105	.097		
G-G	.622	.640	.643	
C-G	.272	.263		

Sample Set C (Family Panel)	p for PDT-SUM	p for PDT-AVE	p for ETDT	Global p 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 ≥ .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 31schizophrenia 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).

Table 3. Results of Case–Control (Using Sample Set B) and Family-Based (Using Sample Set C) Association Studies Between *DAO* and Schizophrenia

Marker No.	Polymorphism[a]	Case-Control *p* Value		PDT *p* Value		ETDT *p* Value	2 SNP-Based Haplotype *p*-value[b] Case-Control/Family-Based					3 SNP-Based Haplotype *p* Value[b] Case–Control/Family-based			
		Allele	Genotype	AVE	SUM										
DAO-01	ATG−6732T>C	.250	.198	.138	.216	0.402	.403/.483								
DAO-02	IVS1−27T>G	.498	.768	.683	.938	0.550		.350/.591				.334/.639			
DAO-03	IVS3−544C>T	.310	.547	.708	1.000	0.618			.198/.549				.412/.710		
DAO-04	IVS4+282T>G	.734	.938	.941	1.000	0.720				.192/.490				.198/.489	
DAO-05	IVS6−55A>G	.309	.565	.547	.870	.454					.046/.685				.011/.689
DAO-06	Stop+38851G>A	.020	.023	.157	.089	.350									

ETDT, extended transmission disequillibrium test; PDT, pedigree disequilibrium test; SNP, single nucleotide polymorphisms.
[a]Second allele is a minor allele.
[b]*p* value represents a global *p* value for both case–control and family-based association tests.

Genetic Association Studies

The two nucleotide variants of *SRR* were first typed using sample set A (50 schizophrenics, 52 control subjects), whose serum data was available (Table 2). Genotypic frequencies for IVS1a+465G>C and genotypic and allelic frequencies for IVS1b+15G>A displayed nominally significant differences between schizophrenia and control subjects.

To follow up these initial findings, a second independent sample set B (570 schizophrenia patients, 570 control subjects) was typed for the two SNPs (Table 2). We failed to replicate positive association results in the second, larger sample groups. The IVS1a+465G>C and IVS1b+15G>A polymorphisms were in substantial LD (Table 2). The reason $D' = 1.00$ in both schizophrenia and control groups is due to the complete linkage between the C allele of IVS1a+465G>C and the G allele of IVS1b+15G>A, and the relatively small values of r^2 could be a result of the major G allele of IVS1a+465G>C being present against a background of both alleles of the IVS1b+15G>A (Table 2). Distributions of haplotypes constructed by the two SNPs did not differ significantly between the schizophrenia and control groups (global $p = .643$) (Table 2).

Table 2 also shows the results of family-based association tests. 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). The two SNPs in *SRR* showed no significant association with schizophrenia by either of the two PDT statistics. We performed another family-based association test, ETDT analysis, on the 80 complete triad families. Again, this did not detect a significant association between either SNP of *SRR* and schizophrenia (Table 2). Moreover, haplotype transmission analysis found no haplotypes that were preferentially transmitted to schizophrenia subjects (specific haplotype *p*s are not shown; global $p = .890$; Table 2).

Genetic analysis of *DAO* demonstrated that the SNPs DAO-02 to DAO-05 form a single haplotype block, with the DAO-01 marker being in moderate LD with the block, with an obvious LD gap between the DAO-06 and the haplotype block (Figure 3). In the sample panel A, significant association between *DAO* and schizophrenia was not detected (Supplement 1). The allelic, genotypic, and haplotypic analyses using the sample sets B and C also failed to reveal genetic association between *DAO* and schizophrenia, except for the DAO-06 marker in the case–control sample set B [allelic $p = .020$, genotypic $p = .023$, global p for the (DAO-04)-(DAO-05)-(DAO-06) haplotype was .011] (Table 3).

The analyzed polymorphisms from the *SRR* and *DAO* genes

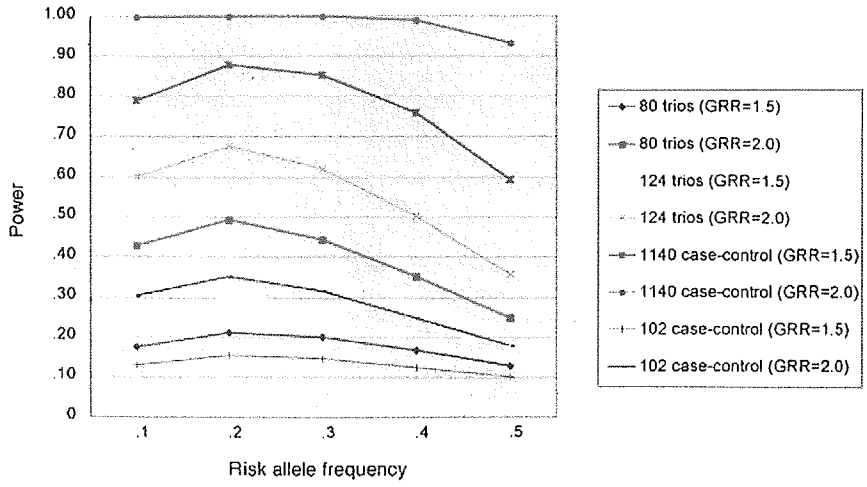

Figure 5. Results of power analysis under varying degrees of risk allele frequency and GRR (genetic relative risk). The family samples used for ETDT (extended transmission disequilibrium test) analysis are designated "80 trios," and those used for PDT (pedigree disequilibrium test) "124 trios"; "1140 case–control" is sample set B and "102 case–control" is sample set A.

Table 4. Relationship Between Ratio of D-Serine to Total Serine (%) and *SRR* Genotypes

Sample	D-Serine % According to IVS1a+465G>C Genotype (Mean ± SE)			*p* Value	D-Serine % According to IVS1b+15G>A Genotype (Mean ± SE)		*p* Value	D-Serine % According to Combined IVS1a+465G>C and IVS1b+15G>A Genotypes (Mean ± SE)				*p* Value
	G/G	G/C	C/C		G/G	G/A		G/G - G/G	G/G - G/A	G/C - G/G	G/C - G/A	C/C - G/G	
Control Group (*n* = 52)	1.39 ± .07 (*n* = 32)	1.07 ± .07 (*n* = 15)	1.41 ± .19 (*n* = 5)	.033	1.27 ± .06 (*n* = 43)	1.43 ± .12 (*n* = 9)	.179	1.38 ± .08 (*n* = 26)	1.49 ± .15 (*n* = 6)	1.02 ± .05 (*n* = 12)	1.30 ± .22 (*n* = 3)	1.41 ± .19 (*n* = 5)	.052
Schizophrenia Group (*n* = 50)	.97 ± .05 (*n* = 28)	1.02 ± .06 (*n* = 22)	None	.383	1.02 ± .04 (*n* = 31)	.94 ± .08 (*n* = 19)	.177	.94 ± .04 (*n* = 17)	1.02 ± .11 (*n* = 11)	1.12 ± .06 (*n* = 14)	.83 ± .09 (*n* = 8)	None	.082
Combined Group (*n* = 112)	1.19 ± .05 (*n* = 60)	1.04 ± .04 (*n* = 37)	1.41 ± .19 (*n* = 5)	.063	1.17 ± .04 (*n* = 74)	1.10 ± .08 (*n* = 28)	.258	1.20 ± .06 (*n* = 43)	1.19 ± .10 (*n* = 17)	1.07 ± .04 (*n* = 26)	.96 ± .11 (*n* = 11)	1.41 ± .19 (*n* = 5)	.145

p values were calculated using the two-tailed Mann–Whitney *U* test in the comparison of two groups or the Kruskal–Wallis test for comparison of three or more groups.

were in Hardy–Weinberg equilibrium in all the sample sets. The results of power analysis under various assumptions of parameters are shown in Figure 5. The case–control sample set B had a power of approximately .6–.9 under GRR (genetic relative risk) = 1.5 and > .9 under GRR = .2, when α = .05 and a dominant mode were assumed. The family sample sets and case–control sample set A showed lower power compared with the sample panel B.

Phenotype–Genotype Correlation Analysis

We examined whether the serum levels of serine enantiomers could be affected by the genotypes of *SRR* or *DAO*. Among the serine-related measures, the ratio of D-serine to total serine (%) was most significantly different between schizophrenia and the control subjects. Table 4 shows the relationship between the ratio of D-serine and the genotypes of two *SRR* polymorphisms. The mean values of this measure were not significantly different among the genotypes, except for the IVS1a+465G>C genotypes in the control group. Other measures including the D-serine, L-serine, and total serine concentrations were not significantly affected by *SRR* genotype (data not shown). Table 5 shows the correlation between the ratio of D-serine levels and genotypes of the six *DAO* polymorphisms. None of these *DAO* genetic markers significantly affected the D-serine parameter.

Discussion

Our previous findings of disturbed serine metabolism, including D-serine reduction and decreased D-serine/total serine ratio, in schizophrenia serum (Hashimoto et al 2003) have here been verified in larger samples. Recently, we also found that the ratio of D-serine to total serine was reduced in cerebrospinal fluids of drug-naïve schizophrenia compared with normal subjects (Hashimoto et al, in press). Therefore, the dysregulation of synthesis, metabolism, or both of D-serine in schizophrenia is likely to underlie the pathophysiology of at least some part of schizophrenia (Hashimoto et al 2004). D-serine is generated from L-serine by serine racemase, a pyridoxal-5'-phosphate-dependent enzyme enriched in brain astrocytes (De Miranda et al 2000; Dunlop and Neidle 1997; Wolosker et al 1999a, 1999b). The recent report of Coyle et al (unpublished data) that disruption of the serine racemase gene in mice resulted in not detectable D-serine in brain confirms the role of this gene. In the study we report here, we first focused on the human *SRR* gene as a potential major determinant for regulating D-serine levels not only in serum but also in brain for the following reasons: 1) serine racemase protein levels are highest in the brain, with a lower level in liver and faint or no detectable expression in other peripheral tissues (Wolosker et al 1999a); 2) liver expresses a large amount of D-amino acid oxidase, which almost completely metabolizes D-serine in liver (Hashimoto and Oka 1997; Wolosker et al 1999a); and 3) administration of D-serine or L-serine leads to the elevation of both stereoisomers of serine in the brain, suggesting a possible shuttling of the compounds through the blood brain barrier (Takahashi et al 1997). These lines of evidence imply that D-serine in the blood may originate from the brain and that reduced serum levels of D-serine in schizophrenia may reflect a decrease in the enzymatic activity of SRR in the brain of patients.

We revealed the existence of four 5' end untranslated exons of the human *SRR* gene in the brain, generating four mRNA isoforms coding for an identical protein sequence. The major transcript uses exon 1b, and our mutation search identified two SNPs in the vicinity of this exon. A database search demonstrated that exon 1b and its flanking intronic sequences that span

Table 5. Relationship Between Ratio of D-Serine to Total Serine (%) and *DAO* Genotypes

Polymorphism and Genotype	% D-Serine According to Genotype (Mean ± SE)		
	Control Group (n = 52)	Schizophrenia Group (n = 50)	Combined Group (n = 112)
DAO-01 (ATG−6732T>C)			
T/T	1.27 ± .38 (n=32)	.99 ± .28 (n=30)	1.13 ± .36 (n=62)
T/C	1.34 ± .40 (n=19)	.99 ± .26 (n=18)	1.17 ± .38 (n=37)
C/C	1.39 ± .00 (n= 1)	1.07 ± .00 (n= 1)	1.23 ± .23 (n= 2)
p	.675	.817	.621
DAO-02 (IVS1−27T>G)			
T/T	1.32 ± .37 (n=13)	.96 ± .27 (n=14)	1.13 ± .37 (n=27)
T/G	1.30 ± .35 (n=31)	1.05 ± .27 (n=24)	1.19 ± .34 (n=55)
G/G	1.25 ± .53 (n= 8)	.90 ± .22 (n=11)	1.05 ± .41 (n=19)
p	.724	.360	.138
DAO-03 (IVS3−544C>T)			
C/C	1.32 ± .37 (n=13)	.96 ± .26 (n=15)	1.13 ± .37 (n=28)
C/T	1.30 ± .35 (n=31)	1.06 ± .27 (n=24)	1.19 ± .34 (n=55)
T/T	1.25 ± .53 (n= 8)	.88 ± .22 (n=10)	1.04 ± .42 (n=18)
p	.724	.179	.103
DAO-04 (IVS4+282T>G)			
T/T	1.18 ± .46 (n=11)	.92 ± .22 n =13)	1.04 ± .37 (n=24)
T/G	1.30 ± .35 (n=30)	1.05 ± .28 (n=23)	1.19 ± .34 (n=53)
G/G	1.4 ± .36 (n=11)	.96 ± .28 (n=13)	1.16 ± .38 (n=24)
p	.225	.462	.125
DAO-05 (IVS6−55A>G)			
A/A	1.32 ± .37 (n=13)	.99 ± .23 (n=14)	1.15 ± .35 (n=27)
A/G	1.30 ± .35 (n=31)	1.04 ± .29 (n=25)	1.18 ± .35 (n=56)
G/G	1.25 ± .53 (n= 8)	.88 ± .22 (n=10)	1.04 ± .42 (n=18)
p	.724	.306	.140
DAO-06 (Stop+36851G>A)			
G/G	1.39 ± .42 (n=12)	.95 ± .33 (n=15)	1.15 ± .43 (n=27)
G/A	1.26 ± .39 (n=23)	1.04 ± .23 (n=25)	1.14 ± .33 (n=48)
A/A	1.29 ± .34 (n=17)	.94 ± .26 (n= 8)	1.18 ± .35 (n=25)
p	.592	.509	.632

p values were calculated using the Kruskal–Wallis test.

IVS1a+465G>C and IVS1b+15G>A are not conserved among species, suggesting human-specific gene regulation. The GC-richness in the genomic sequences of exon 1b and its 5'-flanking region and the lack of a TATA box suggest that mRNA isoform 1b can be transcribed from multiple transcription start sites by contribution of Sp1 motifs. The G-to-C transversion at the IVS1a+465G>C site and G-to-A transition at the IVS1b+15G>A position are predicted to abolish Sp1 binding sites (TFSEARCH: http://mbs.cbrc.jp/research/db/TFSEARCHJ.html). Therefore, these polymorphisms could bear functional consequences. The genetic association analysis using the small sample size of panel A detected nominally significant genotypic or allelic associations (or both) between the two variants and schizophrenia. These initial weak associations could not be replicated in the 10-fold larger case–control panel B or family sample set C, however. These results indicate that the polymorphisms in the 5' portion of *SRR* do not play a major role in the susceptibility to schizophrenia in the Japanese population, although we cannot exclude the possibility that a subset of people with schizophrenia are biologically affected by the *SRR* gene.

Analysis of *DAO*, a gene involved in the metabolic pathway of D-serine, also showed no evidence of association with schizophrenia, with the exception of the DAO-06 SNP. The weak association between this marker and schizophrenia in the large case–control panel B is difficult to interpret, however, because 1) DAO-06 is located more than 36 kb downstream from the last exon of the *DAO* gene, 2) there are no annotated genes within a 20-kb range of the DAO-06 marker, 3) in the small-scale sample set A, DAO-06 showed a trend of allelic association with schizophrenia (p = .094), but the disease-associated alleles were different between the sample sets A and B.

The current study again confirmed the robustness of serum levels of D-serine against the confounding clinical parameters of gender, age, and treatment and duration of illness, suggesting a cogent contribution of genetic variance to D-serine regulation. We failed to detect firm correlation between the genotypes of two 5' SNPs in *SRR* and the D-serine-related measurements, however. The ratio of D-serine to total serine concentrations were slightly different among the genotypes of IVS1a+465G>C in the control group. The statistical difference was due to the reduced mean value in heterozygotes (IVS1a+465G/C) compared with both types of homozygotes (IVS1a+465G/G, IVS1a+465C/C), thus making the results difficult to interpret. It would be more reasonable to interpret the difference as being attributable to statistical fluctuation caused by small sample size (N = 52). Additionally, the *DAO* genetic variants showed no significant correlation with serum D-serine measure.

As stated previously, some enzymes responsible for serine metabolism (e.g. SRR and serine hydroxymethyltransferase) require pyridoxal-5'-phosphate as a cofactor (Schell 2004), it would be intriguing to examine the serum levels of this compound, although Muntjewerff et al (2003) reported no significant differences in levels of pyridoxal-5'-phosphate between schizophrenia and control groups.

In summary, we observed no major genetic effects of *SRR* and *DAO* polymorphisms in the predisposition to schizophrenia and prediction of serum levels of serine isomers in Japanese cohorts. The present results do not, however, exclude the possibility that SRR (DAO) protein or its enzymatic activity is decreased (increased) in schizophrenia by other mechanisms including disturbed functional integrity of D-serine-producing cells (e.g., astrocyes; Hashimoto et al, in press; Steffek et al, unpublished data), but even the genetic contributions of *SRR* and *DAO* in other ethnic populations. In future studies, it would be worth pursuing these possibilities and analyzing other genes involved in the relevant metabolic pathways, for example, genes for serine hydroxymethyltransferase and 3-phosphoglycerate dehydrogenase (committed in L-serine biosynthesis in the phosphorylated pathway; Mitoma et al 2004) and genes for serine racemase-interacting proteins including the glutamate receptor interacting protein (Kim et al 2005) and PICK1 (protein interacting with C kinase 1; Hikida et al, unpublished data).

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Brief Communication

DNA Methylation Status of *SOX10* Correlates with Its Downregulation and Oligodendrocyte Dysfunction in Schizophrenia

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Downregulation of oligodendrocyte-related genes, referred to as oligodendrocyte dysfunction, in schizophrenia has been revealed by DNA microarray studies. Because oligodendrocyte-specific transcription factors regulate the differentiation of oligodendrocytes, genes encoding them are prime candidates for oligodendrocyte dysfunction in schizophrenia. We found that the cytosine–guanine dinucleotide (CpG) island of sex-determining region Y-box containing gene 10 (*SOX10*), an oligodendrocyte-specific transcription factor, tended to be highly methylated in brains of patients with schizophrenia, correlated with reduced expression of *SOX10*. We also found that DNA methylation status of *SOX10* also was associated with other oligodendrocyte gene expressions in schizophrenia. This may be specific to *SOX10*, because the CpG island of *OLIG2*, which encodes another oligodendrocyte-specific transcription factor, was rarely methylated in brains, and the methylation status of myelin-associated oligodendrocytic basic protein, which encodes structural protein in oligodendrocytes, did not account for their expressions or other oligodendrocyte gene expressions. Therefore, DNA methylation status of the *SOX10* CpG island could be an epigenetic sign of oligodendrocyte dysfunction in schizophrenia.

Key words: oligodendrocyte; postmortem; schizophrenia; DNA methylation; epigenetics; *SOX10*

Introduction

Schizophrenia is a severe mental disorder with symptoms such as hallucination, delusion, disorganized thought, and impairment of cognitive functions. Twin, family, and adoption studies have revealed that complex interactions between hereditary and environmental factors are involved in the etiology of schizophrenia (Gottesman, 1991).

Several lines of evidence suggest the alteration of oligodendrocytes in schizophrenia. Electron microscopic studies revealed the ultrastructural alteration of oligodendroglias (Uranova et al., 2004). Immunohistochemical analysis revealed the downregulation of oligodendrocyte proteins in gray matter (Honer et al., 1999; Flynn et al., 2003) and alteration in number and density of oligodendrocytes in layer III of the cortex in schizophrenia (Hof et al., 2003). Although the results remain inconclusive, magnetic resonance imaging studies suggested some changes in white matter in patients with schizophrenia, and these changes are considered related to the above findings (Davis et al., 2003; Stewart and Davis, 2004).

Gene expression analyses using DNA microarray also support the alteration of oligodendrocytes in schizophrenia (Bunney et al., 2003; Mirnics et al., 2004). Coordinated downregulation of a subset of oligodendrocyte-related genes (referred to as oligodendrocyte dysfunction) in prefrontal [Brodmann area (BA) 46 (Hakak et al., 2001; Tkachev et al., 2003) or BA47 (Sugai et al., 2004)] and temporal [BA21 (Aston et al., 2004)] cortices of patients with schizophrenia have been revealed.

Among the downregulated oligodendrocyte genes, we focused on sex-determining region Y-box containing gene 10 (*SOX10*), because a combination of transcription factors such as SOX10 and oligodendrocyte lineage transcription factor 1 (OLIG1) and OLIG2 regulates the differentiation of oligodendrocytes (Kessaris et al., 2001), and that *SOX10* is responsible for terminal differentiation of oligodendrocytes (Stolt et al., 2002). Here, we examined the DNA methylation status of the *SOX10* cytosine–guanine dinucleotide island in brains of patients with schizophrenia. We found that the DNA methylation status of *SOX10* can, but that of *OLIG2* or myelin-associated oligodendrocytic basic protein (*MOBP*) cannot, account for its downregulation and oligodendrocyte dysfunction in schizophrenia.

Materials and Methods

Postmortem brains. Postmortem prefrontal cortices (BA10) were provided by the Stanley Foundation Brain Collection (The Stanley Medical Research Institute, Bethesda, MD). The materials originally were pro-

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vided as frozen tissues, consisted of gray matter and a small portion of white matter, and were coded blindly to diagnostic and demographic variables. Homogenate of the frozen tissue was used for extraction of total RNA and genomic DNA. Information about postmortem brains has been described previously (Torrey et al., 2000). Briefly, controls and patients were matched for age, gender, postmortem interval, and sample pH. We previously obtained gene expression profiles using an Affymetrix U95Av2 chip (Affymetrix, Santa Clara, CA) from 50 subjects, including controls ($n = 15$) and patients with schizophrenia ($n = 13$) (Iwamoto et al., 2004). Genetic and epigenetic analyses were performed after the blinded codes were opened.

Three additional brain samples (BA10), including two controls and one with psychotic disorder, were also provided by the Stanley Foundation Brain Collection. These three specimens were divided into gray and white matter portions. Genomic DNA and total RNA were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA).

Cell culture. A lymphoblastoid cell line, TC42, was established from the lymphocytes of a 28-year-old Japanese male with no history of neuropsychiatric diseases using standard techniques (Kato et al., 2002). SHSY5Y (a human neuroblastoma), 1321N1 (a human astrocytoma; a gift from Dr. Norimichi Nakahata, Tohoku University, Miyagi, Japan), and A2058 (a human melanoma) cell lines were maintained in DMEM containing 10% fetal bovine serum. A melanoma cell line, A2058, was selected as a *SOX10*-expressing cell line (Su et al., 2002) using the SOURCE website, (http://source.stanford.edu) (Diehn et al., 2003). Total RNA and genomic DNA were extracted from the harvested cells using TRIzol reagent.

Expression studies. After the DNase I treatment, 5 μg of total RNA was used for cDNA synthesis by oligo(dT) primer and SuperScript II reverse transcriptase (RT) (Invitrogen). Quantitative RT-PCR (qRT-PCR) using SYBER/GREEN I dye (Applied Biosystems, Foster city, CA) was performed with ABI7900 (Applied Biosystems). The comparative threshold cycle (Ct) method was used for quantification according to the protocol of the manufacture (Applied Biosystems). Measurement of δ Ct was performed at least three times per sample. Amplification of the single product was confirmed by monitoring the dissociation curve and by electrophoresis. In addition to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), we used two other genes [β actin (ACTB) and cofilin 1 (*CFL1*)] for normalization to control for possible fluctuations in quantitative values of the target transcripts. Primer pairs for *GAPDH*, *ACTB*, and *CFL1* have been shown previously (Iwamoto et al., 2004). Primer pairs used for qRT-PCR are as follows: *SOX10*, 5′-CCAGTA CCCGCACCTGCAC-3′ and 5′-CTTTCGTTCAGCAGCCTCCAG-3′; *OLIG2*, 5′-AAGCTTTCCAAGATCGCCACG-3′ and 5′-TAGATC-TCGCTCACCAGTCGCTTC-3′; *MOBP*, 5′-ACTCCGAACACTTCAG-CATACACT-3′ and 5′-GATCCAGTCCTCCTCTTTCTTCTG-3′.

Genetic studies. For mutation screening of *SOX10*, we used genomic DNA extracted from postmortem liver samples of the 13 patients with schizophrenia whose brain RNA was used for the DNA microarray. All exons, exon-intron boundaries, and 1 kb upstream regions from the first exons in the splice variants were sequenced. Primer pairs and PCR conditions are available on request.

Epigenetic studies. To confirm biallelic expression in *SOX10*, we amplified a part of *SOX10* containing a single-nucleotide polymorphism (SNP) (rs139883) with the primers 5′-ACCACTCCTATGACTCC-TGTTTTCTC-3′ and 5′-ATAGAGCCTAGTAAGGGAAGAGGGA-3′ using brain-derived cDNA as a template. PCR products were directly sequenced or cloned using a TOPO TA cloning kit (Invitrogen). Single bacterial colonies were subjected to sequencing analysis.

Bisulfite modification of genomic DNA derived from postmortem brains (BA10) or cell lines was done as follows. After denaturation, 1–2 μg of genomic DNA was treated with 3.6 M sodium bisulfite. The reaction was performed at 55°C for 16 h. Genomic DNA was then purified with a Wizard DNA Clean-up kit (Promega, Madison, MI) and eluted with 50 μl of water. We typically used 2–5 μl of bisulfite-modified DNA for PCR. The CpG islands (Gardiner-Garden and Frommer, 1987) of oligodendrocyte genes were obtained through the University of California, Santa Cruz Genome Browser (http://www.genome.ucsc.edu/index). Primer pairs determined using MethPrimer software (Li and Dahiya, 2002) were as follows: *SOX10*, 5′-TGGGTAAGGTTAAGAAGGAGTAGTAG-3′ and 5′-CTACCTAAACCCACACCATAAAAAC-3′; *OLIG2*-1, 5′-TTTTAAGT-TTTTGTTTTTAGTTGGG-3′ and 5′-AATCTCCTCCCTAACTCTTC-CTCTAT-3′; *OLIG2*-2, 5′-TAGAGGAAGAGTTAG-GGAGGAGATT-3′ and 5′-ACCACCACAAAATCAAATTAAAAAA-3′; *OLIG2*-3, 5′TTA-AAGAAAGGTTTTTATTTTTTATT-3′ and 5′-TCTCTAACCCTCCTTT-TAACTACAC-3′; *MOBP*, 5′-TTAGAAGAAAGAGGAGGATTG-GATT-3′ and 5′-CTTCCAATCTCCCTAAAATACCTTC-3′. After the separation by agarose gel electrophoresis, PCR products were excised, purified, and TA cloned. Single-bacterial colonies were subject to sequencing analysis.

Results

Downregulation of *SOX10* in schizophrenia

Using our previous DNA microarray data (Iwamoto et al., 2004), we confirmed the downregulation of oligodendrocyte-related genes, including *SOX10* in schizophrenia (supplemental Table 1, available at www.neurosci.org as supplemental material). Downregulation of *SOX10* was confirmed by qRT-PCR (Fig. 1*A*). Expression of *SOX10* was not significantly correlated with age ($r = 0.037$; $p = 0.851$; $n = 28$), postmortem interval ($r = -0.168$; $p = 0.392$; $n = 28$), or pH ($r = 0.034$; $p = 0.863$; $n = 28$). Furthermore, expression of *SOX10* did not significantly differ according to gender (male, $n = 17$; female, $n = 11$; $p = 0.327$) or the side of the brain (right, $n = 11$; left, $n = 17$; $p = 0.423$). These results were confirmed when we used qRT-PCR data or the remaining DNA microarray data of patients with other mental disorders for calculation (data not shown).

Mutation screening of *SOX10*

Genetic mutations in *SOX10* cause neurological diseases such as peripheral demyelinating neuropathy, central demyelinating leukodystrophy, Waardenburg syndrome, and Hirschprung disease (known collectively as PCWH) (Inoue et al., 2004). Because some mutations result in the generation of unstable mRNAs that are rapidly degraded by the nonsense-mediated decay (NMD) pathway, downregulation of *SOX10* in schizophrenia may be accounted for by such mutations. To explore this possibility, we performed mutation screening by sequencing all exons, exon-intron boundaries, and 1 kb upstream regions from the first exons of the splice variants in 13 patients with schizophrenia. This analysis was performed using liver-derived genomic DNA, the brain RNA of which was used for the DNA microarray study. We did not find the putative functional SNPs that change amino acid sequences or cause degradation by the NMD pathway (Inoue et al., 2004). There were also no significant differences in the expression levels of *SOX10* among genotypes or haplotypes of polymorphisms detected in these patients (data not shown).

Epigenetic analysis of *SOX10*

To further examine the cause of *SOX10* downregulation, we investigated its DNA methylation status in schizophrenic brains, because hypermethylation is known to correlate with silencing of gene expression in normal and pathological conditions. The brain-derived genomic DNA (BA10) was bisulfite modified and then a part of the CpG island of *SOX10* was amplified (Fig. 2*A*). The amplicon was TA cloned and subjected to sequencing analysis. We performed this analysis in each of 23 subjects (12 controls, 11 schizophrenics). Genomic DNA from the rest of the subjects (three controls, two schizophrenics) did not show sufficiently good quality for methylation analysis. We found that clones contained either densely or nonmethylated alleles (Fig. 1*B*) (supplemental Fig. 1, available at www.neurosci.org as supplemental material). This methylation pattern cannot be explained by monoallelic expression, because expression from both

alleles was confirmed (Fig. 1*C*). We arbitrarily defined a methylated allele as one in which 30% or more of CpG dinucleotides are methylated in the examined region (Fig. 1*B*). We then determined for each subject the percentage of the total methylated alleles. Patients with schizophrenia showed a tendency of higher percentage of methylated alleles and lower expression levels of *SOX10* compared with control subjects. There was an inverse correlation between the percentage of methylated alleles and expression level of *SOX10* ($r = -0.586$; $p = 0.003$; $n = 23$), suggesting that downregulation of *SOX10* was associated with an increased percentage of the methylated allele in brains (Fig. 1*D*). The inverse correlation between percentage of methylated alleles and expression level was not dependent on the definition of methylated allele (Table 1). There were no significant differences of DNA methylation status among genotypes or haplotypes of polymorphisms detected in these patients (data not shown). Additionally, we found that the methylation status of *SOX10* was also related to downregulations of other oligodendrocyte genes that were differentially expressed in schizophrenia (Table 2).

To test whether the DNA methylation status of *SOX10* is different between gray and white matters, we divided another set of postmortem samples into gray and white matter portions and then examined their methylation status. The percentage of methylated alleles was significantly greater in gray matter than in white matter ($p = 0.02$; *t* test) (Fig. 2*B*) (supplemental Fig. 2, available at www.neurosci.org as supplemental material), consistent with the *SOX10* expression in oligodendrocytes. We next examined the *SOX10* methylation status in human cell lines. The *SOX10*-expressing cell line had nonmethylated alleles, whereas the *SOX10*-nonexpressing cell lines had only methylated alleles (Fig. 2*C*) (supplemental Fig. 3, available at www.neurosci.org as supplemental material). Together, these results suggest that expression of *SOX10* is associated with the presence of nonmethylated CpG island alleles.

Epigenetic analysis of *OLIG2* and *MOBP*

To test whether correlation between methylation and gene expression was also observed in other genes, we examined the methylation status of *OLIG2* and *MOBP*. *OLIG2* plays a role in the specification of oligodendrocytes and motor neurons (Lu et al., 2002), and MOBP is one of the major protein components of myelin. Both genes

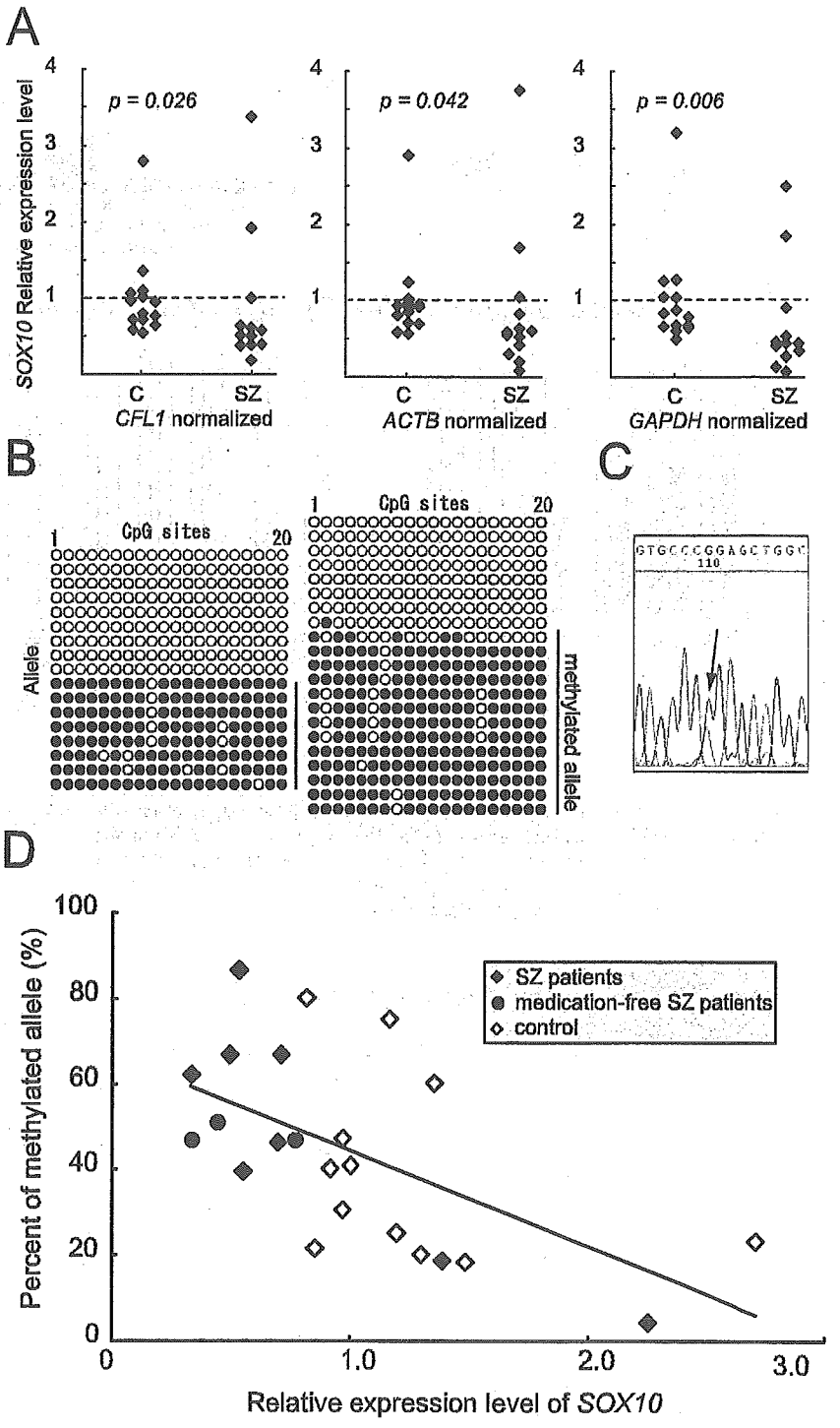

Figure 1. Expression and epigenetic analyses of *SOX10*. *A*, qRT-PCR of *SOX10*. *p* values were calculated by the Mann–Whitney *U* test. C, Control subjects ($n = 14$); SZ, schizophrenic patients ($n = 13$). The original data set (Torrey et al., 2000) was composed of 15 control subjects and 15 schizophrenic patients. One control subject was omitted from qRT-PCR data analysis, because we could not obtain appropriate amplification curves. Samples of two schizophrenic patients were omitted from DNA microarray analysis and qRT-PCR, because they showed poor quality, as revealed by denatured gel electrophoresis and the results of Test2chip (Affymetrix). *B*, Examples of DNA methylation status in a control subject (left) and a schizophrenic patient (right). Results of all subjects can be found in supplemental Figure 1 (available at www.jneurosci.org as supplemental material). Open circle, Nonmethylated CpG; closed circle, methylated CpG. *C*, Biallelic expression of *SOX10*. The arrow indicates an SNP at the 3′-untranslated region (reverse orientation of rs139883) in the postmortem brain cDNA sequence. Biallelic expression was also confirmed by cloning and sequence analysis of three independent subjects who had heterozygous alleles with regard to rs139883. *D*, Inverse correlation between methylation status and expression level of *SOX10*.

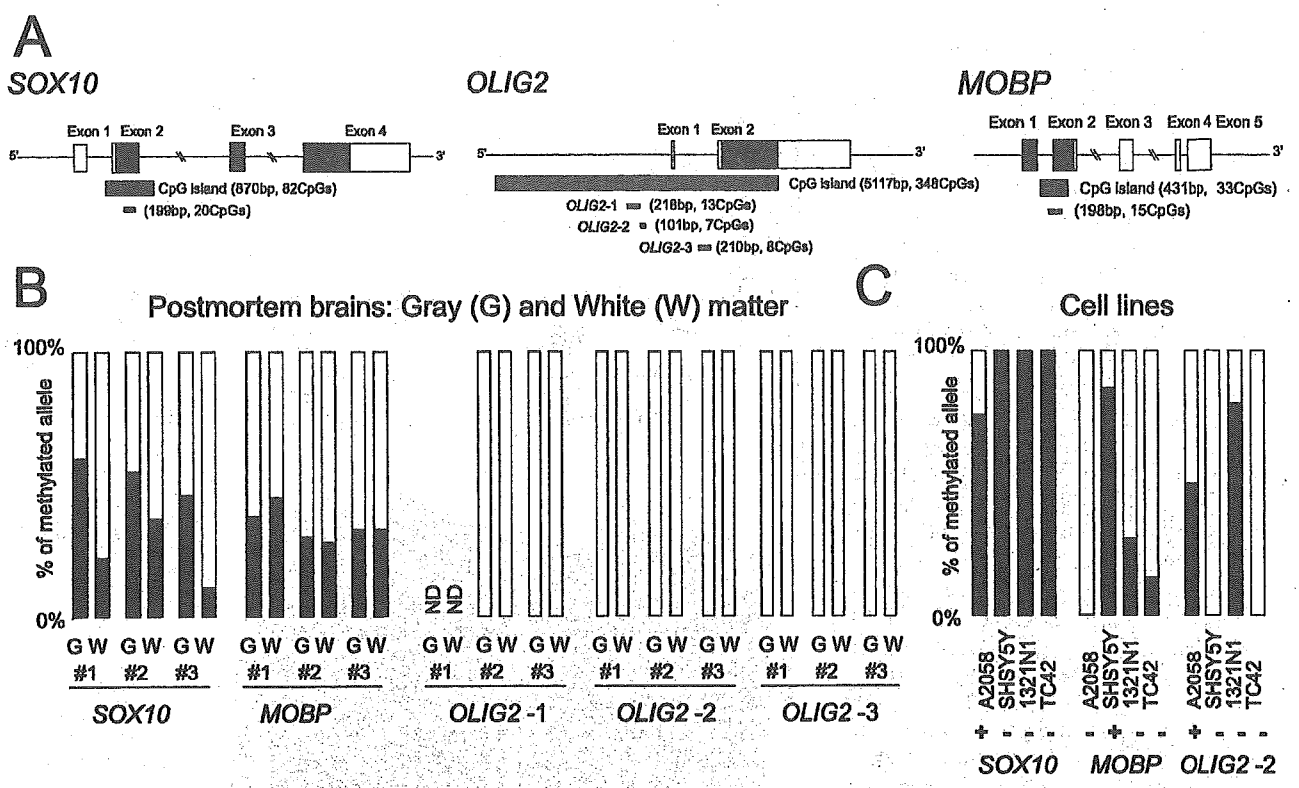

Figure 2. DNA methylation status of oligodendrocyte genes. *A*, Genomic structure and CpG island of *SOX10*, *OLIG2*, and *MOBP*. Exons are denoted by boxes, with untranslated regions in white and translated regions in black. PCR-amplified regions for methylation analysis are underlined. *B*, DNA methylation status in postmortem brains. The percentage of methylated alleles is indicated in black. ND, Not determined. *C*, DNA methylation status in human cell lines. A2058, SHSY5Y, 1321N1, and TC42 are melanoma, neuroblastoma, astrocytoma, and lymphoblastoid cell lines, respectively. Expression levels of each oligodendrocyte gene were measured (+, detected; −, not detected). The relative expression levels in relation to *GAPDH* are as follows: 0.03363 (*SOX10* in A2058), 0.00025 (*OLIG2* in A2058), and 0.00005 (*MOBP* in SHSY5Y).

Table 1. Definition of methylated allele and correlation with expression levels

	Definition of a methylated allele						
	20%	30%	40%	50%	60%	70%	80%
SOX10							
r	−0.534	−0.586	−0.579	−0.574	−0.485	−0.419	−0.366
p	0.009	0.003	0.004	0.004	0.019	0.047	0.086
MOBP							
r	0.139	0.005	0.005	0.019	0.007	0.104	−0.021
p	0.528	0.981	0.981	0.931	0.974	0.636	0.924

A methylated allele was defined as an allele that gave a percentage or more of methylated CpGs of the total number of CpGs in the examined region. There were 20 and 15 CpGs in *SOX10* and *MOBP*, respectively, in the examined region. Pearson's *r* and significance (*p*) values are given.

were consistently downregulated in schizophrenia, as revealed by a previous DNA microarray study (Tkachev et al., 2003) and by our results. Because the CpG island of *OLIG2* spans >5 kb, we examined its methylation status in three regions (*OLIG2-1* to *OLIG2-3*) (Fig. 2*A*). We found that the CpG island of *OLIG2* was rarely methylated in gray or white matter in brains (Fig. 2*B*) (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). We further examined the methylation status in several control and schizophrenia samples and also found that none of the subjects had a densely methylated CpG allele (supplemental Fig. 4, available at www.neurosci.org as supplemental material). In the case of *MOBP*, both gray and white matter portions contained the methylated alleles (Fig. 2*B*) (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). However, there are no apparent differences in the percentage of methylated alleles between gray and white matter. In addition, the methylation status of *MOBP* did not correlate with its expression level (Table 1) or the expression levels of other oligodendrocyte genes (Table 2) (supplemental Fig. 5, available at www.neurosci.org as supplemental material). In cell lines, the DNA methylation status of the CpG islands of *OLIG2* and *MOBP* were not associated with their expression patterns, suggesting that their expressions were not regulated through DNA methylation in these cell lines (Fig. 2*C*) (supplemental Fig. 3, available at www.jneurosci.org as supplemental material).

Discussion

Accumulating evidence suggests that complex mental disorders may be mediated through aberrant epigenetic processes (Costa et al., 2003; Abdolmaleky et al., 2004; Petronis, 2004). However, there are few studies that document the epigenetic status directly in the brains of patients. To date, DNA methylation of the reelin promoter was suggested to be involved in its downregulation in schizophrenia (Veldic et al., 2004; Abdolmaleky et al., 2005), and site-specific DNA methylation of catechol-*O*-methyltransferase promoter was proposed to be altered in schizophrenia (Murphy et al., 2005). Our present findings provide the first evidence concerning the epigenetic aspects of oligodendrocyte dysfunction in schizophrenia. We found that the DNA methylation status of

Table 2. Correlation between methylation status and expression levels of oligodendrocyte genes

	Methylation status			
	SOX10 ($n = 23$)		*MOBP* ($n = 23$)	
	r	p	r	p
SOX10	−0.586	0.003	−0.139	0.527
OLIG2	−0.533	0.009	−0.130	0.554
MAG	−0.592	0.003	−0.109	0.620
PLP1	−0.640	0.001	−0.141	0.520
MOBP	−0.527	0.010	0.005	0.981
OMG	−0.074	0.738	−0.073	0.740
PMP22	−0.605	0.002	−0.146	0.507
MAL	−0.618	0.002	−0.138	0.529
CNP	−0.485	0.019	−0.147	0.505
TF	−0.531	0.009	0.044	0.842
GSN	−0.513	0.012	−0.061	0.783

Pearson's correlation coefficient (r) and p values are given. CNP, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; GSN, gelsolin; MAL, myelin and lymphocyte protein; OMG, oligodendrocyte-myelin glycoprotein; PLP1, myelin proteolipid protein; PMP22, peripheral myelin protein 22; TF, transferrin.

SOX10 inversely correlates with expression levels of *SOX10* and other oligodendrocyte genes. Considering that expression of oligodendrocyte genes are regulated by a combination of oligodendrocyte-specific transcription factors (Kessaris et al., 2001) and the role of SOX10 in the oligodendrocyte differentiation (Stolt et al., 2002), it would be reasonable to assume that the majority of the oligodendrocyte-expressed genes, including those listed in Table 2, are under the direct or indirect control of SOX10. It is not known whether observed oligodendrocyte dysfunction was accompanied by substantial loss or increase of specific cell populations. However, variations in the sampling step of postmortem brains were not likely to be the cause for this dysfunction, because not all of oligodendrocyte-specific genes or glial genes were downregulated in schizophrenia by independent DNA microarray studies, including ours, and some reports revealed the oligodendrocyte dysfunction in gray matter of patients with schizophrenia (Honer et al., 1999; Hakak et al., 2001; Flynn et al., 2003).

Our findings at the mRNA levels were in good accordance with previous reports at the protein levels such as downregulation of 2′, 3′-cyclic nucleotide 3′-phosphodiesterase in schizophrenia (Flynn et al., 2003). However, the results of some genes such as myelin basic protein (Honer et al., 1999) or myelin-associated glycoprotein (MAG) (Flynn et al., 2003) were not compatible with mRNA findings. This was partly caused by the presence of complex splicing isoforms (Tkachev et al., 2003).

We cannot completely rule out the effect of medication on oligodendrocyte gene expressions in this study. Our sample set included three medication-free patients with schizophrenia (including a patient who was treated with electroconvulsive treatment). Consistent with a previous report (Tkachev et al., 2003), these medication-free patients also showed the typical downregulations of oligodendrocyte genes (Fig. 1*D*), suggesting that these expression changes were not caused by the medication. In addition, expression and DNA methylation levels of *SOX10* were not significantly correlated with a lifetime antipsychotic dose (fluphenazine-equivalent dose) in the schizophrenia group ($r = -0.078$ and $r = 0.066$, respectively).

Oligodendrocyte dysfunction has also been reported in bipolar disorder (Tkachev et al., 2003) and major depression (Aston et al., 2005). Our analysis of BA10 (Iwamoto et al., 2004) failed to detect oligodendrocyte dysfunction in bipolar disorder and major depression, whereas that of BA46 (Iwamoto et al., 2005) revealed the downregulation of oligodendrocyte genes in both diseases (data not shown). Therefore, contrary to the case of schizophrenia, there might be regional differences in oligodendrocyte dysfunction in other mental disorders. Alternatively, the discrepancy between our data sets with respect to bipolar disorder may be caused by the difference in patient population.

We do not know which factors affect the percentage of methylated *SOX10* alleles in schizophrenic brains at this stage. Mutation screening suggests that genetic variations in *SOX10* were not involved in its expression level or DNA methylation status. The increased DNA methylation of *SOX10* in gray matter, compared with that in white matter, and results from cell line experiments suggest the dysfunction and/or decreased number of *SOX10*-expressing cells in brains of patients with schizophrenia. Considering previous DNA microarray results (Hakak et al., 2001; Tkachev et al., 2003; Aston et al., 2004) along with our results, which show the downregulation of a subset of oligodendrocyte genes, specific gene expression deficits within oligodendrocytes are probable. Because postmortem samples included both gray and a small portion of white matter, it is not clear whether possible defects were restricted in gray matter.

Importantly, the DNA methylation status of *MOBP* and *OLIG2* cannot explain their expression levels or expressions of other oligodendrocyte genes. Although the expression of *SOX10*, a gene that is important for oligodendrocyte differentiation, is regulated through global DNA methylation of the CpG island, other oligodendrocyte genes may not be tightly regulated at that level. Therefore, the DNA methylation status of the *SOX10* CpG island provides a clue in elucidating the regulation of oligodendrocyte gene expressions, and it could be an epigenetic sign of oligodendrocyte dysfunction in schizophrenia.

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Short Communication

Human netrin-G1 isoforms show evidence of differential expression

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Abstract

The recently identified netrins-G1 and -G2 form a distinct subgroup within the UNC-6/netrin gene family of axon guidance molecules. In this study, we determined the size and structure of the exon/intron layout of the human netrin-G1 (*NTNG1*) and -G2 (*NTNG2*) genes. Northern analysis of both genes showed limited nonneuronal but wide brain expression, particularly for *NTNG2*. Reverse transcriptase PCR detected nine alternatively spliced isoforms including four novel variants of *NTNG1* from adult brain. A semiquantitative assay established that major expression was restricted to isoforms G1c, G1d, G1a, and G1e in the brain and to G1c in the kidney. There is also evidence of developmental regulation of these isoforms between fetal and adult brain. In conclusion, *NTNG1* may use alternative splicing to diversify its function in a developmentally and tissue-specific manner.

Keywords: Laminet-1; Laminet-2; Alternative splicing; Differential expression; Semiquantitative RT-PCR

The developing nervous system is dependent on the actions of various secreted factors and membrane proteins that allow axons to find their correct targets. The netrin family was originally defined by netrin 1 and netrin 2, which were isolated from vertebrates [1–3]. They are secreted proteins, structurally related to the short arms of laminin γ [4]. Netrin 4 is also a secreted protein, but is more similar to the laminin β chains [5,6]. Recently, two diverged molecules, netrin-G1 (Ntng1) and netrin-G2 (Ntng2), also called laminet-1 and laminet-2, respectively, have been identified from the mouse and included as family members [7–9]. These molecules differ from classical netrins by three main features: (1) the presence of a glycosyl phosphatidylinositol lipid (GPI) site for membrane anchorage, (2) the generation of multiple isoforms, and (3) the failure to bind classical netrin receptors [7–9]. No orthologues for these genes have been found in *Caenorhabditis elegans* or *Drosophila melanogaster*, prompting the suggestion that netrins-G1 and -G2 may provide a function in cell architecture that is unique to vertebrates. Supporting this theory is the finding that netrin-G1 shows genetic association with schizophrenia [10]. We set out to complete the genomic mapping and cDNA structure determination for human netrin-G1 and all its isoforms. We also examined the tissue distribution of mRNA for *NTNG1* and *NTNG2*, as well as the expression of *NTNG1* alternatively spliced transcripts.

First, mouse netrin-G1a cDNA sequence NM_030699 and G1d sequence AB038664, as well as human netrin-G1 expressed sequence tag (EST) clones BC030220 and AB023193, were aligned with human genomic BAC clones RP11-270C12, RP11-396N10, and RP11-436H6 (GenBank Accession Nos. AC114491, AL590427, and AL513187, respectively), using the NCBI BLAST 2 sequences algo-

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rithm (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). These comparisons identified a total of 10 exons for *NTNG1* (Fig. 1), with the intron/exon boundaries shown in Supplementary Fig. 1. All of the splice junctions conform to the basic GT/AG rule although there are deviations from the extended consensus sequence starting at exon 4. Translation starts in exon 2 and encodes a maximally sized predicted protein of 581 amino acids. The context of the initiator methionine (GAAUUUAGA*A*UGU, in which A of the *A*UG represents position 1) maintains the −3 and −9 positions relative to the Kozak consensus (GCCGCGA/GCC*A*UGG) [11]. Six potential start sites, all in a poor context, lie upstream. This suggests that *NTNG1* may be regulated at the translational stage. Translation of each exon sequence revealed that exons 2 and 3 code for the VI domain, exons 4 and 5 for the V-1 domain, exon 6 and 7 for the unknown domain [7], exons 8 and 9 for domains V-2 and V-3, respectively, and exon 10 for the C′ domain, which contains the GPI anchor (Fig. 1).

Northern analysis using *NTNG1* exon 3 as a probe detected two groups of transcripts. The upper band at 4.4 kb may represent a partially spliced transcript, AB023193, which comprises spliced exons 1 to 5 with a retained intron 5. The lower diffuse band at 3.2 kb should represent the alternatively spliced transcripts. Both sized transcripts are present in the brain and kidney with possible weak expression in the spleen, liver, small intestine, placenta, and lung (Fig. 2). In the brain, expression was strongest in the cerebral cortex followed by the occipital pole, frontal lobe, temporal lobe, and putamen. Strong expression has also been detected in the thalamus and inferior colliculus [7].

For the determination of *NTNG2* structure, the mouse netrin-G2 cDNA sequence NM_133500.1 and a human *NTNG2* EST sequence NM_032536.1 were aligned with human BAC clones RP11-479K20, RP11-203M2, RP11-5N16, and RP11-738I14 (AL159997.14, AL353701.15, AL353631.17, and AL354735.14, respectively) in the manner previously stated. This analysis detected eight exons

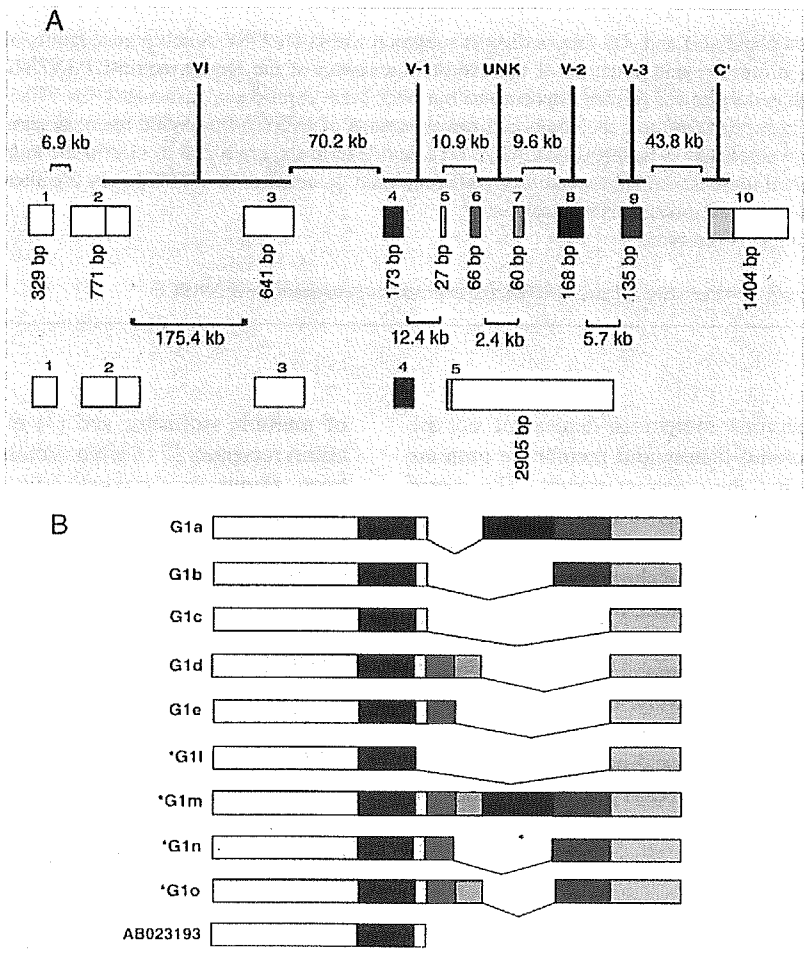

Fig. 1. Genomic organization and isoforms of *NTNG1*. (A) Upper diagram shows the genomic layout of *NTNG1* exons and their encoded domains. The lower diagram shows the exons that encode the truncated transcript AB023193. The span of each domain is indicated by an inverted "T". Brackets denote intron sizes. Colored boxes denote coding exons, and white boxes, untranslated regions. (B) Exonic composition of the nine alternatively spliced isoforms and the database-derived human transcript AB023193. Asterisks denote newly detected isoforms. Diagram is not to scale.

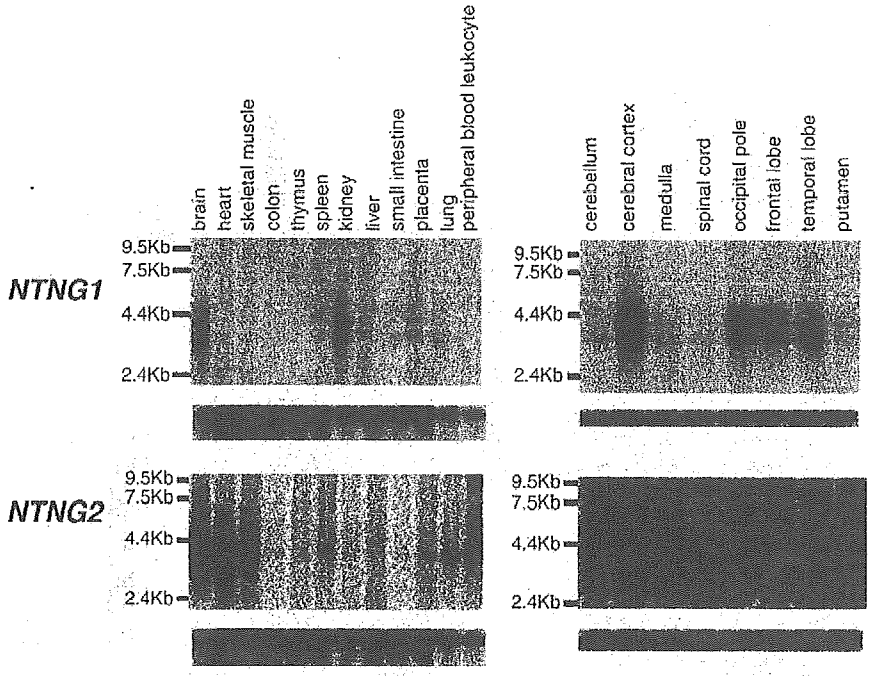

Fig. 2. Northern analyses of *NTNG1* and *NTNG2*. The blots were purchased from Clontech. Probes for both genes were derived from exon 3 (*NTNG1*-F, GTTTGATTTTGAAGGAAGACA, 3′ end at nt 338, -R, AAAAACGCGAACCTGTC, 3′ end at nt 680, BC030220; and *NTNG2*-F, CCACCTACTGGCA-GAGCATCA, 3′ end at nt 331, -R, CGATGTTGGAGATGGCGTAGAAGT, 3′ end at nt 821, AB058760). β-Actin was used as a control probe.

for *NTNG2* (data shown in Supplementary Fig. 2, since it was superseded by the sequence OTTHUMG00000020835 located at http://www.ensembl.org/Homo_sapiens/). All splice junctions conform to the GT/AG rule but few conserve the longer consensus sequence (Supplementary Fig. 1). The initiator methionine context maintains the −6 and essential −3 position (UGCGCAGCC*A*UGC, in which A of the *A*UG represents position 1) and three upstream start sites also maintain the −3 position, suggestive of translational control as for *NTNG1* [11]. Exons 2 and 3 code for domain VI, and exons 4, 5, 6, and 7 code for domains V-1, V-2, V-3, and C′, respectively, producing a predicted protein of 530 amino acids (Supplementary Fig. 2). An exon 3-derived probe identified two diffuse bands, spanning 4.4 to 3.6 kb, in the brain and peripheral blood leukocytes (Fig. 2). A diffuse band of approximately 3.6 kb was observed in heart and skeletal muscle with very low signal in placenta and lung tissues. In the brain, two transcripts were observed in all regions tested, with the possible exception of the cerebellum and medulla, where only the smaller sized transcript was present. This differential detection of *NTNG2* transcripts suggests alternative splicing of the gene although this feature was not examined in this study.

A fluorescently labeled probe derived from exons 1 to 5 of netrin-G1 showed a single hybridization to chromosome 1 at p13.3 (Supplementary Fig. 3), in keeping with its database assignment. A netrin-G2 probe (exons 2–6) showed fluorescent signal at 9q34 in all cells, with weak signal at 12q24.3 in 20% of the cells (Supplementary Fig. 3). The probe sequence showed no significant similarity to *NTN4*, which maps to 12q22–q23, nor to related family members *NTN1* and *NTN2L* (17p13.1 and 16p13.3, respectively) [5,12,13], indicating a novel but related sequence of netrin-G2 at this locus.

Previous studies reported six mouse Ntng1 isoforms that retain the GPI lipid anchor [7,9]. To detect human alternative splice forms, we performed RT-PCR analysis using adult whole brain-derived cDNA (Clontech, Palo Alto, CA, USA) with primers designed to exons 4 and 10. These exons were chosen because they flank the variable region observed in the mouse and select for transcripts containing the GPI lipid anchor [7]. Amplicons were cloned and sequenced to determine their precise size and exonic composition. A total of nine human isoforms denoted as G1a–G1o, in keeping with the original mouse nomenclature [7,9], were identified (Fig. 1). Mouse isoforms G1f, G1h, G1i, and G1j were not detected in this study since they lack the GPI domain-encoding exons. Isoforms G1a, G1b, G1c, G1e, and G1f have already been detected in the mouse [8,9]. The large number of *NTNG1* isoforms detected in human brain reflects findings that between 40 and 60% of genes undergo alternative splicing, with the brain showing the highest levels of exon skipping [14,15]. The exon skipping seen in *NTNG1* results in essentially the same amino acid sequence for each included domain but with single amino acid changes in some isoforms at points where exon fusions form a new triplet (Supplementary Fig. 4). These spliced variants are conserved among several species including mouse, rat, cattle, and chicken, implying functional gene diversification. This is supported by the semiquantitative detection of differential

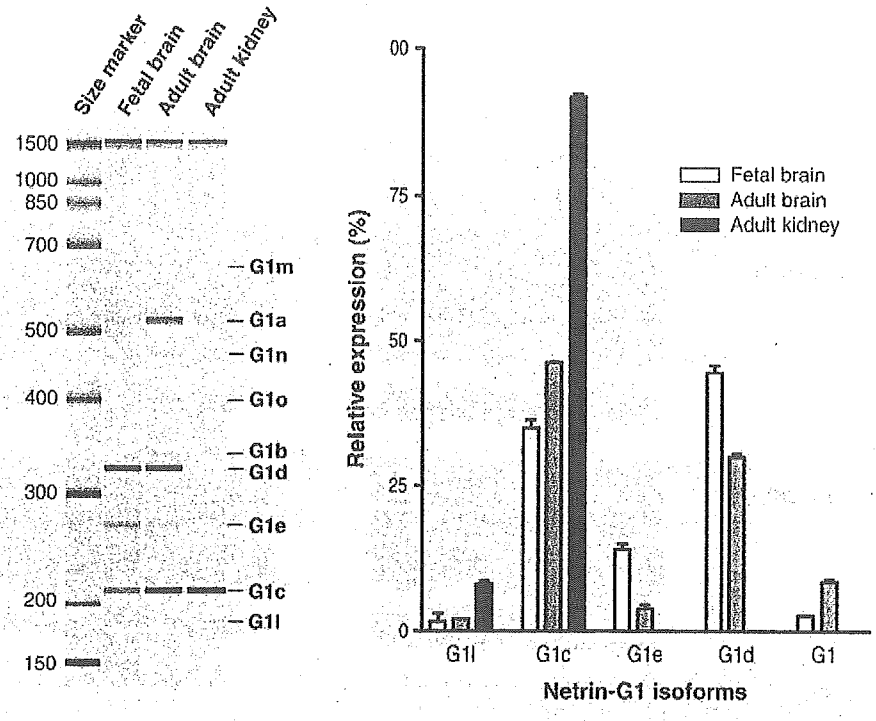

Fig. 3. Tissue-dependent expression patterns of *NTNG1* isoforms. Left shows an Agilent gel (Agilent Technologies, Palo Alto, CA, USA) containing PCR products representing alternatively spliced *NTNG1* isoforms derived from human fetal brain (22 weeks), adult brain, and adult kidney (Clontech). The exon 4 primer is F, CCCCATCCCCAAAGGCACTGC, and exon 10 primer, R, GTCGGAGCCGCAGCTGCCAGC. The isoform bands were distinguished by size comparison with previously sequenced clones. The bar chart shows the relative expression of the major *NTNG1* isoforms detected in human fetal brain, adult brain, and adult kidney. For the semiquantitative assay, PCR amplicons were loaded onto an Agilent 2100 Bioanalyzer utilizing a DNA 1000 LabChip kit (Agilent Technologies). The relative expression of each isoform was calculated by determining the fraction of each isoform from the total quantity of detected isoforms. Values are an average of two independent experiments.

expression between the brain and the kidney (Fig. 3). The isoform amplicons were identified by direct comparison of the computer-generated sizes with previously sequenced clones. The relative expression of each isoform was calculated by first normalizing total expression of netrin-G1 in each cDNA pool (using primers to exon 3) and then determining the ratio of each isoform relative to the total isoform pool. In the kidney, isoform G1c is the major transcript, with low levels of G1l. Isoform G1c was shown to bind to the netrin-G1 ligand (NGL-1), promoting the outgrowth of thalamic neurons in culture [16]. Interestingly, Northern analysis shows no expression of *NGL1* in the kidney [16], so in this tissue, G1c appears to require a different ligand. Although no human locus for kidney disease maps to the *NTNG1* position at 1p13.3, this region is syntenic with mouse chromosome 3, where a modifier locus for renal vascular disease lesions has been identified [17].

Additionally, there is evidence of developmental regulation of *NTNG1* in the brain (Fig. 3). Of the nine isoforms detected in adult brain, only five isoforms were present in fetal brain, with G1c and G1d being the major species. Fetal brain shows higher levels of G1d and the minor transcript G1e relative to adult brain, as well as lower levels of G1c and the minor transcript G1a. While G1a most closely resembles the classical netrins in structure, G1c is the most abundantly expressed isoform and it differs from G1d by the inclusion of an unknown domain coded for by exons 6 and 7. These exons have not been previously identified in human *NTNG1* ESTs, but are conserved among mouse, rat, and chicken. No specific sequences or regulatory elements that drive alternative splicing in *NTNG1* have been identified to date, nor have any mutations been associated with specific isoforms. The truncated AB023193 encodes a protein that would terminate after domain V-1. Its easy detection by Northern analysis suggests that this transcript may not undergo nonsense-mediated decay [18]. It remains to be seen how or whether this isoform, which is similar in sequence to G1c, plays a role in cellular migration.

Netrin-G1 null animals show reduced prepulse inhibition, strongly suggesting that the gene may be important in the maintenance of neuronal plasticity associated with sensory motor gating and or cognitive functioning [19]. Reduced prepulse inhibition is a behavioral paradigm associated with schizophrenia [20], raising the possibility that *NTNG1* plays a role in the pathophysiology of this disease. Indeed, an association study from our laboratory suggests that *NTNG1* does show association with schizophrenia, possibly via a mechanism that alters the ratio of isoform expression [21]. This could be analogous to pathologies associated with the tau protein. Mutations that affect splicing, particularly the

inclusion of exon 10, lead to skewed ratios of tau isoforms and, by multistep mechanisms, to several neurodegenerative disorders such as Alzheimer disease [22,23]. Further analysis is necessary to determine the cellular specificity of *NTNG1* isoforms and their roles in the development of sensory motor systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2005.04.004.

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Association of An Orexin 1 Receptor 408^{Val} Variant with Polydipsia–Hyponatremia in Schizophrenic Subjects

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Background: *Primary polydipsia is a common complication in patients with chronic psychoses, particularly schizophrenia. Disease pathogenesis is poorly understood, but one contributory factor is thought to be dopamine dysregulation caused by prolonged treatment with neuroleptics. Both angiotensin-converting enzyme (ACE) and orexin (hypocretin) signaling can modulate drinking behavior through interactions with the dopaminergic system.*

Methods: *We performed association studies on the insertion/deletion (I/D) sequence polymorphism of ACE and single nucleotide polymorphisms within the prepro-orexin (HCRT), orexin receptor 1 (HCRTR1), and orexin receptor 2 (HCRTR2) genes. Genotypes were determined by polymerase chain reaction amplification, followed by either electrophoretic separation or direct sequencing.*

Results: *The ACE I/D polymorphism showed no association with polydipsic schizophrenia. Screening of the orexin signaling system detected a 408 isoleucine to valine mutation in HCRTR1 that showed significant genotypic association with polydipsic–hyponatremic schizophrenia (p = .012). The accumulation of this mutation was most pronounced in polydipsic versus nonpolydipsic schizophrenia (p = .0002 and p = .008, for the respective genotypic and allelic associations). The calcium mobilization properties and the protein localization of mutant HCRTR1 seem to be unaltered.*

Conclusion: *Our preliminary data suggest that mutation carriers might have an increased susceptibility to polydipsia through an undetermined mechanism.*

Key Words: Schizophrenia, drinking behavior, neuroleptics, hypocretin, angiotensin-converting enzyme, dopamine system

Polydipsia, or the excessive intake of fluids, is commonly observed in patients with psychoses, particularly those with chronic schizophrenia (de Leon et al 1994; Vieweg et al 1985). Simple polydipsia often goes unrecognized until patients develop the more severe condition, water intoxification, which manifests both neurological and psychiatric symptoms (Goldman 1991). This disorder is comparable to neuroleptic-induced tardive dyskinesia and malignant syndrome in the management of psychiatric illnesses and is responsible for nearly 20% of premature deaths among schizophrenic subjects (Vieweg et al 1985).

Little is known about causative genetic factors, but the existence of genetically polydipsic mice suggests that the condition might have a genetic basis (Silverstein and Sokoloff 1958). Other studies have suggested alcohol dependence, male gender, and smoking as co-associating factors (Ahmed et al 2001; Shinkai et al 2003). Polydipsia is thought to be a side effect of chronic neuroleptic treatment, generating a hypersensitivity to dopamine that alters hypothalamic function. The observation that clozapine, an atypical antipsychotic with limited dopamine D2 receptor activity, alleviates the symptoms of polydipsia, lends weight to this theory (Canuso and Goldman 1999). Additionally, the behavioral pattern of increased drinking episodes and clustering of these episodes without an increase in the volume ingested in each session suggests that polydipsia might represent a stereotypy, a compulsive and repetitive behavior often observed in rodents after dopaminergic stimulation (Shutty and Song 1997); however, there is no genetic evidence linking dopamine-related genes with polydipsia.

Verghese et al (1993) suggested that normal drinking behavior is modulated by the interaction of dopamine D2 receptors with angiotensin. This precursor molecule from the renin–angiotensin system is converted in two stages to angiotensin II (ATII), a powerful dipsogen (Simpson and Routtenberg 1978). The rate-limiting enzyme in this catalysis is the angiotensin-converting enzyme (ACE), and inhibitors of this enzyme have been shown to reduce water intake in both rats and humans (Greendyke et al 1998; Rowland et al 1994). Association studies based on an *ACE* insertion/deletion (I/D) polymorphism that controls ATII levels, however, are inconclusive. The first study described a modest association with simple polydipsia, and the second failed to confirm this finding (Ouyang et al 2001; Shinkai et al 2003).

Orexins (also known as hypocretins) play a role in animal feeding and drinking behavior. Kunii et al (1999) reported that central administration of orexin A in rats stimulated an acute increased water intake, with a longer-lasting effect than ATII. Similar studies in rats have noted an increase in both feeding and drinking behavior, as well as hyperlocomotion and stereotypy, after orexin administration (Dube et al 1999; Hagan et al 1999; Sakurai 1999; Sakurai et al 1998). Orexin neurons are specifically localized in the lateral hypothalamic area and the medial part of the zona incerta, both anatomical regions thought to be involved in the regulation of drinking and feeding behaviors (Gonzalez-

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