Table 1b
TGFBR2 mRNA expression in medication-free schizophrenic ($N = 19$) and control subjects ($N = 19$)

		Male ($N = 9$)	Female ($N = 10$)	Total ($N = 19$)
Schizophrenia (S1–S19)	Age	28.1 ± 5.6	32.4 ± 11.5	30.4 ± 9.3
	The TGFBR2 mRNA expression before treatment	Isoform A + isoform B		
		0.99 ± 0.23	1.11 ± 0.18	1.05 ± 0.20*
		Isoform B		
		1.00 ± 0.24	1.19 ± 0.34	1.11 ± 0.30*
Control	Age	27.6 ± 4.8	33.4 ± 10.4	30.6 ± 8.6
	The TGFBR2 mRNA expression	Isoform A + isoform B		
		0.79 ± 0.17	0.83 ± 0.16	0.81 ± 0.16
		Isoform B		
		0.78 ± 0.12	0.88 ± 0.16	0.83 ± 0.15

The mean TGPBR2 mRNA levels of medication-free schizophrenia patients were significantly higher than those of age-and sex-matched controls (isoform A + isoform B, $P < 0.001$, isoform B; $P = 0.003$, paired T-test). No correlation between TGFBR2 mRNA levels and baseline BPRS scores were observed (isoform A + isoform B; $P = 0.23$, isoform B; $P = 0.97$, Spearman's correlation efficient).
* $P < 0.01$, compared with the control group.

All subjects signed written informed consent to participate in the expression and genetic association studies approved by the institutional ethics committees.

3. Quantitative real-time PCR

Total RNA was extracted from the peripheral leukocytes using the PAXgene Blood RNA kit (Qiagen, Tokyo, Japan) according to the manufacturer's recommendations. One microgram of total RNA was used for cDNA synthesis by QuantiTect Reverse Transcription Kit (Qiagen, Japan) after assessing RNA quality and quantity with NanoDrop (NanoDrop Technologies, DE, USA). Expression of the TGFBR2 gene transcript was quantified by real-time PCR with the TaqMan Gene Expression Assay (Applied Biosystems, CA, USA). TGFBR2 gene has two splicing variants (isoform A, isoform B) (Lin et al., 1992; Nikawa, 1994). Suzuki et al. indicated that both isoforms of TGFBR2 gene mouse homolog are expressed in all tissues studied (Suzuki et al., 1994) and Hirai et al. showed that the isoform B is a major type of human TGFBR2 mRNA determined by RT-PCR (Hirai and Fujita, 1996). We measured the expression levels of isoform B separately as well as the transcript combinations of isoform A + isoform B using ABI probe/primers (Hs00559661_m1, Hs00947893_m1). GAPDH gene expression was used as an internal control and measurement of threshold cycle (Ct) was performed in triplicate. Data were collected and analyzed with Sequence Detector Software version 2.1 (Applied Biosystems) and the standard curve method. Relative gene expression was calculated as the ratio of TGFBR2 to GAPDH gene and the mean of the three replicate measures was assigned to each individual. Chronbach's alpha coefficient of three replicate measures was 0.980 and standard error of measurement was 0.122. The expression of the TGFBR2 mRNA in the peripheral leukocytes was not changed among blood samples collected at several points during the day time or over several weeks in the same control subject.

4. Genotyping

Genotyping was performed using commercially available TaqMan probes for TGFBR2 gene (C_29354774_10, C_29354775_10, C_27491740_10, C_1612565_10, C_11565984_20, C_1612508_10, C_11566050_10, C_8778140_10, C_25809090_10, C_15882489_10) with Applied Biosystems 7500 Fast Real Time PCR System according to the protocol recommended by the manufacturer (Applied Biosystems, CA, USA). We selected these 10 single nucleotide polymorphic (SNP) markers for genotyping from the public databases (dbSNP Home page) according to International Hap Map Project (http://www.hapmap.org/index.html.en). The heterozygocities of these 10 SNPs, rs7625858 (C/T), rs7648606 (C/T), rs3087465 (A/G), rs4522809 (C/T), rs12487185 (A/G), rs1864615 (A/G), rs3773652 (A/G), rs1367609 (A/C), rs3773663 (A/G) and rs2276767 (A/C) in Japanese population are reported as 0.23, 0.10, 0.18, 0.38, 0.37, 0.45, 0.48, 0.49, 0.42 and 0.09, respectively.

5. Statistical analysis

Statistical calculations were carried out using the SPSS Statistical Software Package 11.5 (SPSS, Tokyo, Japan). Expressional differences between patients and age-and sex-matched control subjects were calculated using the paired T-test after checking equal variances by Kolmogorov–Smirnov test. Changes before and after treatment were also analyzed with the paired T-test. Spearman correlation coefficients were used to evaluate the correlations between TGFBR2 mRNA levels and BPRS scores. Analysis of covariance (ANCOVA) was performed to determine the independent and combined effect of sex, diagnosis and age with the expression of TGFBR2 between groups. All significance levels were two-tailed. Allele and genotype frequencies of patients and control subjects were compared using Fisher's exact test. The SNPAlyze 3.2Pro software (DYNACOM, Japan) was used to estimate haplotype

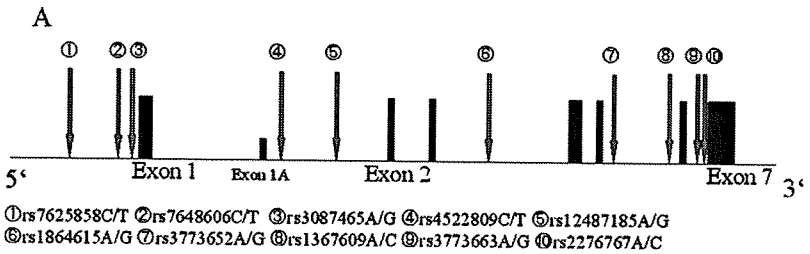

Fig. 1. Graphic representation of the TGFBR2 gene and the SNPs analyzed in the present study. Isoform B is major spliced variant without exon 1A. The amino acid sequence of isoform A contains an inset of 26 amino acids after Ser31, replacing Val132 of TGFBR2 isoform B.

frequencies, LD, and permutation P values. Pair-wise linkage disequilibrium (LD) indices, D' and r2, were calculated in the control subjects. The criterion for significance was set at $P < 0.05$ for all tests. Data are presented as mean ± SD. Our sample size had a post hoc power of 0.81 to detect an effect size of $w = 0.22$ at the 0.05 significance level, as calculated by software program G Power (Erdfelder et al., 1996) (see Fig. 1).

6. Results

6.1. TGFBR2 mRNA expression in medication free schizophrenic and control subjects (Tables 1a and 1b)

Relative expression levels of TGFBR2 mRNA (isoform A + isoform B) in 19 medication-free patients were 1.05 ± 0.20, while 0.81 ± 0.16 in healthy volunteers, showing a statistical difference (paired T-test: $P < 0.001$, Kolmogorov–Smirnov test: $P = 0.200$, Fig. 2). No correlation between TGFBR2 mRNA levels and baseline BPRS scores were observed (Spearman's correlation efficient: $P = 0.23$). The same result was also obtained in the mRNA expression levels of TGFBR2 isoform B (data shown in Tables 1a and 1b).

6.2. TGFBR2 mRNA expression in schizophrenia after several weeks antipsychotic treatment (Tables 2a and 2b)

The TGFBR2 mRNA levels after antipsychotic treatment for several weeks were investigated in 13 subjects who were able to be followed up among 19 medication-free patients. Mean chlorpromazine-equivalent doses were 490.4 ± 510.1 mg/day and mean duration of treatment was 68.6 ± 23.9 days. BPRS scores were significantly improved after antipsychotic treatment for several weeks (at baseline: 43.3 ± 19.6, after treatment: 35.1 ± 13.4; paired T-test: $P = 0.002$, Kolmogorov–Smirnov test: $P = 0.200$) and the mean TGFBR2 mRNA levels (isoform A + isoform B) also showed a significant decrease toward healthy control levels after antipsychotic treatment (at baseline: 1.04 ± 0.18, after treatment: 0.88 ± 0.23; paired T-test: $P = 0.027$, Kolmogorov–Smirnov test: $P = 0.200$). The TGFBR2 mRNA levels after treatment were not different from controls' (paired T-test: $P = 0.14$). No correlation between TGFBR2 mRNA levels and BPRS scores after treatment were observed (Spearman's correlation efficient: $P = 0.37$). The changes of BPRS scores did not show significant correlation with the change of the mRNA levels (Spearman correlation efficient: $P = 0.86$).

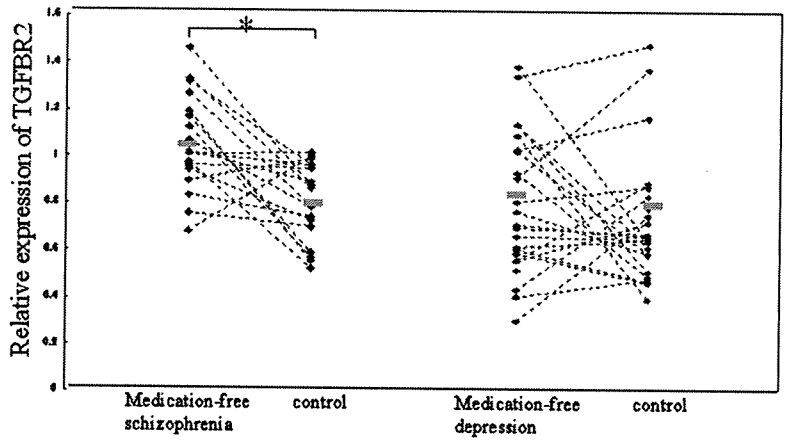

Fig. 2. Compared with the normal control group, the mean TGFBR2 mRNA level (isoform A + isoform B) in the leukocytes of medicaction-free schizophrenic patients ($N = 19$) was significantly higher than that of age- and sex-matched controls (patients: 1.05 ± 0.20, controls: 0.81 ± 0.16, paired T-test: $P < 0.001$). The mean TGFRBR2 mRNA level (isoform A + isoform B) in the leukocytes of medication-free major depressive patients ($N = 25$) showed no significant difference compared with sex- and age-matched controls (patients: 0.89 ± 0.31, controls: 0.84 ± 0.28, paired T-test: $P = 0.452$). *$P < 0.01$, compared with the control group.

The same result was also obtained in the mRNA expression levels of TGFBR2 isoform B (data shown in Tables 2a and 2b).

6.3. TGFBR2 mRNA expression in medication free major depression and control subjects

Relative expression levels of TGFBR2 mRNA (isoform A + isoform B) in 25 medication-free major depressive patients were 0.89 ± 0.31, while 0.84 ± 0.28 in healthy volunteers, showing no significant statistical difference (paired T-test: $P = 0.452$, Fig. 2). TGFBR2 mRNA expression levels of isoform B also showed the same result.

7. Genetic association analysis (Tables 3 and 4)

There were no significant deviations in all 10 SNPs from Hardy–Weinberg equilibrium in either patients or control subjects. Allele and genotype frequencies of the eight SNPs are shown in Table 4. There were no associations between these SNPs and schizophrenia neither in the allelic frequencies nor in the genotypic distributions. Permutation test of rs7625858–rs7648606 ($D' = 0.895$), rs7648606–rs3087465 ($D' = 0.866$) and rs3773663–rs2276767 ($D' = 0.945$) showed no significant difference in estimated frequencies of these haplotypes between the controls and patients (permutation $P = 0.19$, 0.27, 0.96, each).

Table 2a
Demographic data for schizophrenic patients after short-term antipsychotic treatment studied ill TGFBR2 mRNA expression analysis ($N = 13$)

	Age (y.o)	Gender	Duration of treatment (day)	Medication (at second point)	BPRS score
S1	25	M	90	Olz 10 mg	34
S2	24	M	134	Ris 3 mg	37
S3	24	M	54	Ris 3 mg	20
S4	27	M	55	Sulpiride 100 mg	27
S5	36	M	57	Olz 20 mg	23
S6	39	M	74	Olz 20 mg	36
S7	27	M	59	Olz 5 mg	47
S8	20	F	57	Ris3 mg, Lp25 mg	36
S9	23	F	71	Ris 2 mg	34
S10	34	F	85	Ris 2 mg	20
S11	47	F	47	Olz 15 mg	40
S12	15	F	44	Ris 2 mg	31
S13	26	F	65	Olz 20 mg, Ris 12 mg	71

Thirteen subjects (S1–S13) in Tables 2a and 2b were samples who were able to be followed up among 19 medication-free patients in Tables 1a and 1b. The age (years old: y.o) represent the age of the subject when the leukocytes were drawn. M = male, F = female, Olz: olanzapine, Ris: risperidone, LP: levomepromazine.

Table 2b
TGFBR2 mRNA expression in schizophrenics before treatment and after several weeks antipsychotic treatment ($N = 13$) and control subjects ($N = 13$)

		Male ($N = 7$)	Female ($N = 6$)	Total ($N = 13$)
Schizophrenia (S1–S13)	Age	28.9 ± 6.1	27.5 ± 11.5	28.2 ± 8.6
	The TGFBR2 mRNA expression before treatment	Isoform A + isoform B		
		1.00 ± 0.20	1.08 ± 0.16	1.04 ± 0.18*
		Isoform B		
		0.97 ± 0.21	1.13 ± 0.39	1.04 ± 0.30*
	The TGFBR2 mRNA expression after treatment	Isoform A + isoform B		
		0.75 ± 0.23	1.03 ± 0.10	0.88 ± 0.23
		Isoform B		
		0.61 ± 0.19	0.86 ± 0.17	0.72 ± 0.22
Control	Age	28.1 ± 5.2	29.2 ± 10.0	28.6 ± 7.5
	The TGFBR2 mRNA expression	Isoform A + isoform B		
		0.76 ± 0.18	0.77 ± 0.17	0.77 ± 0.17
		Isoform B		
		0.78 ± 0.14	0.82 ± 0.13	0.80 ± 0.13

BPRS scores were significantly improved after antipsychotic treatment for several weeks (at baseline: 43.3 ± 19.6, after treatment: 35.1 ± 13.4; paired T-test: $P = 0.002$).

The mean TGFBR2 mRNA levels showed a significant decrease toward healthy control levels after antipsychotic treatment (isoform A + isoform B; $P = 0.027$, isoform B; $P = 0.003$, paired T-test).

The TGFBR2 mRNA levels after treatment were not different from controls' (isoform A + isoform B; $P = 0.14$, isoform B; $P = 0.20$, paired T-test).

* $P < 0.05$, compared with the control group.

Table 3
Linkage disequilibrium (LD) indices (lower left are r2, upper right are D')

	rs 7625858	rs 7648606	rs 3087465	rs 4522809	rs 12487185	rs 1864615	rs 3773652	rs 1367609	rs 3773663	rs 2276767
rs 7625858	–	0.89465	0.58411	0.39018	0.38766	0.11919	0.00178	0.08145	0.06098	0.00053
rs 7648606	0.24556	–	0.8664	0.35761	0.31141	0.419	0.25127	0.18866	0.10999	0.59183
rs 3087465	0.30864	0.25458	–	0.0239	0.06625	0.00499	0.06456	0.03747	0.12032	0.03609
rs 4522809	0.01817	0.02333	0.00006	–	0.79095	0.69391	0.1183	0.18935	0.03031	0.81976
rs 12487185	0.02622	0.0121	0.00069	0.42727	–	0.76359	0.06257	0.1694	0.0435	0.49541
rs 1864615	0.00601	0.00907	0	0.13551	0.23876	–	0.04822	0.09866	0.07076	0.40741
rs 3773652	0	0.0034	0.00066	0.00943	0.00386	0.00224	–	0.14808	0.08763	0.43636
rs 1367609	0.00191	0.00315	0.00037	0.01748	0.02032	0.00568	0.01578	–	0.40153	0.87012
rs 3773663	0.00081	0.00081	0.00429	0.00034	0.0015	0.00259	0.00413	0.12234	–	0.94548
rs 2276767	0	0.00281	0.00053	0.02974	0.01588	0.02651	0.01238	0.08098	0.10863	–

Table 4
Genetic studies of TGFBR2 with schizophrenia in ease-control samples

Snp	Group	Genotype			n	Hardy–Weinberg P-value		Allele		P-value
rs7625858		T/T	C/T	C/C				T	C	
	sch	166	94	16	276	0.702	0.732	426	126	0.469
	cont	177	87	15	279	0.420		441	117	
rs7648606		T/T	C/T	C/C				T	C	
	sch	227	45	4	276	0.508	0.465	499	53	0.238
	cont	239	38	2	279	0.944		516	42	
rs3087465		A/A	A/G	G/G				A	G	
	sch	16	98	163	277	0.933	0.224	130	424	0.095
	cont	13	82	184	279	0.432		108	450	
rs4522809		T/T	T/C	C/C				T	C	
	sch	123	122	31	276	0.964	0.649	368	184	0.403
	cont	131	122	25	278	0.757		384	172	
rs12487185		A/A	A/G	G/G				A	G	
	sch	57	126	94	277	0.269	0.476	240	314	0.223
	cont	48	124	106	278	0.319		220	336	
rs1864615		A/A	A/G	G/G				A	G	
	sch	36	123	117	276	0.780	0.385	195	357	0.260
	cont	47	117	108	272	0.154		211	333	
rs3773652		A/A	A/G	G/G				A	G	
	sch	44	142	92	278	0.447	0.466	230	326	0.626
	cont	47	128	104	279	0.559		222	336	
rs1367609		A/A	C/A	C/C				A	C	
	sch	75	133	70	278	0.552	0.192	283	273	0.338
	cont	58	151	69	278	0.114		267	289	
rs3773663		A/A	A/G	G/G				A	G	
	sch	58	132	85	275	0.699	0.588	248	302	1.0
	cont	52	145	80	277	0.401		249	305	
rs2276767		A/A	A/C	C/C				A	C	
	sch	3	43	232	278	0.799	1.0	49	507	1.0
	cont	4	42	233	279	0.355		50	508	

sch, schizophrenia; cont, control subjects. P-values are calculated by Fisher's exact test.
There were no associations between these SNPs and schizophrenia neither in the allelic frequency not in the genotypic distributions.

8. Discussion

In the present study, relative expression levels of the TGFBR2 mRNA (isoform A + isoform B, isoform B) in both medication-free schizophrenic patients and major depressive patients were investigated. In addition, the association between 10 polymorphisms in the TGFBR2 locus and schizophrenia was investigated. To the best of our knowledge, this is the first study to investigate the role of TGFBR2 in the pathogenesis of schizophrenia.

First, our data showed that the mRNA expression level of TGFBR2 gene in the peripheral leukocytes was significantly higher in medication-free schizophrenics but not in medication-free depression. The results suggest that the expressional change of TGFBR2 gene in schizophrenia may be disease-specific and not due to non-specific stress from psychiatric condition. The BPRS scores were significantly improved after several week-antipsychotic treatment and the mean TGFBR2 mRNA levels showed a significant decrease toward healthy control levels after treatment. The

decrease of the TGFBR2 mRNA expression after treatment may be a consequence of pharmacological effects of antipsychotics or clinical improvement. These results suggest that altered expression of TGFBR2 mRNA in the peripheral leukocytes from schizophrenic patients may not be trait-oriented but state-related change. Be contrary to our anticipation, the mRNA expression level of TGFBR2 gene was not up-regulated in schizophrenia who took antipsychotic medications. TGFBR2 may be associated with reportedly low susceptibility to cancer in unmedicated but not medicated schizophrenia. Other tumor suppressor genes or oncogenes may have strong influence on tumor resistance associated with schizophrenia. In spite of the limited number of medication-free schizophrenic samples, the fact that altered mRNA expression of TGFBR2 gene in schizophrenia before treatment may have pathophysiological significance because peripheral lymphocytes could reflect the metabolism of brain cells (Gladkevich et al., 2004). Further expression study using human brain tissue is needed in order to reveal the pathological role of TGFBR2 gene to schizophrenia.

Second, we investigated the genetic association between TGFBR2 gene and schizophrenia in Japanese population. The TGFBR2 gene is located at 3p22, which has been previously reported to be linked with schizophrenia. However we did not find any association of 10 SNPs in TGFBR2 gene (rs7625858, rs7648606, rs3087465, rs4522809, rs12487185, rs1864615, rs3773652, rs1367609, rs3773663 and rs2276767) with schizophrenia. Haplotype analyses in the TGFBR2 gene did not reveal any significance, either. Further studies with denser polymorphisms and a larger sample set will be needed although our sample sizes were suitable for genetic comparison (power > 0.8).

In conclusion, our investigation revealed that the mean TGFBR2 mRNA levels (isoform A + isoform B, isoform B) in medication-free schizophrenic patients were significantly higher than those of age- and sex-matched controls and showed a significant decrease toward healthy control levels after antipsychotic treatment. There were no associations between the TGFBR2 gene and schizophrenia. We conclude that the TGFBR2 gene itself does not link to schizophrenia but that the TGFBR2 mRNA levels in the peripheral leukocytes may be a potential state marker for schizophrenia.

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BIOLOGICAL PSYCHIATRY - ORIGINAL ARTICLE

Genetic association analysis of tagging SNPs in alpha4 and beta2 subunits of neuronal nicotinic acetylcholine receptor genes (*CHRNA4* and *CHRNB2*) with schizophrenia in the Japanese population

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Abstract Several lines of evidence suggest that nicotinic cholinergic dysfunction may contribute to the cognitive impairments in schizophrenia. The majority of high affinity nicotine binding sites in the human brain have been implicated in heteropentameric alpha4 and beta2 subunits of neuronal nicotinic acetylcholine receptors; therefore, these two neuronal nicotinic acetylcholine receptors genes (*CHRNA4* and *CHRNB2*) are considered to be attractive candidate genes for the pathophysiology of schizophrenia. To represent these two genes in a gene-wide manner, we first evaluated the linkage disequilibrium structure using our own control samples. Thirteen SNPs (7 SNPs for *CHRNA4* and 5 SNPs for *CHRNB2*) were selected as tagging SNPs. Using these tagging SNPs, we then conducted genetic association analysis of case-control samples (738 schizophrenia and 753 controls) in the Japanese population. No significant association was detected in the allele/genotype-wise or haplotype-wise analysis. Our results suggest that *CHRNA4* and *CHRNB2* do not play a major role in Japanese schizophrenia.

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Introduction

Cognitive impairments in areas such as attention, executive function, language and memory, for which there is not much hope of recovery with treatment, have been implicated as endophenotypes for schizophrenia (Green 1996), and such impairments may be partially mediated by nicotinic acetylcholine receptors (Levin and Simon 1998). A recent study has reported an association of such cognitive impairments with abnormalities in the neuronal network in the prefrontal cortex, superior temporal gyrus and cerebellum in schizophrenics (Bonilha et al. 2008). The smoking rate of schizophrenics is much higher than that of healthy individuals with lifetime history (Hughes et al. 1986). Schizophrenics may smoke to compensate for their cognitive dysfunctions, since nicotine has cognitive-enhancing properties (Kumari and Postma 2005).

The majority of high affinity nicotine binding sites in the brain consist of heteropentameric alpha4 and beta2 subunits of neuronal nicotinic acetylcholine receptors (nAChRs) (Flores et al. 1997). Several lines of evidence support an association between abnormalities in alpha4 and beta2 subunits and schizophrenia. Firstly, beta2 subunits in the ventral tegmental area in mutant mice showed alternation of dopamine release in the nucleus accumbens and changes in cognitive functions such as navigation and exploratory behaviour compared with wild mice (Maskos et al. 2005). Alpha4 subunit mutant mice also showed increased anxiety-like behaviour and a reduction of substantia nigra dopaminergic neurons on ageing (Labarca et al. 2001; Ross et al. 2000). These animal study results

may reflect dopamine and neurodevelopmental hypotheses of the pathophysiology of schizophrenia, and these behaviors may be major symptoms of schizophrenia (Lang et al. 2007). Secondly, a postmortem study reported fewer alpha4/beta2 subunits in the hippocampi of schizophrenics (Freedman et al. 1995). Thirdly, De Luca et al. reported that the alpha4 gene (*CHRNA4*) and beta2 subunit gene (*CHRNB2*) showed genetic interactions with schizophrenia in a family-based association study in the Canadian population (De Luca et al. 2006), and *CHRNA4* and *CHRNB2* were also associated with smoking among schizophrenics (Faraone et al. 2004; Voineskos et al. 2007).

Therefore, we conducted a genetic association study of *CHRNA4* (located on 20q13) and *CHRNB2* (located on 1q21) with schizophrenia in the Japanese population. In this study, we applied the "gene-wide" approach recommended by Ikeda to consider the population differences (Ikeda et al. 2008), first evaluating the linkage disequilibrium (LD) structure of these genes and selecting tagging SNPs ("tag SNPs"). These tag SNPs were then used to present the LD properties of the gene in the Japanese population in the following association analysis.

Materials and methods

Subjects

The subjects in the association analysis were 738 schizophrenia patients (395 males and 343 females; mean age ± standard deviation (SD) 47.4 ± 16.6 years; age of onset 26.0 ± 9.55 years) and 753 healthy controls (326 males and 427 females; 37.3 ± 14.3 years). Patients were grouped according to the following DSM-IV subtypes of schizophrenia: Paranoid Type ($n = 216$), Disorganized Type ($n = 221$), Catatonic Type ($n = 29$), Residual Type ($n = 142$), Undifferentiated Type ($n = 130$). All healthy controls are identical to those in our previous paper (Kishi et al. 2008). The subjects for LD evaluation were 96 controls, who were also among the sample used in the association analysis. All subjects were unrelated to each other and ethnically Japanese. The patients were diagnosed according to DSM-IV criteria with the consensus of at least two experienced psychiatrists on the basis of unstructured interviews and a review of medical records. All healthy controls were also psychiatrically screened based on unstructured interviews that included current and past psychiatric history. None had serious medical complications such as cirrhosis, renal failure, heart failure or other Axis-I disorders according to DSM-IV. No structured methods were used to assess psychiatric symptoms in the controls that included hospital staff, their families and medical students.

After explaining the study to all subjects, written informed consent was obtained from each. This study was approved by the Ethics Committee at Fujita Health University and Nagoya University School of Medicine.

SNP selection and LD evaluation

Methods for selection of tagging SNPs were described in our previous paper (Kishi et al. 2008). Briefly, we first consulted the HapMap database (release#20/phaseII, Jan 2006, www.hapmap.org) to select the tag SNPs; however, no dense marker sets were listed in HapMap (2SNPs and 5SNPs in *CHRNA4* and *CHRNB2*, respectively). Therefore, we accessed another information source, the JSNP database (Haga et al. 2002; Hirakawa et al. 2002), and picked up 11 SNPs to evaluate the denser LD structure of *CHRNA4* (we tried to select SNPs with minor allele frequencies (MAFs) of more than 0.05 to increase the power).

At first, we genotyped all these SNPs using our own 96 control samples to evaluate the LD structure in the Japanese population. We then selected tag SNPs with the criteria of r^2 threshold greater than 0.8 in "pair-wise tagging only" mode using the "Tagger" program (Paul de Bakker, http://www/broad.mit.edu/mpg/tagger) in Haploview 3.2 for the following association analysis (Barrett et al. 2005; Gabriel et al. 2002).

SNP genotyping

For rapid genotyping of SNPs, we used TaqMan assays (Applied Biosystems) and direct sequencing (Table 1). Detailed information, including primer sequences and reaction conditions, is available on request.

Statistical analysis

The genotypic deviation from Hardy-Weinberg equilibrium (HWE) was evaluated by χ^2 test (SAS/Genetics, release 8.2, SAS Japan Inc, Tokyo, Japan).

Marker-trait association was evaluated by the χ^2 test (allele and genotype-wise analyses), and the log-likelihood test (haplotype-wise analysis; the haplotype frequencies were estimated with the expectation-maximization algorithm) (SAS/Genetics, release 8.2). In this haplotype-wise analysis, the information of the "LD block" (criteria based on 95% confidential intervals on the *D'* values) in the LD evaluation step were used.

Power calculation was performed using a statistical program prepared by Ohashi et al. (2001). The significance level for all statistical tests was 0.05.

Table 1 tag SNPs and association analysis of *CHRNA4* and *CHRNB2*

Gene symbol	SNP ID[a]	Phenotype	MAF	Genotype deviation				*P* value		
				N	M/M	M/m	m/m	HWE	Genotype	Allele
CHRNA4	SNP A-1 rs755203	SCZ	0.418	732	250	352	130	0.752	0.972	0.809
		CON	0.414	747	259	358	130	0.742		
	SNP A-2 rs2273506	SCZ	0.105	732	585	141	6	0.431	0.124	0.0542
		CON	0.127	747	564	176	7	0.0939		
	SNP A-6 rs2273504	SCZ	0.465	732	214	355	163	0.493	0.741	0.560
		CON	0.454	747	232	351	164	0.152		
	SNP A-9 rs1044396	SCZ	0.287	732	379	286	67	0.222	0.572	0.476
		CON	0.275	747	393	297	57	0.932		
	SNP A-10 rs1044397	SCZ	0.390	731	278	336	117	0.360	0.826	0.539
		CON	0.401	747	273	349	125	0.454		
	SNP A-12 rs2236196	SCZ	0.116	731	573	147	11	0.656	0.167	0.309
		CON	0.128	747	563	177	7	0.0874		
	SNP A-13 rs4522666	SCZ	0.422	732	240	366	126	0.501	0.567	0.650
		CON	0.430	747	247	357	143	0.490		
CHRNB2	SNP B-1 rs4845652	SCZ	0.116	735	579	141	15	0.0697	0.401	0.194
		CON	0.101	749	607	132	10	0.359		
	SNP B-2 rs2072658	SCZ	0.201	738	467	246	25	0.283	0.930	0.772
		CON	0.205	747	466	256	25	0.155		
	SNP B-3 rs2072659	SCZ	0.143	734	542	174	18	0.370	0.623	0.606
		CON	0.136	751	559	179	13	0.759		
	SNP B-4 rs2072660	SCZ	0.252	735	410	279	46	0.873	0.585	0.352
		CON	0.238	753	440	268	45	0.622		
	SNP B-5 rs3811450	SCZ	0.119	736	576	145	15	0.106	0.218	0.981
		CON	0.119	751	580	163	8	0.354		

P value for association of SNPs in *CHRNA4* and *CHRNB2* with schizophrenia

[a] tag SNPs

SCZ schizophrenia, *CON* controls, *MAF* minor allele frequency of 96 controls, *N* number, *M* major allele, *m* minor allele, *HWE* Hardy–Weinberg equilibrium

Results

For the initial LD evaluation, 13 SNPs (2SNPs from HapMap, 11SNPs from JSNP) and five SNPs (5SNPs from HapMap) for *CHRNA4* and *CHRNB2*, respectively, were genotyped for 96 controls. The LD structure results can be seen in our previous paper (Kishi et al. 2008). Seven and five SNPs were then selected as tag SNPs for each gene (Kishi et al. 2008). The genotypic distributions of all SNPs were in HWE.

No significant associations were found between any tag SNPs in *CHRNA4* and *CHRNB2* and Japanese schizophrenia in the allele/genotype-wise analysis (Table 1) or in the haplotype analysis (*CHRNA4* Block I-P: 0.502, Block II-P: 0.432, *CHRNB2* Block I-P: 0.564). To further investigate these associations, we included an explorative analysis of gender effects, because recent studies showed that genetic factors underlying nicotine addiction probably play a different role in female and male smokers (Feng et al. 2004; Greenbaum et al. 2006; Li et al., 2005). This suggests the existence of sex-specific genetic components in nicotine use disorders or these genes. In the present analysis, we found that SNP A-10 and B-4 were significantly associated with male and female schizophrenics, respectively (SNP A-10 and male schizophrenia P: 0.0389, SNP B-4 and female schizophrenia P: 0.0262). However, these results were no longer statistically significant after Bonferroni correction (SNP A-10 and male schizophrenia P: 0.545, SNP B-4 and female schizophrenia P: 0.262).

In the power analysis, we obtained power of more than 80% for the detection of association when we set the genotype relative risk at 1.23–1.36 and 1.26–1.35 for *CHRNA4* and *CHRNB2*, respectively, under a multiplicative model of inheritance.

Discussion

In this study, we found no significant association between two major cholinergic receptor genes, *CHRNA4* and *CHRNB2*, and schizophrenia in the Japanese population.

Although nominal significant associations between two SNPs (A-10 and B-4) and subgroups divided by gender were detected in the explorative analyses, these associations might have been the result of type I error due to multiple testing.

A recent family-based association study showed gene–gene interactions between *CHRNA4* and *CHRNB2* (De Luca et al. 2006). Therefore, we applied the recently recommended strategy of "gene-based" association analysis for the purpose of detecting susceptibility genes for schizophrenia (Neale and Sham 2004), and conducted a case-control association analysis by selecting the tag SNPs. As in the original study, we did not detect an association between these genes and schizophrenia. To evaluate the interactions with each SNP in these genes, we then analyzed the gene–gene interactions with the use of the Multifactor Dimensionality Reduction (MDR) method (Hahn et al. 2003). This analysis, however, revealed no interactions with schizophrenia (data not shown).

Voineskos et al. showed a significant association of rs3746372 in *CHRNA4* with heavy smoking in schizophrenia (Voineskos et al. 2007). The rs3746372 is located upstream 39593 bp from the initial exon and rs3746372. In this study, we selected tag SNPs in an association analysis after performing a LD evaluation that covered *CHRNA4*, including the promoter region, using our control samples. Although we confirmed LD between the SNPs selected in this study and rs3746372 according to the HapMap database, this LD was not found to be tight. Since we thought that rs3746372 might not be included in *CHRNA4*, we did not perform an association analysis of this SNP.

A very recent study reported that two functional SNPs (rs6122429 and rs2236196) in CHRNA4 were associated with luciferase activity (Winterer et al. 2007), and the question of whether these SNPs were associated in our schizophrenic samples should be examined. Of these SNPs, rs2236196 was included in this study, but rs6122429 was not. To evaluate whether our tag SNPs represent this rs6122429, located in the 5′ flanking region of CHRNA4, we genotyped rs6122429 using our 96 control samples. We found that this SNP was in LD with our SNP1 ($r^2 = 0.85$), and thus speculate that rs6122429 is not associated with schizophrenia in the Japanese population.

Our study is reasonable in terms of its design (selecting tag SNPs to represent each gene) and sample size large enough to gain high power. However, a couple of limitations should be noted. First, our samples were not age- or gender-matched. Although we included subgroup analyses divided by gender, careful interpretation is needed with respect to the association of schizophrenia itself. Second, we did not include a mutation scan to detect rare variants with functional effects. However, it is difficult to evaluate the association for a rare variant (e.g. MAF < 0.05). A larger sample size will be required for conclusive results in mutation search and association analysis. Lastly, several investigations have suggested that alternation of nAChRs may reflect the cognitive dysfunction and nicotine dependence associated with schizophrenia (Faraone et al. 2004; Levin and Simon 1998; Voineskos et al. 2007). Moreover, because the heritability of nicotine dependence and schizophrenia is reported to be about 40–70% (Li et al. 2003; Maes et al. 2004; Swan et al. 1990) and 80% (Cannon et al. 1998), respectively, we considered that the influence of genetic factors was about the same. Therefore, further study will be required to investigate the relationship between these genes and cognitive function or/and high smoking rate in schizophrenia, because we did not have information on smoking history in our samples or evaluate cognitive function.

In conclusion, our results suggest that *CHRNA4* and *CHRNB2* do not play a major role in schizophrenia in the Japanese population.

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Genetic Variations of Human Neuropsin Gene and Psychiatric Disorders: Polymorphism Screening and Possible Association with Bipolar Disorder and Cognitive Functions

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Human neuropsin (NP) (hNP) has been implicated in the progressive change of cognitive abilities during primate evolution. The hNP gene maps to chromosome 19q13, a region reportedly linked to schizophrenia and bipolar disorder. Therefore, hNP is a functional and positional candidate gene for association with schizophrenia, mood disorders, and cognitive ability. Polymorphism screening was performed for the entire hNP gene. The core promoter region was determined and whether or not transcriptional activity alters in an allele-dependent manner was examined by using the dual-luciferase system. Allelic and genotypic distributions of five single-nucleotide polymorphisms (SNPs) were compared between patients with schizophrenia ($n=439$), major depression ($n=409$), bipolar disorder ($n=207$), and controls ($n=727$). A possible association of the hNP genotype with memory index (assessed with Wechsler Memory Scale, revised, WMS-R) and intelligence quotient (IQ assessed with Wechsler Adult Intelligence Scale, revised; WAIS-R) was examined in healthy controls ($n=166$). A total of 28 SNPs, including nine novel SNPs, were identified. No significant effects on transcriptional activity were observed for SNPs in the promoter region. A significant allelic association was found between several SNPs and bipolar disorder (for SNP23 at the 3′ regulatory region; odds ratio 1.48, 95% confidential interval 1.16–1.88, $P=0.0015$). However, such an association was not detected for schizophrenia or depression. Significant differences were observed between SNP23 and attention/concentration sub-scale score of WMS-R ($P=0.016$) and verbal IQ ($P<0.001$). Genetic variation of the hNP gene may contribute to molecular mechanisms of bipolar disorder and some aspects of memory and intelligence.
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Keywords: neuropsin; polymorphism screening; association study; bipolar disorder; memory; intelligence

INTRODUCTION

Neuropsin (NP, MIM: 605644), also called as kallikrein 8 (KLK8), is one of the secreted-type serine proteases, which was first cloned by our group in mice (Chen *et al*, 1995). NP mRNA is expressed specifically in the limbic system of mouse brain and is localized at the highest concentration in pyramidal neurons of the hippocampal CA1-3 sub-fields. Direct hippocampal stimulation and kindling induced by amygdaloid stimulation caused a significant bilateral change in NP mRNA level in the hippocampal pyramidal neurons. The activity-dependent changes and the specific localization indicate that NP is involved in hippocampal plasticity (Chen *et al*, 1995). Indeed, NP has a regulatory effect on Schaffer-collateral at the early phase of long-term potentiation (LTP) (Komai *et al*, 2000). Mice lacking NP were significantly impaired in the Morris water maze and Y maze, suggesting that NP has an important role in learning and memory (Tamura *et al*, 2006). The human NP (hNP) gene was cloned by Yoshida *et al* (1998), and then localized to chromosome 19q13.3–q13.4 (Gan *et al*, 2000; Harvey *et al*, 2000). It consists of six exons and the first exon is non-translational. Four alternative splicing variants have been identified (Mitsui *et al*, 1999; Magklara *et al*, 2001). The regular form is called type1, and type 2 contains a 135-bp

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insertion of 5′ upstream region of exon 3 (Mitsui *et al*, 1999). Interestingly, type 2 is a hominoid-specific splicing form (Li *et al*, 2004) and is expressed as abundantly as the type 1 in human brain (Mitsui *et al*, 1999). These findings points to the possibility that type 2 hNP may contribute to progressive change of cognitive abilities during primate evolution. Moreover, dysfunctions in hNP may be involved in psychiatric diseases of cognitive abilities, including schizophrenia and mood disorders.

Family, twin, and adoption studies clearly suggest that genetic components play an important role in the pathogenesis of schizophrenia and mood disorders (reviewed by Shih *et al*, 2004). These psychiatric diseases demonstrate substantial cognitive deficits such as learning and memory (reviewed by Sharma and Antonova, 2003; Robinson *et al*, 2006; Green, 2006). A genome screen of linkage with bipolar disorder pedigrees provided evidence for susceptibility locus on chromosome 19q13 (Badenhop *et al*, 2002). Another genome scan in schizophrenia and bipolar pedigrees obtained an LOD ratio score of 1.5 at 19q13 in schizophrenic families (Macgregor *et al*, 2004). Therefore, the hNP gene is a good candidate gene for association with schizophrenia and mood disorders. Here we performed, for the first time, a polymorphism screening and association analysis of the hNP gene with schizophrenia, major depression, and bipolar disorder in a Japanese sample. A possible association of hNP with memory and intelligence in healthy subjects was also examined. In addition, we determined a core promoter region of the hNP gene and examined whether transcriptional activity varies in an allele-dependent manner.

MATERIALS AND METHODS

Subjects

Subjects for the association study were 439 patients with schizophrenia (240 males, mean age of 44.6 years (SD 14.0)), 409 patients with major depression (136 males, 53.3 years (15.9)), 207 patients with bipolar disorder (80 males, 50.2 years (14.7)), and 727 healthy controls (324 males, 43.5 years (16.4)). Among these, 104 patients with bipolar disorder and 108 controls were recruited around Shiga prefecture, approximately 350 km to the west of Tokyo, while the remaining 1570 subjects were recruited around Tokyo. Consensus diagnosis by at least two psychiatrists, one of whom was in charge of the patients, was made for each patient according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) criteria (American Psychiatric Association, 1994), on the basis of unstructured interviews and information from medical records. Control subjects were healthy volunteers who had no current or past contact to psychiatric services. Among them 213 controls were screened by the Japanese version of the Mini-International Neuropsychiatric Interview (Sheehan *et al*, 1998; Otsubo *et al*, 2005) by a research psychiatrist, whereas the remaining controls were not screened by such a structured interview. Participants were excluded if they had prior medical histories of central nervous system disease or severe head injury, or if they met the criteria for substance abuse or dependence, or mental retardation. All subjects were biologically unrelated Japanese. After description of the study, written informed consent was obtained from every subject. The study protocol was approved by institutional ethics committees.

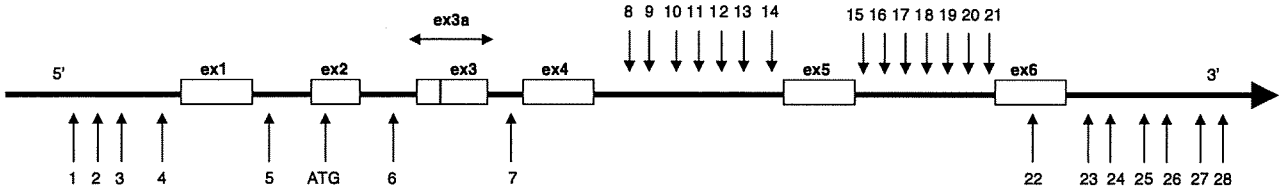
Neuropsychological Test Measures

Among controls, 166 (53 males, 37.6 years (12.4)) were subject to memory and intelligence tests to detect possible association with the hNP genotype. These individuals were all screened by the Mini-International Neuropsychiatric Interview with respect to their psychiatric history and confirmed that they had no current or past history of psychiatric illness. To assess memory and intelligence, Japanese full versions of the Wechsler Memory Scale-Revised (WMS-R) (Sugishita, 2001; Wechsler, 1987) and the Wechsler Adult Intelligence Scale-Revised (WAIS-R) (WAIS-R, Shinagawa *et al*, 1990; Wechsler, 1981), respectively, were administered. Testing and scoring were performed by psychologists who were blind to genotypic data.

Polymorphism Screening and Genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to standard procedures. The genomic structure of hNP was determined from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and the University of California at Santa Cruz (UCSC) database (http://genome.ucsc.edu/cgi-bin/hgBlat). To screen for polymorphisms, we used direct sequencing with the Genome Lab-DTCS (Dye Terminator Cycle Sequencing) kit and CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). The entire 7428-bp genomic region containing all the exons, introns, the 1078-bp 5′ flanking region upstream to exon 1, and the 655-bp 3′ flanking region downstream to exon 6 were amplified from the genomic DNA of 24 randomly selected schizophrenic subjects. Sequences of 24 sets of primers for the polymorphism screening are listed in Supplementary Table S1.

The examined 7428-bp region seemed to constitute of single haplotype blocks (Supplementary Figure S1). We genotyped five single-nucleotide polymorphisms (SNPs) using TaqMan 5′-exonuclease allelic discrimination assay. They were A-807>G (ss73688625, SNP3), G-658>C (rs1722550, SNP4), IVS2-101T>A (rs1701947, SNP6), IVS3-10A>G (rs1701946, SNP7), and A5229>G (rs1612902, SNP23) (Figure 1). SNPs 3 and 4 were chosen from the 5′ regulatory region since they may have some effects on transcriptional activity, and SNPs 1, 2, 4, and 5 were in absolute linkage disequilibrium (LD) (ie, genotypes were completely the same) with each other. SNPs 6 and 7 were chosen because they were SNPs located close to the splicing sites of exon 3a (ie, an exon specific to type 2 hNP) and exon 4, respectively, and may have some effects on splicing. SNP23 was chosen from the 3′ region, since SNPs15, 16, 17, 19, 21, 23, 24, 25, 27, and 28 were in absolute LD with each other. TaqMan probes and Universal PCR master mix were obtained from Applied Biosystems (Foster City, CA). Thermal cycling conditions for polymerase chain reaction (PCR) were 1 cycle at 95°C for 10 min followed by 50 cycles of 92°C for 15 s and 60°C for 1 min. After



1. rs2569456	g.G-1302>T	15. rs1654539	g. IVS5+106G>A
2. rs6509509	g.C-948>T	16. rs1722559	g. IVS5+184C>T
3. ss73688625	g.A-807>G	17. rs1701914	g. IVS5+187T>C
4. rs1722550	g.G-658>C	18. rs1722560	g. IVS5+673T>G
5. ss73688633	g.IVS1+25,26 ins/del CA	19. rs1722561	g. IVS5+764T>C
6. rs1701947	g.IVS2-236T>A (exon3)	20. rs1701915	g. IVS5-442A>G
7. rs1701946	g.IVS3-10A>G	21. rs1627239	g. IVS5-355G>T
8. ss73688628	g.IVS4+596G>A	22. ss73688626	g. G5047>A (Val 286 Ile)
9. rs7245714	g.IVS4+866C>T	23. rs1612902	g. A5229>G
10. ss73688629	g.IVS4+1034C>T	24. rs1654542	g. T5308>C
11. ss73688630	g.IVS4-951A>G	25. rs1654543	g. A5435>G
12. rs12610313	g.IVS4-624C>A	26. ss73688627	g.A5579>G
13. ss73688631	g.IVS4-585C>T	27. rs1897605	g. T5646>C
14. ss73688632	g.IVS4-306T>C	28. rs1897606	g. C5777>T

Figure 1 Genomic structure and identified polymorphisms in the human NP gene. A total of 28 SNPs, including one insertion/deletion (ins/del) polymorphism, were identified. The A of the translational start ATG is designated + 1. Nine SNPs were novel and have been registered in the dbSNP (ss-tagged numbers). ex, exon; ex3a, exon3 in hNP type2.

amplification, the allele-specific fluorescence was measured with ABI PRISM 7900 Sequence Detection Systems (Applied Biosystems). Genotype data were read blind to the case–control status. Ambiguous genotype data were not included in the analysis.

Promoter Assay in Primary Cultured Neurons

Primary dissociated cultures were prepared from the brain cortex of postnatal 2-day-old rats (SLC, Shizuoka, Japan) as described previously (Numakawa *et al*, 2002). To generate plasmids for luciferase gene reporter assay, two differentially sized (964 and 128 bp) fragments of the 5′ flanking region of hNP were amplified by PCR with primers 5′-CGA CGCGTGCGTGTGCTGGGTTTGAA-3′ (forward) and 5′-GA AGATCTCTAGAGCCTGGGGAGCTTCT-3′ (reverse) for the 964-bp fragment, and 5′-CGACGCGTCCTCCTCTCCCTAGC CTCAG-3′ (forward) and 5′-GAAGATCTCTAGAGCCTGGG GAGCTTCT 3′ (reverse) for the 128-bp fragment. These primers were designed to incorporate *Mlu*I (forward) and *Bgl*II (reverse) restriction sites, and the PCR product was inserted into the multiple cloning site upstream of the luciferase coding region in the pGL3-Basic vector (Promega, Madison, WI). The inserted sequence was confirmed with the auto sequencer CEQ8000 in both directions using primers 5′-TCTCCATCAAAACAAAACGAA-3′and 5′-TTCC ATCTTCCAGCGGATA-3′.

Among the four SNPs (SNPs 1–4; see Figure 1) in the 5′ upstream region (ie, putative promoter region) of the hNP gene, the genotypes of SNPs 1, 2, and 4 were completely the same for all the 24 schizophrenic subjects, and we found a significant association of bipolar disorder with SNP4 but not SNP3 (see results). In addition, haplotypes containing the A allele (the major allele), but not the G-allele, of SNP3 showed some evidence for association with bipolar disorder in haplotype analysis (see Table 2). We therefore made two allele-specific promoter fragments (haplotypes consisting of SNPs 1-2-3-4 were G-C-A-G and T-T-A-C) of 964- and 128 bp upstream from the transcription initiation site, which were subject to the luciferase reporter gene assay. The plasmid constructs were transfected into cultured neurons at 5 days *in vitro*. Cells on 24-well plates were co-transfected with 3200 ng of pGL3-Basic firefly luciferase reporter vectors, which included allele-specific promoter fragments of 964 and 128 bp, and 100 ng of phRL-TK *Renilla* luciferase vector (Promega, Tokyo, Japan) as an internal control using Lipofectamine 2000 regent (Invitrogen, Tokyo, Japan). As negative control, an empty pGL3-Basic vector was simultaneously transfected in all the experiments. At 24 h after transfection, luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega) and a Lumat LB 9507 luminometer (Berthold, Bad Wildbad, Germany), as described previously (Tadokoro *et al*, 2004; Okada *et al*, 2006). Firefly and *Renilla* luciferase activities were quantified sequentially as relative light unit (RLU) by addition of their respective substrates according to the protocol of the supplier. The ratio of firefly RLU to *Renilla* RLU of each sample was automatically computed. The activity of each construct was expressed at the relative value compared with that of pGL3-Promoter (as a positive control), and these relative values were computed by *t*-test. Primary cultured cells were prepared six times and transfection was performed quadruplicate for each cell culture.

Statistical Analysis

Deviations of genotype distributions from Hardy–Weinberg equilibrium were assessed with χ^2-test for goodness of fit. Genotype and allele distributions of each SNP were compared between patients and controls using χ^2-test for independence. The association of the hNP genotype with

memory and intelligence was examined by multiple analysis of variance (MANOVA) controlling for possible confounders (age, sex, and education years). These tests were performed with the SPSS software version 11 (SPSS Japan, Tokyo, Japan). The LD (D') between polymorphisms was examined using the Haploview program (http://www.broad.mit.edu/mpg/haploview/) (Barrett *et al*, 2005) and haplotype-based association analyses were performed with COCAPHASE software version 2.4 (http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/; Dudbridge *et al*, 2000). The expectation-maximization (EM) and 'droprare' options were used. Haplotypes with frequencies less than 3% were considered to be rare. We examined associations by permutation procedure (10 000 replications) to determine the empirical significance. All P-values reported are two-tailed. Statistical significance was considered when $P<0.05$.

RESULTS

Polymorphism Identification and Genotyping

The 7428-bp genomic region containing all the exons, introns, 5′ flanking, and 3′ flanking regions of hNP were screened for polymorphisms in 24 schizophrenic patients. A total of 28 SNPs, including one insertion/deletion (ins/del) polymorphism, were identified (Figure 1). Among them 19 SNPs had already been listed in the NCBI dbSNP database, whereas nine SNPs were novel. Four SNPs were located in 5′ upstream, one SNP in exon 6, six SNPs in 3′ downstream, and the remaining 17 polymorphisms in introns. There was only one SNP that resulted in an amino-acid change, SNP22 (G5047>A; Val286Ile: the number of the amino acid is according to NP_653088), which gave rise to a restriction site for *Acy*I and was located at an evolutionarily conserved (rodents through humans) residue. This non-synonymous polymorphism was found in only one schizophrenic patient. Additional genotyping was performed for 178 individuals with schizophrenia; however, there was no individual carrying the 286Ile allele, indicating that this amino-acid change is a rare mutation. The LD between SNPs is shown in Supplementary Figure S1, indicating that the entire genomic region consists of single haplotype block. Genotypes for SNPs 1, 2, 4, and 5, those for SNPs 8 and 9, those for SNPs 10 and 13, and those for SNPs15, 16, 17, 19, 21, 23, 24, 25, 27, and 28, respectively, were completely the same as each other for the 24 individuals.

Promoter Assay

We identified four SNPs in the 1078-bp 5′ upstream region of the hNP gene, and SNPs 1, 2, and 4 were found to be associated with bipolar disorder, memory, and intelligence quotient (IQ) (see below). Furthermore, to our knowledge, there is no information in the literature on the location of core promoter of the hNP gene. We therefore performed a promoter assay using the dual-luciferase system (Promega) in rat cultured cortical neurons and examined whether transcriptional activity alters in an allele-dependent manner. As shown in Figure 2a, pGL3-Basic vectors containing 128- and 964-bp fragments, which consisted of major alleles for the four SNPs, demonstrated substantially higher RLEs

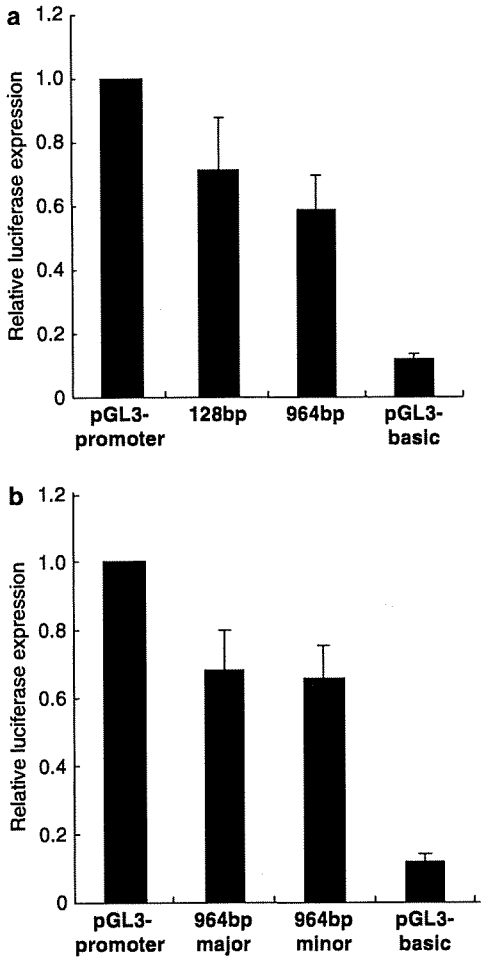


Figure 2 Promoter assay. (a) RLE for pGL3-Basic vector with insertion of 128 and 964 bp of the hNP 5′ flanking regions in comparison with pGL3-Basic vector, which dose not contain a promoter sequence. The RLE for pGL3-promoter vector containing SV40 promoter (positive control vector) was assigned a value of 1. Both 128- and 964-bp fragments showed substantially higher RLE compared with pGL3-basic vector without promoter sequence. (b) Comparison of RLE between the major (G-C-A-G for SNPs 1-2-3-4) and minor (T-T-A-C) alleles. No significant difference was found between the two alleles.

(relative luciferase expression) than that of pGL3-basic empty vector, suggesting that the core promoter region is located within the 128-bp fragment. We then cloned the 964-bp allele-specific promoter fragments (SNP1-2-3-4; major allele: G-C-A-G, minor allele: T-T-A-C) and compared RLEs between the two alleles (Figure 2b); however, we found no significant difference in the RLE between the two haplotype fragments. These results suggest that SNPs 1, 2, and 4 might not influence the transcriptional activity of the hNP gene.

Association with Psychiatric Diseases

We genotyped five SNPs (SNPs 3, 4, 6, 7, and 23) to examine possible association with schizophrenia, major depression, and bipolar disorder. Genotype and allele distributions in the diagnostic groups are shown in Table 1. Genotype

Table 1 Genotype and Allele Distributions of the five SNPs of the hNP Gene in Patients with Schizophrenia, those with Major Depression, those with Bipolar Disorder, and the Controls

SNP	Diagnosis	N	Genotype frequency (GF)			Allele frequency (AF)		Odds ratio (95% CI)	χ^2-Test *vs* controls		
									GF *vs* HW	GF (df = 2)	AF (df = 1)
SNP3			A/A	A/G	G/G	A	G				
	Controls	696	462 (0.66)	208 (0.30)	26 (0.04)	1132 (0.81)	260 (0.19)		0.67		
	SZ	421	277 (0.66)	126 (0.30)	18 (0.04)	680 (0.81)	162 (0.19)	1.06 (0.85–1.33)	0.45	$\chi^2 = 0.21$ $P = 0.90$	$\chi^2 = 0.11$ $P = 0.74$
	MD	382	276 (0.72)	90 (0.24)	16 (0.04)	642 (0.84)	122 (0.16)	1.18 (0.93–1.51)	0.02	$\chi^2 = 4.94$ $P = 0.08$	$\chi^2 = 2.48$ $P = 0.12$
	BD	202	139 (0.69)	56 (0.28)	7 (0.03)	334 (0.83)	70 (0.17)	1.09 (0.82–1.46)	0.65	$\chi^2 = 0.4$ $P = 0.81$	$\chi^2 = 0.36$ $P = 0.54$
SNP4			G/G	G/C	C/C	G	C				
	Controls	683	388 (0.57)	243 (0.36)	52 (0.08)	1019 (0.75)	347 (0.25)		0.11		
	SZ	406	234 (0.58)	150 (0.37)	22 (0.05)	618 (0.76)	194 (0.24)	1.1 (0.90–1.36)	0.75	$\chi^2 = 1.97$ $P = 0.37$	$\chi^2 = 0.62$ $P = 0.43$
	MD	371	219 (0.59)	126 (0.34)	26 (0.07)	564 (0.76)	178 (0.24)	1.1 (0.89–1.36)	0.19	$\chi^2 = 0.5$ $P = 0.78$	$\chi^2 = 0.58$ $P = 0.47$
	BD	198	91 (0.46)	90 (0.45)	17 (0.09)	272 (0.69)	124 (0.31)	1.33 (1.04–1.7)	0.43	$\chi^2 = 7.47$ $P = 0.023$	$\chi^2 = 5.35$ $P = 0.019$
SNP6			T/T	T/A	A/A	T	A				
	Controls	711	316 (0.44)	306 (0.43)	89 (0.13)	938 (0.66)	484 (0.34)		0.27		
	SZ	422	195 (0.46)	192 (0.45)	35 (0.08)	582 (0.69)	262 (0.31)	1.17 (0.97–1.41)	0.20	$\chi^2 = 4.86$ $P = 0.09$	$\chi^2 = 2.15$ $P = 0.14$
	MD	378	171 (0.45)	164 (0.43)	43 (0.11)	506 (0.67)	250 (0.33)	1.06 (0.88–1.29)	0.70	$\chi^2 = 0.31$ $P = 0.86$	$\chi^2 = 0.21$ $P = 0.65$
	BD	197	70 (0.36)	99 (0.50)	28 (0.14)	239 (0.61)	155 (0.39)	1.25 (0.99–1.58)	0.46	$\chi^2 = 5.03$ $P = 0.08$	$\chi^2 = 3.8$ $P = 0.051$
SNP7			A/A	A/G	G/G	A	G				
	Controls	718	325 (0.45)	314 (0.44)	79 (0.11)	964 (0.67)	472 (0.33)		0.81		
	SZ	433	209 (0.48)	190 (0.44)	34 (0.08)	608 (0.70)	258 (0.30)	1.16 (0.96–1.40)	0.31	$\chi^2 = 3.26$ $P = 0.20$	$\chi^2 = 2.36$ $P = 0.12$
	MD	387	182 (0.47)	163 (0.42)	42 (0.11)	527 (0.68)	247 (0.32)	1.05 (0.87–1.28)	0.55	$\chi^2 = 0.33$ $P = 0.85$	$\chi^2 = 0.21$ $P = 0.65$
	BD	203	72 (0.35)	103 (0.51)	28 (0.14)	247 (0.61)	159 (0.39)	1.31 (1.04–1.65)	0.36	$\chi^2 = 6.3$ $P = 0.042$	$\chi^2 = 5.56$ $P = 0.018$
SNP23			A/A	A/G	G/G	A	G				
	Controls	714	428 (0.60)	241 (0.34)	45 (0.06)	1097 (0.77)	331 (0.23)		0.16		
	SZ	421	267 (0.63)	135 (0.32)	19 (0.05)	669 (0.79)	173 (0.21)	1.17 (0.94–1.44)	0.71	$\chi^2 = 2.25$ $P = 0.32$	$\chi^2 = 2.13$ $P = 0.14$
	MD	388	240 (0.62)	127 (0.33)	21 (0.05)	607 (0.78)	169 (0.22)	1.08 (0.87–1.34)	0.44	$\chi^2 = 0.56$ $P = 0.75$	$\chi^2 = 0.56$ $P = 0.45$
	BD	204	98 (0.48)	86 (0.42)	20 (0.10)	282 (0.69)	126 (0.31)	1.48 (1.16–1.88)	0.86	$\chi^2 = 9.82$ $P = 0.0073$	$\chi^2 = 10.07$ $P = 0.0015$

Abbreviations: 95% CI, 95% confidence interval; BD, bipolar disorder; df, degrees of freedom; hNP, human neuropsin; HW, Hardy–Weinberg; MD, major depression; SNP, single-nucleotide polymorphism; SZ, schizophrenia.Significant *p*-values are gray colored.

distributions of these SNPs did not deviate significantly from Hardy–Weinberg equilibrium, except for SNP3 in patients with major depression ($P = 0.02$). There was no significant difference in genotype or allele distribution for any SNP between patients and controls for schizophrenia or major depression. However, there was a significant difference in genotype distributions between patients with bipolar disorder and controls for three SNPs, that is, SNPs 4, 7, and 23. Allele frequencies for these SNPs also differed significantly between the two groups. *P*-values, odds ratios, and their 95% confidence interval (CI) are shown in Table 1. Then we performed haplotype-based analysis with a two-marker sliding window method. We obtained no evidence of a significant association for schizophrenia or major depression (data not shown). With respect to bipolar disorder, we obtained significant individual *P*-values for all combinations of two markers; however, significant global *P*-value (0.0068) was obtained only when haplotype consisted of SNPs 7 and 23 (Table 2). Furthermore, overall global *P*-value ($P = 0.083$), considering all multiple testing for all the combinations of two-marker haplotypes, just failed to reach statistical significance. Thus, we did not obtain any stronger evidence for association in the haplotype-based analysis than in the single-marker analysis of SNP23 ($P = 0.0015$).

Association with Memory and IQ

Among the 166 controls whose memory scale and IQ were measured, SNP23 (A/G) was successfully genotyped in 163 individuals. Mean (SD) index scores of verbal memory, visual memory, general memory, attention and concentration, and delayed recall in the 163 controls were 110.9 (13.7), 109.9 (9.0), 112.2 (12.1), 103.9 (13.5), and 112.1

Table 2 Two-Marker Haplotype Analysis in Patients with Bipolar Disorder and Controls.

Markers					Haplotype frequency			P-value	
SNP3	SNP4	SNP6	SNP7	SNP23	BD	Controls	Individual	Global[a]	Overall global[a]
A	C				0.31	0.26	0.028	0.073	
	C	A			0.30	0.24	0.030	0.10	
		A	G		0.39	0.33	0.028	0.11	
			G	G	0.30	0.23	0.0068	0.014	0.083

Abbreviations: BD, bipolar disorder; SNP, single-nucleotide polymorphism.
[a]Global *P*-value for each combination of two markers and overall global significance for all combinations of two markers were calculated by permutation of 10 000 simulations.

(12.0), respectively. Mean (SD) full-scale IQ, verbal IQ, and performance IQ were 109.3 (11.6), 107.3 (12.9), and 110.3 (11.7), respectively. Since SNP23 showed the strongest association with bipolar disorder (G-allele was the risk allele) among the 5 SNPs examined, memory and IQ were compared between those who carried the G-allele (carrier, G/G or A/G, $N=64$) and those who did not (non-carrier, A/A, $N=99$) (Figure 3). Since the number of individuals with G/G genotype was very small ($N=8$), they were combined with those with the A/G genotype. With respect to sub-scales of WMS-R, the mean score of attention/concentration was significantly lower in carriers than in non-carriers ($P=0.016$); however, there were no significant differences between the two groups for the remaining sub-scales (verbal memory, visual memory, general memory, and delayed recall). With respect to WAIS-R, there was a significant difference in full-scale IQ ($P=0.018$) between the two groups. When verbal and performance IQ were examined separately, there was a highly significant difference in verbal IQ ($P<0.001$), but not in performance IQ, between the two groups. The mean verbal IQ (SD) for carriers and non-carriers was 103.4 (12.9) and 109.8 (12.3), respectively. As for the other SNPs, similar results are obtained (data not shown) because of the tight LD across the SNPs.

DISCUSSION

In the present study, we performed polymorphism screening and identified 28 SNPs, including nine novel SNPs, in the 7428-bp region of the whole hNP gene, including the 5′ and 3′ flanking regions. Then we performed promoter assay and determined a core promoter region of the hNP gene, although failing to find significant effects of SNPs on transcriptional activity. Association analysis using five SNPs as markers revealed significant difference in genotype and allele distributions for some of the SNPs between patients and controls for bipolar disorder, but not for schizophrenia or major depression. When a possible association of the SNPs with memory and IQ was examined in healthy control subjects, we found significant differences in attention/concentration sub-scale score of the WMS-R and verbal IQ between genotypes.

Among the 28 SNPs identified, there was only one SNP in the exons; SNP22 was a Val286Ile missense mutation in exon 6, which was detected in a patient with schizophrenia. Additional genotyping for 178 schizophrenic subjects did not find anyone carrying this variant, indicating that this is a rare mutation. Thus, whether this mutation is pathogenic or not is unclear. Since we examined only 24 individuals for polymorphism screening, we may have missed some rare mutations as the SNP22.

Our promoter assay in rat primary cultured neurons suggested that the core promoter is present in the 128-bp 5′ upstream region of the hNP gene. Since the RLE of pGL3-vector containing the 964-bp fragment was somewhat lower than that of pGL3-vector containing 128-bp fragment, a silencer-like region may be present between 128- and 964-bp positions upstream of the hNP gene. Then we examined whether transcriptional activity differs in an allele-dependent manner; however, we found no significant difference between alleles. These results suggest that SNPs 1, 2, and 4 might not influence the transcriptional activity of the hNP gene. According to the TFSEARCH database (http://mbs.cbrc.jp/research/db/TFSEARCHJ.html), these SNPs are not located on any of the binding sites of transcriptional factors, which is in line with our finding of no significant difference between the alleles.

In our association study with psychiatric diseases, we found, for the first time, significant differences in genotype and allele distributions between patients with bipolar disorder and controls. The best *P*-value was obtained for SNP23 in allele distribution ($P=0.0015$, odds ratio 1.48, 95% CI 1.16–1.88). This *P*-value remained significant even after correcting the critical *P*-value for Bonferroni's multiple testing (15 comparisons: 5 SNPs × 3 diseases). Haplotype-based analysis also yielded nominally significant results particularly when SNP23 was included in markers of analysis. These results suggest that SNP23 or other unknown SNPs in LD with SNP23 confers susceptibility to bipolar disorder. Since hNP is a part of a gene cluster (kallikreins), there remains a possibility that variations of some other kallikrein gene might be truly responsible to giving susceptibility to bipolar disorder. The results are in line with a previous study reporting a susceptibility locus for bipolar disorder on chromosome 19q13 (Badenhop *et al*, 2002). A possible limitation is that a portion of patients with bipolar disorder and controls were recruited in a geographically different area (ie, Shiga prefecture but not in Tokyo), which may have resulted in a population stratification; however, the minor allele frequency of SNP23 was very similar in controls from Shiga and those from Tokyo (0.233 in Shiga and 0.232 in Tokyo), suggesting that the effect of stratification is unlikely. Another limitation might be that

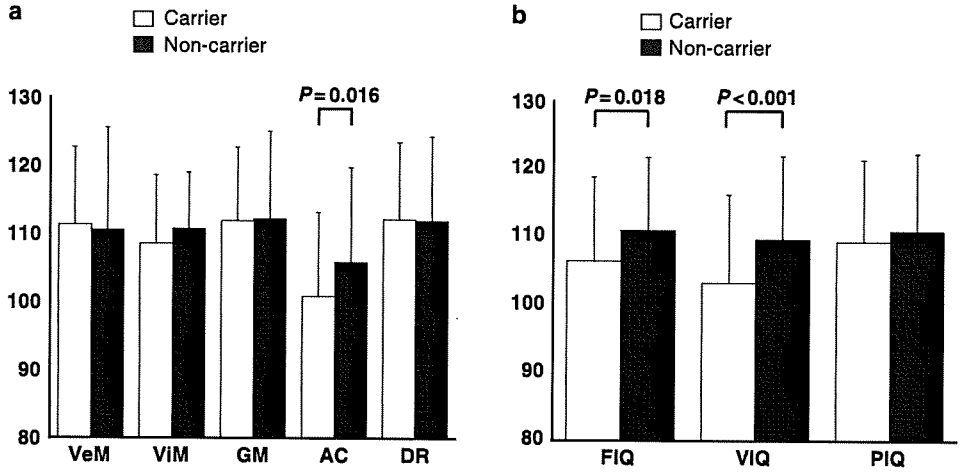


Figure 3 Relationship of memory and IQ with genotype of SNP23. Memory and IQ were compared between those who carried the G allele (carrier: G/G or A/G) of SNP23 and those who did not (non-carrier: A/A). (a) Memory and genotype. VeM, verbal memory; ViM, visual memory; GM, general memory; AC, attention and concentration; DR, delayed recall. (b) IQ and genotype. FIQ, full-scale IQ; VIQ, verbal IQ; PIQ, performance IQ.

we did not conduct structured interview for diagnosis of the patients. However, consensus diagnosis was made by at least two psychiatrists one of whom was in charge of the patients; thus, the possibility of misdiagnosis might be minimal. In addition, our sample size (207 bipolar disorder subjects and 727 controls) was not very large, and thus further investigations in other samples are required to draw any conclusion. With respect to schizophrenia or major depression, we did not obtain any evidence for association with hNP.

Interestingly, we found significant association of memory and IQ with the hNP gene in healthy subjects. Carrying the G-allele of SNP23, the risk allele for bipolar disorder, was associated with lower score in attention/concentration assessed with the WMS-R ($P=0.016$) and lower verbal IQ assessed with WAIS-R ($P<0.001$). The evidence for the former association (with attention/concentration) was weak and it would not be significant any more after correcting for multiple testing; however, the latter association (with verbal IQ) was highly significant and remained significant even when multiple testing was taken into consideration. Since bipolar disorder shows a wide range of cognitive deficits, including memory and IQ (Schretlen *et al*, 2007; Daban *et al*, 2006), the observed impact on intelligence may have some relevance to susceptibility to bipolar disorder. However, given that deficits in intelligence and memory are generally worse in schizophrenia than in bipolar disorder, alterations in hNP may have some effects specific to molecular mechanisms of bipolar disorder.

NP is a secretory serine protease that degrades cell adhesion molecule L1 (CAM-L1) (Matsumoto-Miyai *et al*, 2003) and is possibly involved in the synaptogenesis and maturation of orphan and small synapses (Nakamura *et al*, 2006). Furthermore, NP has been shown to be involved in activity-dependent synaptic plasticity, that is, LTP and kindling epileptogenesis (Komai *et al*, 2000; Okabe *et al*, 1996). As mentioned above, the type 2 splice variant has been shown to be expressed as abundant as the type 1 in human brain (Mitsui *et al*, 1999) and the hominoid-specific form (Li *et al*, 2004), which occurred through a human-specific T-to-A mutation (c.71–127T>A) during primate evolution (Lu *et al*, 2007). Taken together, NP is involved in synaptic plasticity via modulation of synaptic structure, and may play an important role in brain function of higher order such as learning, memory, and mental disorders. With respect to psychiatric diseases, indeed, altered expression levels of CAM-L1 mRNA and protein have been reported in postmortem brains of depressed patients (Laifenfeld *et al*, 2005). In line with this, chronic antidepressants increase expression levels of CAM-LI in rats (Sairanen *et al*, 2007; Laifenfeld *et al*, 2002). It would be intriguing to examine the expression levels of NP in postmortem brains of psychiatric patients.

We found that SNP23 is most associated with bipolar disorder among the examined SNPs. Haplotype-based analysis did not yield any stronger results, suggesting that SNP23 may be responsible for giving susceptibility to bipolar disorder. In addition, SNP23 showed strong impact on verbal IQ in healthy subjects. SNP23 is located 69 bp downstream to the 3′ end of exon 6 (the final exon). Thus SNP23 is on the 3′ regulatory region of the hNP gene. Growing evidence has shown that 3′ regulatory regions of human genes play an important role in regulating mRNA 3′ end formation, stability/degradation, nuclear export, subcellular localization, and translation, and are consequently rich in regulatory elements. Indeed, several diseases have been reported to be associated with variants in the 3′ regulatory region (Chen *et al*, 2006). Notably, the major allele (A) of SNP23 differs from the corresponding base (G) in monkeys or apes (ie, rhesus macaques or chimpanzees), according to the UCSC database, and thus it is not evolutionally conserved. It is interesting that carriers of the G allele were found to be poorer in memory and IQ subscales than individuals with A/A genotype in the present study. Although SNP23 is not located on obvious motifs or conserved sequence elements, it is also possible that this human-specific mutation may contribute to the higher memory and intelligence functions in humans. If our results

are replicated in other samples, it is important to elucidate the possible functional effects of SNP23 on regulation of hNP mRNA, which may contribute to understanding of the pathogenesis of bipolar disorder and brain function of higher order specific to human beings.

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DISCLOSURE/CONFLICT OF INTEREST

All authors declare that they have no conflict of interests to disclose.

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