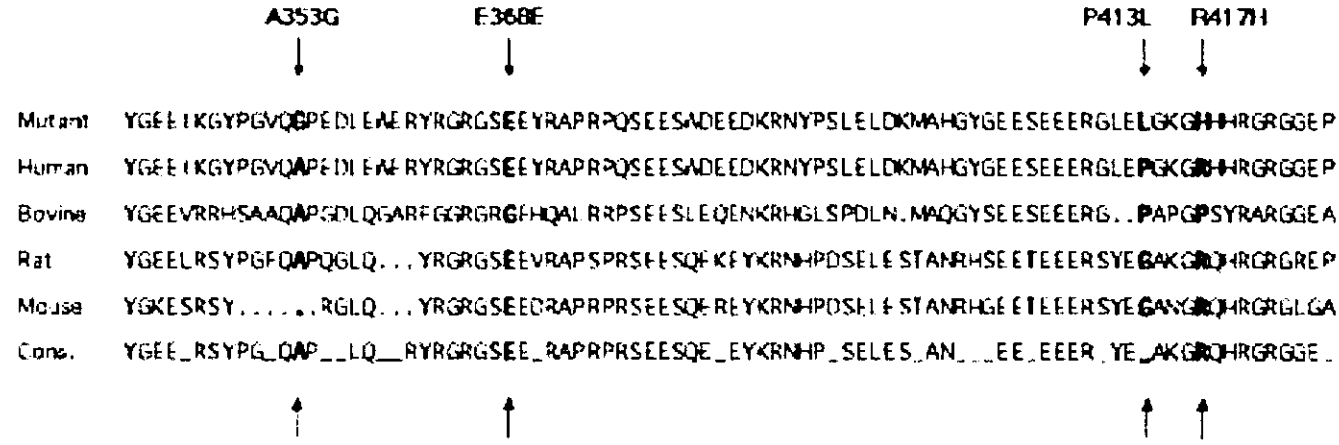

Figure 2. Comparison of the amino acid sequences of chromogranin B from human, bovine, rat, and mouse. Cons. is calculated consensus sequence. Dots (.) denote deletions in the sequence.

T(P413L) (rs742710), 1250G/A(R417H) (rs742711), IVS4+726G/T (rs236154), IVS4+804G/A (rs236155), and IVS5+84C/A (rs2821), are listed in the database for single nucleotide polymorphisms (dbSNP) in the NCBI (http://www.ncbi.nlm.nih.gov/SNP/). Three SNPs, −1401A/G, IVS2+22A/G, and 1499G/A(R500K), not listed in the dbSNP database were identified in the mutation search of *CHGB* in the Chinese Han population (Zhang et al 2002). Seven SNPs, −1095G/C (rs989462), −1042G/A (rs6085321), 402G/C(K177N) (rs236150), 727G/A(T423A) (rs236151), 1466C/T(L489L) (rs6107717), IVS4+9T/C (rs617000), and IVS5+277A/T (rs 236156), which are listed in the dbSNP database, were not detected in the Japanese population. One SNP, −58T/C, was newly identified in this study. Among these SNPs, 16 at the coding or regulatory regions were selected for further association study.

Linkage disequilibrium was found in many pairs of the 16 SNPs (Table 2). The genotype and allele frequencies of all SNPs are shown in Table 3. The genotype distributions did not deviate significantly from those expected according to the Hardy-Weinberg equilibrium for polymorphisms. Statistically significant differences in allele distributions were found between schizophrenic patients and healthy control subjects for 1058C/G ($p = 5.9 \times 10^{-6}$, empirical $p = 8.2 \times 10^{-5}$, OR = .510, 95% confidence interval [CI] = .38–.68) and 1104A/G ($p = 6.2 \times 10^{-7}$, empirical $p = 1 \times 10^{-5}$, OR = .474, 95% CI = .35–.64), and a trend of association was found between 1250G/A and schizophrenia ($p = .005$, empirical $p = .05$, OR = 1.51, 95% CI = 1.13–2.01) (Table 2). The genotype distributions of these three SNPs suggest the presence of a gene dose-response effect: a linear trend in disease risk with number of copies of the high-risk allele (uncorrected $p = 1.3 \times 10^{-5}$, uncorrected $p = 2.0 \times 10^{-6}$, and uncorrected $p = .006$, respectively). The frequency of the risk GG genotype of A353G in the control subjects was .28 and its OR for schizophrenia was 2.1 (95% CI = 1.4–3.3) compared with other genotypes. Two SNPs, 1058C/G and 1104A/G, were in almost complete linkage disequilibrium (Table 2), and almost the same p-value was obtained for the haplotype associations with schizophrenia (uncorrected $p = 6.1 \times 10^{-7}$). Other haplotypes are also associated with schizophrenia; however, they are mainly due to 1058C/G and 1104A/G polymorphisms (Table 4). Linkage disequilibrium was significant ($p < .05$) between SNPs associated with schizophrenia, namely 1058C/G (D′ = .43), 1104A/G (D′ = .44), and 1250G/A (D′ = .37) in the *CHGB* and the 109-bp allele of D20S95.

Although it has been reported that the significant association with schizophrenia ($p < .001$) was observed at two SNPs of 433G/A and 533A/G in the Chinese Han population, these findings were not replicated in our present study. The power of this study to replicate the findings from the Chinese-Han population was 90%.

Discussion

Our hypothesis that the chromogranin B gene (*CHGB*) is a plausible positional candidate for association with schizophrenia can be strongly supported by the results of our additional dense mapping analyses examined in the present study. This hypothesis is further supported by the findings in our subsequent association study between *CHGB* and schizophrenia: a significant association of 1058C/G and 1104A/G, and a trend of association of 1250G/A. These SNPs were in linkage disequilibrium with the 109-bp allele of D20S95. Association study may lead to false-positive and false-negative signals because of genetic stratification or population subdivision. Our subjects were all of Japanese descent living in the same area and we have no evidence of population stratification in our case or control samples. As shown in a previous report, Japanese is a relatively homogeneous population with no stratification (Daimon et al 2003; Kakiuchi et al 2003). However, unknown population stratification would not be excluded.

There is another Chinese study showing a significant association between some SNPs of *CHGB* and schizophrenia. Although our study and a study of Zang et al (2002) found significant associations between *CHGB* SNPs and schizophrenia, the associated SNPs differed between the two studies. Significant associations with schizophrenia were found at two SNPs, 433G/A ($p = .004$, the G allele was associated with schizophrenia, OR = 1.60, 95% CI = 1.17–2.18) and 533A/G ($p = .0005$, the A allele was associated with schizophrenia, OR = 1.73, 95% CI = 1.28–2.18), in the Chinese Han population (Zhang et al 2002). We failed to find these associations in the present study, 433G/A (OR = 1.01, 95% CI = .75–1.36) and 533A/G (OR = .87, 95% CI = .65–1.16). The ORs between the two studies significantly differed ($p = .04$ for 433G/A and $p = .001$ for 533G/A, respectively). The alleles

Table 4. Haplotypes Showing Most Significant Association with Schizophrenia per Each Window

Haplotype Window	433 G/A	533A/G	598A/C	695G/A	1058C/G	1104A/G	1238C/T	1250G/A	1499G/A	Haplotype Frequency (%) Schiz	Control	*p* Values Global	Permutation Global	Individual
Window 2					C	A				.31	.49	6.6×10^{-6}	.00009	6.6×10^{-6}
Window 3					C	A	C			.31	.49	5.9×10^{-5}	.0007	1.0×10^{-5}
Window 4				G	C	A	C			.27	.43	.0007	.01	2.0×10^{-5}
Window 5			A	G	C	A	C			.27	.40	.003	.04	.0001
Window 6		A	A	G	C	A	C			.03	.12	.005	.04	.002

Schiz, schizophrenia

that found to be associated with schizophrenia in the Chinese population, the G allele of the 433G/A polymorphism and the A allele of the 533A/G polymorphism, were on the haplotypes negatively associated with schizophrenia in our Japanese population. Linkage disequilibrium between 1058C/G and 433G/A or 533A/G were not observed (D′ = .47, D′ = .63, respectively). Our Japanese study tested more SNPs, and the most interesting SNPs were not tested in the larger Chinese sample. The SNPs significantly associated with schizophrenia in the Chinese population, however, also were nonsynonymous and of potential interest. Although it is possible that the apparent discrepancy between our study and that of Zhang et al (2002) occurred by chance, it is also possible that the discrepancy is due to ethnic differences or differences in the clinical characteristics of schizophrenic patients. We have observed that *CHGB* SNPs are associated with extrapyramidal side effects and clinical symptoms (unpublished data). Therefore, further studies are necessary to address the discrepancy between the Japanese and Chinese studies. Associations between *CHGB* and schizophrenia have been found in two independent studies of East Asian populations, indicating that *CHGB* is a plausible association with susceptibility to schizophrenia and is one of the genes contributing to the linkage of 20p12.3-p11 to schizophrenia found by meta-analysis (Lewis et al 2003).

The granin family plays an important role in the sorting and aggregation of secretory products in the trans-Golgi network (Ozawa and Takata 1995). Chromogranin A, chromogranin B (secretogranin I), and chromogranin C (secretogranin II) are well known. Chromogranin B also localizes to the nucleus and controls transcription of many genes, including those for transcription factors (Yoo et al 2002). Recently, chromogranin B was reported to have an important role in the intracellular calcium signaling in neurons by interacting with IP3 receptor in a pH-dependent manner (Thrower et al 2003). Human chromogranin B consists of 657 amino acids (Benedum et al 1987). Since the function of chromogranin B is still a matter of debate, it is acceptable that no functional analysis was performed for the 1058G/C(A353G). However, the amino acid 353A and 417R are relatively conserved among species, suggesting that the substitution has some impact on the protein function (Bauer and Fischer-Colbrie 1991) (Figure 2).

Because two of three SNPs we found to be associated with schizophrenia were nonsynonymous, they might be related directly to the susceptibility to schizophrenia. It will be important to replicate the finding in independent samples of schizophrenia of the Japanese population. However, it is possible that these SNPs are just in linkage disequilibrium with other polymorphisms responsible for giving the disease susceptibility. Our findings represent an initial step toward an understanding of the possible etiologic role that *CHGB* plays in schizophrenia. It will be interesting to consider the functional changes in the chromogranin B proteins derived from its genetic variations that may have roles in the clinical phenotypes of schizophrenia. Further research is needed with transgenic mice to clarify the exact role of chromogranin B in the pathophysiology of schizophrenia, especially to identify the functional changes derived from the genetic variations of *CHGB*. Clinical studies are also required to evaluate the psychopathological features in schizophrenic patients who have biochemical changes in chromogranin B levels and *CHGB* variations.

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Appendix 1. Supplemental Data for Table 1. Case-Control Association Analyses with Microsatellite Markers on Chromosome 20p

	Individual Allele					Global		
Marker	Allele (bp)	Case	Control	Chi-square	*p*	LRS	DF	*p*
D20S835						8.5	10	.58
	195	.169	.193	.712	.399			
	197	.003	.000	1.501	.221			
	199	.009	.013	.313	.576			
	201	.015	.013	.031	.861			
	203	.142	.138	.029	.864			
	205	.349	.388	1.197	.274			
	207	.087	.073	.505	.478			
	209	.017	.026	.633	.426			
	211	.119	.096	.987	.321			
	213	.087	.060	2.004	.157			
	215	.003	.000	1.501	.221			
D20S873						7.9	7	.34
	185	.000	.008	3.913	.048			
	187	.291	.270	.430	.512			
	189	.003	.010	1.685	.194			
	191	.100	.105	.044	.834			
	193	.026	.018	.466	.495			
	195	.537	.545	.040	.842			
	197	.034	.042	.288	.592			
	199	.009	.003	1.236	.266			
D20S882						21.3	7	.003
	72	.037	.031	.245	.620			
	74	.335	.247	7.420	.006			
	76	.126	.181	4.759	.029			
	78	.355	.316	1.316	.251			
	80	.136	.219	9.711	.002			
	82	.007	.005	.169	.681			
	84	.002	.000	1.353	.245			
	86	.002	.000	1.353	.245			
D20S95						116.6	11	.000
	95	.000	.002	1.148	.284			
	97	.009	.103	48.970	.000			
	99	.124	.111	.411	.522			
	101	.093	.022	26.940	.000			
	103	.052	.032	2.672	.102			
	105	.100	.192	17.630	.000			
	107	.339	.320	.436	.509			
	109	.098	.027	24.160	.000			
	111	.141	.148	.098	.754			
	113	.028	.020	.720	.396			
	115	.011	.020	1.480	.224			
	117	.004	.003	.065	.798			
D20S905						24.9	11	.009
	76	.003	.003	.001	.982			
	78	.038	.042	.080	.777			
	80	.008	.016	.937	.333			
	82	.014	.016	.063	.802			
	84	.264	.311	2.014	.156			
	86	.429	.376	2.187	.139			
	88	.223	.174	2.848	.092			
	90	.014	.053	9.457	.002			
	92	.000	.005	2.714	.099			
	94	.008	.000	4.268	.039			
	96	.000	.003	1.356	.244			
	98	.000	.003	1.356	.244			
D20S194						17.7	21	.67
	185	.003	.000	1.442	.230			
	187	.011	.003	2.105	.147			
	189	.011	.013	.065	.799			
	191	.003	.005	.290	.591			
	193	.025	.044	2.167	.141			
	195	.060	.065	.069	.793			
	197	.047	.047	.000	.991			
	199	.069	.063	.117	.733			
	201	.052	.076	1.707	.191			
	203	.088	.073	.569	.451			
	205	.146	.130	.373	.541			
	207	.107	.138	1.659	.198			
	209	.146	.117	1.325	.250			
	211	.060	.060	.001	.975			
	213	.096	.081	.553	.457			
	215	.025	.034	.549	.459			
	217	.016	.026	.826	.364			
	219	.019	.013	.458	.499			
	221	.008	.003	1.162	.281			
	223	.003	.003	.001	.970			
	225	.000	.005	2.672	.102			
	227	.005	.003	.397	.529			
D20S192						12.1	12	.44
	273	.000	.003	1.275	.259			
	275	.003	.000	1.507	.220			
	277	.012	.005	.939	.333			
	281	.015	.010	.261	.610			
	283	.003	.003	.007	.935			
	285	.436	.471	.929	.335			
	287	.020	.031	.838	.360			
	289	.088	.081	.115	.735			
	291	.114	.122	.121	.728			
	293	.298	.255	1.677	.195			
	295	.003	.016	3.436	.064			
	297	.006	.003	.468	.494			
	299	.003	.000	1.507	.220			
D20S156						3.7	5	.59
	181	.007	.000	1.097	.295			
	183	.149	.130	.297	.586			
	185	.518	.506	.050	.822			
	187	.298	.351	1.282	.257			
	189	.021	.013	.380	.538			
	191	.007	.000	1.097	.295			
D20S892						20.8	12	.05
	199	.006	.000	2.980	.084			
	201	.006	.000	2.980	.084			
	203	.106	.076	2.110	.146			
	205	.017	.036	2.622	.105			
	207	.011	.044	7.635	.006			
	209	.299	.284	.199	.656			
	211	.060	.047	.656	.418			
	213	.121	.130	.151	.698			
	215	.313	.307	.030	.863			
	217	.043	.057	.772	.380			
	219	.014	.013	.025	.876			
	221	.003	.003	.005	.944			
	223	.000	.003	1.292	.256			
D20S846						4.0	8	.85
	269	.349	.318	.832	.362			
	271	.009	.005	.300	.584			
	273	.261	.294	.991	.320			
	275	.071	.086	.565	.452			
	277	.017	.013	.202	.653			
	279	.153	.159	.041	.839			
	281	.102	.091	.261	.610			
	283	.034	.034	.000	.986			
	285	.003	.000	1.477	.224			

LRS, likelihood ratio statistics; DR, degrees of freedom.

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Letter to the Editors

Failure to find association between PRODH deletion and schizophrenia☆

Dear editors,

A deletion of the 22q11.2 region, which occurs in 1 of 4000 live births (Wilson et al., 1994), is a relatively common genetic disorder in humans. The 22q11.2 deletion has been found in 0.3–2% of adult patients with schizophrenia (Arinami et al., 2001; Karayiorgou et al., 1995) and in 6% of childhood-onset schizophrenia (Usiskin et al., 1999). It was reported that 24% of patients with VCFS met the criteria for schizophrenia (Murphy et al., 1999). Therefore, the risk of schizophrenia for patients with the 22q11.2 deletion may be 25–30 times higher than the general population risk of 1%. However, it is difficult to determine which of the specific genes in this genomic region may be associated with psychiatric problems.

The proline dehydrogenase gene (PRODH) is located in this region and has been suggested to increase susceptibility to schizophrenia because of abnormal findings in a PRODH-mutant mouse, allelic association with schizophrenia (Liu et al., 2002), and detection of a family with schizophrenia and a 350-kb deletion that includes PRODH (Jacquet et al., 2002). During our ongoing screening study for polymorphisms associated with schizophrenia in the 22q11.2 region, we identified one Japanese family with three members who carried the PRODH deletion. The father and two daughters of the family were hemizygous for at least a 100-kb region extending from single nucleotide polymorphism (SNP) rs416659 (dbSNP of National Center for Biotechnology Information (NCBI)) to D22S1638 because they each carried only one allele of the rs416659 and rs1210635 SNPs and D22S1638 and the father's allele was not transmitted to the daughters. The father's paternity was confirmed by testing of more than 400 microsatellite markers, which were used for a genome-wide linkage scan (Takahashi et al., 2003). The father's null allele should have been transmitted to the daughters.

To confirm the deletion, we devised a PCR-based homologous gene quantitative amplification screening method to detect the PRODH deletion. A primer set that amplifies an intronic region of PRODH and its counterpart, intronic region of ψPRODH was designed with the BLAST 2 (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). PCR was done with the following primers: forward, 5′-AGCTCAGTGCCCATGTCAGT and reverse, 5′-ACTGCCCTGTCTGCCTGTAG. The reverse primer was 5′-labeled with the fluorescein dye 6-FAM. The PCR product sizes from PRODH (NCBI accession number AC008103) and ψPRODH (AC007663) were 229 and 239 bp, respectively. After denaturation at 95 °C for 10 min, amplification consisted of 26 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 30 s followed by a final extension of 72 °C for 7 min. PCR product was mixed with ROX labeled GeneScan 400 HD. Electrophoresis was carried out with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Peak height of each PCR product from PRODH and ψPRODH was measured with the GeneScan and Genotyper programs (Applied Biosystems). The peak ratio of ψPRODH/PRODH was calculated. To monitor the quality of each experiment, samples from individuals with the PRODH deletion were amplified simultaneously.

The sequences of these regions are highly homologous, but the PCR product from PRODH was 10 bp shorter than that from ψPRODH (Fig. 1). In this

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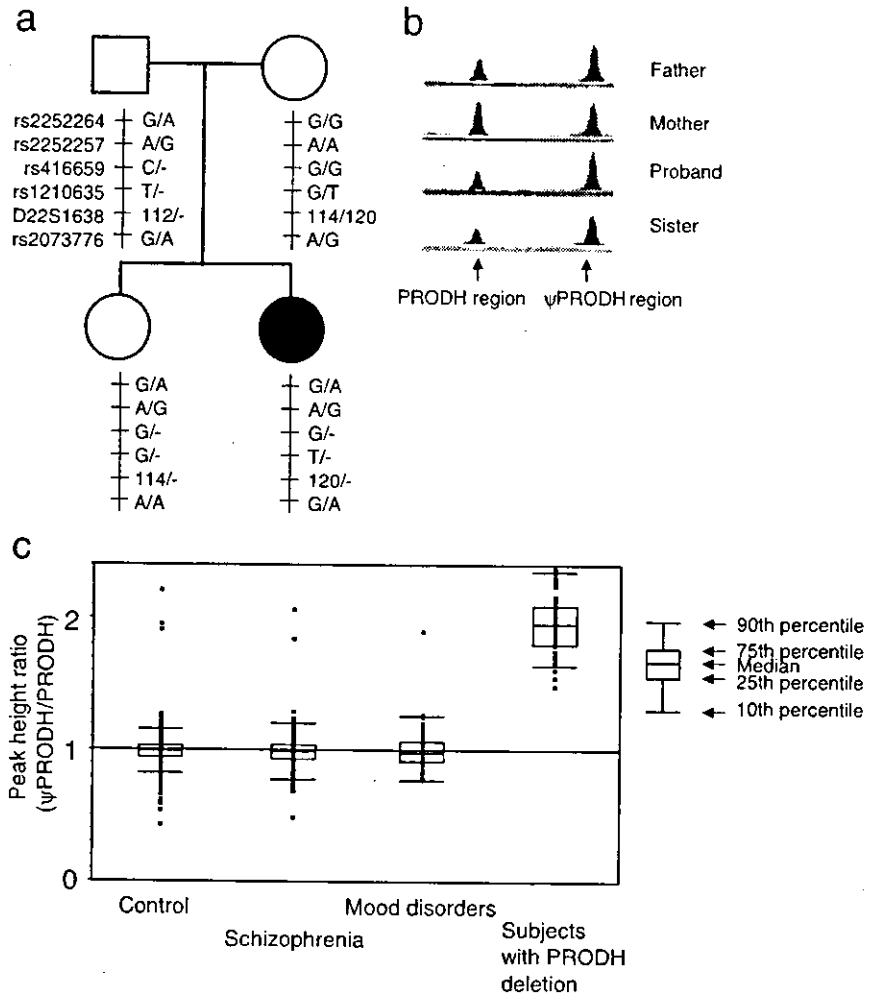

Fig. 1. A family with the PRODH deletion and detection of the deletion by a homologous gene quantitative amplification. (a) A family with the PRODH deletion and genotypes of the markers within and around the deletion. Closed circle indicates the proband with schizophrenia. Other family members are psychosis-free. Alleles of a microsatellite marker are indicated as size (bp). Minus (−), deleted (null) allele. (b) Electropherogram of deletion screening in members of the family. (c) Distribution of the peak height ratio of ψPRODH to PRODH. Six individuals among controls and patients with schizophrenia and mood disorders had a ratio of approximately 2, indicating the presence of the deletion. The distribution of subjects with PRODH deletion is that of simultaneously amplified products from three samples with the deletion to monitor the quality of each experiment.

family, the peak of the PCR product from PRODH was half as high as that from ψPRODH in the father and the two daughters, whereas the height of the two peaks was equal in the mother, supporting our finding of the deletion in the father and daughters. These data also showed that the devised homologous gene quantitative amplification method was useful for detecting the PRODH deletion.

Because the deletion did not co-segregate with schizophrenia in our family or a family reported by Jacquet et al. (2002), we tried to perform association analysis with this simple method. We screened for the PRODH deletion in patients with schizophrenia and mood disorder and controls. All subjects were unrelated Japanese. A total of 1505 unrelated Japanese subjects were screened, and six subjects carried the PRODH deletion (Fig. 1). To confirm the PRODH deletion in these six subjects, three polymorphic markers flanking PRODH, rs416659, rs1210635, and D22S1638, were analyzed. Only one allele of each marker was detected in the six subjects. The minor allele frequencies of rs416659 and rs1210635 were

0.2047 and 0.4648, respectively, in Japanese (IMS-JST Japanese SNP database, http://snp.ims.u-tokyo.ac.jp/index.html), and heterozygosity of D22S1638 was 0.8123 (our unpublished data). Although the chance that a Japanese individual is homozygous for these three markers is 6.5%, the chance that all six of the subjects were homozygous for the three markers is 7.5×10^{-8}, supporting our quantitative PCR finding that the six subjects carried the PRODH region deletion. The region includes at least PRODH and DGCR6. The deletion was found in 2 of 509 patients with schizophrenia, 1 of 107 patients with mood disorders, and 3 of 889 control subjects. Thus, 1 in approximately 250 Japanese individuals (95% confidence interval, 1 in 115 to 1 in 547) carries this deletion, indicating that the deletion is 10-fold more prevalent than the 22q11.2 deletion. However, the findings of the present study indicate that haploinsufficiency for PRODH and DGCR6 is not likely to account for the at least 10-fold increased risk of schizophrenia in individuals with a 22q11.2 deletion.

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No association was found between a functional SNP in ZDHHC8 and schizophrenia in a Japanese case–control population

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Abstract

ZDHHC8 is a new and attractive candidate for a schizophrenia-susceptibility factor. First, several lines of linkage studies showed that 22q11, on which ZDHHC8 is located, is a "hot" region. Second, fine linkage disequilibrium mapping revealed a significant association around ZDHHC8. Moreover, a very recent study reported that one single nucleotide polymorphism (SNP: rs175174) in ZDHHC8 might affect the splicing process, the ZDHHC8 knock-out mice showed the gender-specific phenotype, and the transmission disequilibrium test (TDT) using this SNP also showed significant association with human female schizophrenia. Thus, we attempted a replication study of this SNP using relatively large Japanese case–control samples (561 schizophrenics and 529 controls). No association was found between schizophrenia and controls even after dividing samples by gender. Because our sample size provided quite high power, ZDHHC8 may not play a major role in Japanese schizophrenia. And our results did not support the gender-specific effect of this SNP.

Keywords: Chromosome 22q11; Gender difference; Candidate gene

The 22q11 region (OMIM: #600850 SCZD4) is associated with increased risk for schizophrenia [2]. Two independent meta-analyses of linkage studies showed the linkage around 22q11 [1,5], although one negative result was also reported [8]. This chromosome region contains at least three genes, COMT [12], PRODH2 and DGCR6 [7], implicated as susceptibility genes for schizophrenia.

Recently, ZDHHC8 was reported as a new and attractive candidate gene on 22q11 from the evidence of a genetic association study and animal study [6,9]. In the initial genetic association study, Liu et al. showed that three single nucleotide polymorphisms (SNPs) in ZDHHC8 were associated with schizophrenia. One of these SNPs (rs175174), which was located in intron 4 of ZDHHC8, showed the most highly significant *P* value [6]. This intronic SNP seemed to modify ZDHHC8 expression by causing imperfect splicing, intron retention and reduced enzyme activity. In addition, *Zdhhc8* knockout mice had a gender-dependent dimorphic deficit in prepulse inhibition similar to schizophrenia and reactivity to the psychomimetic *N*-methyl-D-aspartate (NMDA) receptor blocker dizocilpine. In the light of these findings, the transmission disequilibrium test (TDT) divided samples according to gender differences, revealing that human female schizophrenia was significantly associated with this SNP [9]. Thus, we here provide a replication study of rs175174 in ZDHHC8 using Japanese case–control samples.

A total of 561 patients with schizophrenia (259 female; mean age ± standard deviation (S.D.) 49.6 ± 16.4 years: 302

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male; 47.0 ± 14.9) and 529 controls (270 female; 39.7 ± 15.4 years: 259 male; 34.9 ± 12.4 years) were genotyped for association analysis of rs175174. Moreover, in additional linkage disequilibrium (LD) mapping around this SNP, 95 schizophrenic patients (50 female and 45 male) and 96 controls (44 female and 52 male), part of each sample used in association analysis, were genotyped for three SNPs. The general characterization of these subjects and a description of their psychiatric assessment according to identical criteria were published elsewhere [13]. After explaining the study to all subjects, written informed consent was obtained from each. This study was approved by the Ethics Committee of the Nagoya University Graduate School of Medicine and Fujita Health University.

Genomic DNA was extracted from peripheral blood of all subjects. For rapid genotyping of SNPs, rs175174 and additional three SNPs for LD mapping (rs175169, rs175175 and rs2292570), polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) assays were developed. The information of PCR primers is available on request. The PCR reactions of all SNPs were carried out in a 10 μl volume containing 10 ng genomic DNA, 0.4 M of each primer, 200 μM of dNTP, 1 × PCR Gold Buffer, 1.5 mM $MgCl_2$ and 0.25 U of Amplitaq Gold™ (Applied Biosystems Japan Ltd., Tokyo, Japan), using the GeneAmp™ PCR system 9700 (Applied Biosystems Japan Ltd.). PCR cycling conditions consisted of an initial denaturation step at 95 °C for 9 min, followed by 45 cycles of 95 °C for 15 s, 56 °C for 20 s, 72 °C for 30 s, and ending with a final extension step at 72 °C for 7 min. PCR product was digested using appropriate restriction enzymes according to the manufacture's recommendation (New England Biolabs, England, UK) (Table 1). DNA fragments were resolved by electrophoresis in a 6% acrylamide gel stained with ethidium bromide.

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by χ^2 test. Marker-trait association analysis was also evaluated by χ^2 test (SPSS 10.0J, SPSS Japan Inc., Japan). To evaluate pairwise LD matrices among SNPs (by D' and r^2), we used the software HAPLOVIEW version 2.05 (developed in Mark Daly's lab., URL; http://www.broad.mit.edu/personal/jcbarret/haploview/index.php). This software also defined "LD blocks" by reasonable criteria based on 95% confidential bounds on D' values [4]. Power calculation was performed using a statistical program prepared by Ohashi et al. [10]. The significance level for all statistical tests was 0.05.

In view of the gender differences in gene effects, we included analyses of samples divided according to the gender. Both in cases and controls, genotype frequencies of total, female and male samples were not significantly different from HWE.

In association analysis, we could not find associations of rs175174 with schizophrenia in either male or female (Table 2).

Next, to test whether rs175174 is representative for ZDHHC8 or not, we performed LD mapping using three additional SNPs around ZDHHC8 (Fig. 1). LD matrices between each pair of SNPs showed strong LD both in cases and controls (Table 1). Even after dividing samples according to the gender, all LD patterns showed the same trends (data not shown). These findings may suggest that the LD pattern of ZDHHC8 is a block-like pattern and that rs175174 is the "representative SNP" of this gene.

The power based on genotype relative risk (GRR) was calculated to evaluate the non-significant results due to type II error. When we set the GRR at 1.28, 1.42 and 1.40 in all, female and male samples, respectively (multiplicative model), our sample size provided powers of more than 80%.

We could not replicate an original positive association using TDT of ZDHHC8 with schizophrenia by the present case–control association analysis among Japanese. Nor could we replicate the gender-specific effect of the risk SNP. In this association analysis, our sample sizes provide enough power to deny the hypothesis. We also performed the fine LD mapping of Japanese samples and showed that the LD pattern of ZDHHC8 was the same block-like pattern as one of the samples from the United States and South Africa. The results provide evidence that not only rs175174 but also ZDHHC8 would not be a susceptibility factor for schizophrenia in either Japanese females or males. The discrepancy between Japanese and the samples from the United States and South Africa may derive from ethnic differences.

A couple of limitation should be addressed to discuss the present results. Initially, the mean age of controls is much younger than that of patients in the present study. This means that a number of young controls, although not more than five subjects given a lifetime morbidity risk of 0.8–1.0%, may go on to develop schizophrenia. This confounding factor might weaken the power of the present study. Another limitation

Table 1
SNPs in LD mapping and pairwise LD matrices

SNP ID	D'				Restriction enzyme
	rs175169	rs175174	rs175175	rs2292570	
rs175169		0.97 (0.78)	1.0 (0.29)	1.0 (0.67)	*Bsl*I
rs175174	0.97 (0.80)		1.0 (0.36)	1.0 (0.58)	*Bse*RI
rs175175	1.0 (0.26)	1.0 (0.31)		1.0 (0.21)	*Alw*NI
rs2292570	0.93 (0.76)	0.97 (0.70)	1.0 (0.23)		*Tsp*RI

Upper diagonal figures are D' (r^2) of controls and lower diagonal figures are D' (r^2) of schizophrenia.

Table 2
Association analysis of rs175174

Samples	Number	G/G	G/A	A/A	*P* value (genotype)	MAF[a]	*P* value (allele)
Total							
SCZ	561	238	245	78		0.357	
CON	529	205	259	65	0.213	0.368	0.618
Female							
SCZ	259	114	106	39		0.355	
CON	270	112	130	28	0.133	0.344	0.714
Male							
SCZ	302	124	139	39		0.359	
CON	259	93	129	37	0.457	0.392	0.260

SCZ: schizophrenia; CON: control.
[a] Minor allele frequency.

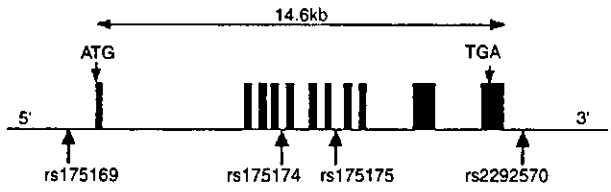

Fig. 1. Genomic structure of ZDHHC8 and SNPs used in association analysis and LD mapping. Vertical bars represent exons of ZDHHC8, and each number under arrows represents SNP ID.

which must be exercised is that the other candidates related to the neurodevelopmental and neuroprotective effect of ZDHHC8 would be in locus heterogeneity [11]. For example, ZDHHC8 encodes a putative transmembrane palmitoyltransferase modulating numerous classes of neuronal proteins including proteins important for neuronal development, neurotransmitter receptors such as NMDA [3]. Thus, the combined effect between ZDHHC8 and the other genes might be a stronger predisposing factor. Further genetic analysis including related candidate genes would definitely be required for a conclusive result.

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Association of Neural Cell Adhesion Molecule 1 Gene Polymorphisms with Bipolar Affective Disorder in Japanese Individuals

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Background: *Although the pathogenesis of mood disorders remains unclear, heritable factors have been shown to be involved. Neural cell adhesion molecule 1 (NCAM1) is known to play important roles in cell migration, neurite growth, axonal guidance, and synaptic plasticity. Disturbance of these neurodevelopmental processes is proposed as one etiology for mood disorder. We therefore undertook genetic analysis of* NCAM1 *in mood disorders.*

Methods: *We determined the complete genomic organization of human* NCAM1 *gene by comparing complementary deoxyribonucleic acid and genomic sequences; mutation screening detected 11 polymorphisms. The genotypic, allelic, and haplotype distributions of these variants were analyzed in unrelated control individuals (*n *= 357) and patients with bipolar disorder (*n *= 151) and unipolar disorder (*n *= 78), all from central Japan.*

Results: *Three single nucleotide polymorphisms, IVS6+321>C, IVS7+11G>C and IVS12+21C>A, displayed significant associations with bipolar disorder (for allelic associations, nominal* p *= .04,* p *= .02, and* p *= .004, respectively, all* p *> .05 after Bonferroni corrections). Furthermore, the haplotype located in a linkage disequilibrium block was strongly associated with bipolar disorder (the* p *value of the most significant three-marker haplotype is .005).*

Conclusions: *Our results suggest that genetic variations in* NCAM1 *or nearby genes could confer risks associated with bipolar affective disorder in Japanese individuals.*

Key Words: NCAM1, association study, linkage disequilibrium, haplotype, neurodevelopment

Affective disorder is a common psychiatric disease, afflicting approximately 10% of the population worldwide. Once the disease develops, episodes tend to recur throughout life, and prophylaxis is difficult to achieve in some cases with the therapeutic agents currently available. The etiologic bases remain unknown, although twin, family, and adoption studies have provided evidence for the involvement of heritable risk factors (Cardno et al 1999; Craddock and Jones 1999; Mendlewicz and Rainer 1977; Taylor et al 2002). Positive findings from linkage analyses and case–control association studies have also been reported (Berrettini 2000, 2001, 2002; Craddock et al 2001; Kato 2001).

Neural cell adhesion molecule 1 (NCAM1) is a member of the immunoglobulin gene superfamily and is widely expressed in the central nervous system. In addition, three major protein isoforms, of 180 kd, 140 kd, and 120 kd, are well known to possess multiple neurobiological functions in the brain (Kiss and Muller 2001; Ronn et al 1998). The genomic organization has already been partially reported (Saito et al 1994). The 180-kd and 140-kd isoforms of NCAM1 are transmembrane proteins, whereas the 120-kd isoform is linked to the plasma membrane via a glycosyl phosphatidyl-inositol (GPI) lipid anchor. Glycosyl phosphatidyl-inositol is attached to the C-terminal amino acid encoded by exon 15, the exon skipped in the transmembrane forms of NCAM 140 and NCAM 180. The difference between NCAM 140 and NCAM 180 involves the use of exon 18 by the latter isoform (Ronn et al 1998). Furthermore, some alternatively spliced exons exist between exons 7 and 13 (Barton et al 1988; Gower et al 1988; Saito et al 1994; van Duijnhoven et al 1992; also see Figure 1 legend). Several lines of evidence have supported the idea that dysregulation of NCAM1 isoforms in the brain might be involved in the pathophysiology of neuropsychiatric disorders, particularly bipolar affective disorder (Vawter 2000a). Secreted exon (SEC)-NCAM1 is increased in the hippocampus of patients with bipolar disorder (Vawter et al 1999), whereas variable alternative spliced exon (VASE)-NCAM1 is increased in the prefrontal cortex and hippocampus of patients with bipolar disorder (Vawter et al 1998). Furthermore, Poltorak et al (1996) reported elevated concentrations of NCAM1 protein in the cerebrospinal fluid of patients with mood disorder.

In this study, we performed genetic analysis of *NCAM1* as a compelling candidate for involvement in mood disorders.

Methods and Materials

Subjects

Mood disorder samples comprised unrelated patients with bipolar disorder (n = 151; 66% bipolar I, 34% bipolar II) and 78 patients with unipolar disorder (n = 78). Patients with bipolar disorder comprised 80 men (mean age, 49.0 ± 11.9 years) and 71 women (mean age, 48.9 ± 12.4 years). Patients with unipolar disorder comprised 33 men (mean age, 48.1 ± 10.7 years) and 45 women (mean age, 50.7 ± 10.9 years). All patients were diagnosed according to DSM-IV criteria for mood disorder (American Psychiatric Association 1994), to give a best-estimate lifetime diagnosis with consensus from at least two experienced psychi-

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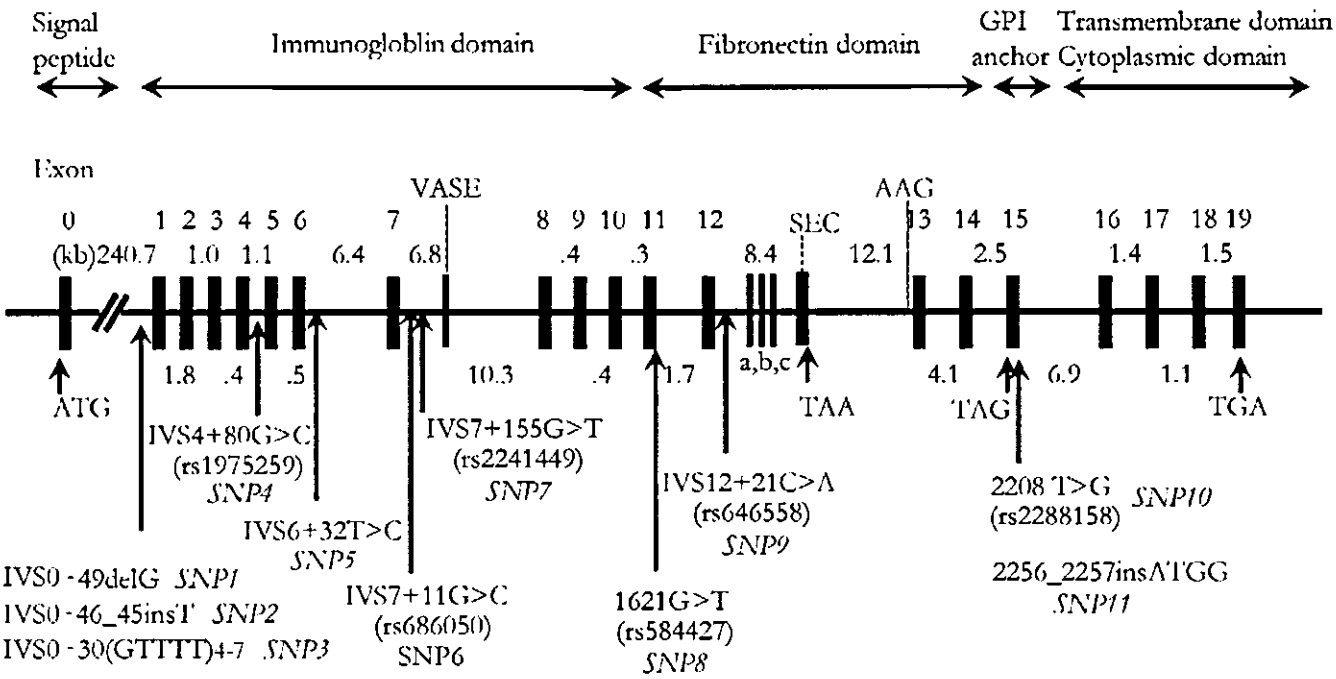

Figure 1. Genomic structure and locations of polymorphic sites for human NCAM1. Exons 0 through 14 are common in all neural cell adhesion molecule (NCAM) isoforms. In addition to the common exons, NCAM 180 uses exons 16, 17, 18, and 19; NCAM 140 uses exons 16, 17, and 19; and NCAM 120 uses exon 15. Locations of the initiation codon (ATG) and stop codons (TAA and TAG), and sizes (kilobases [kb]) of introns are provided. GPI, glycosyl phosphatidylinositol; VASE, variable alternative spliced exon; SEC, secreted exon; a, b, c, and triplet AAG, mini-exons; rs number is the National Center for Biotechnology Information single nucleotide polymorphism (SNP) cluster identification number from the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/).

atrists. The interview parameters included those described in the Structured Clinical Interview for DSM-IV Axis I Disorders (First et al 1997). All available medical records and family informant reports were also taken into consideration. Control subjects were recruited from among hospital staff and company employees documented to be free from psychoses: they included 173 men (mean age, 50.5 ± 13.5 years) and 184 women (mean age, 52.8 ± 11.0 years). All of our samples were collected from central Japan.

The present study was approved by the Ethics Committees of Tokyo Institute of Psychiatry, RIKEN Brain Science Institute, and Okayama University, and all participants provided written informed consent.

Determination of Genomic Structure

The complete genomic structure of *NCAM1* was determined by comparing complementary deoxyribonucleic acid (DNA) sequence (GenBank accession nos. NM_000615, M22094, S73101, XM_084656, X53243, and AK057509) and the University of California, Santa Cruz (UCSC) April 2003 draft assembly of the human genome (UCSC Genome Bioinformatics web site: http://genome.ucsc.edu/). We newly identified the location of exons VASE (S73101), SEC (M22094), 15 (M22094), 18 (XM_084656, AK057509), and 19 (AK057509). These sequences were not included in the UCSC's gene prediction program. "A" from the ATG initiation codon was considered +1.

Screening for Polymorphisms and Genotyping of Variants

Genomic DNA was isolated from blood samples according to standard methods. All exons and splice boundaries of *NCAM1*, except for some minor exons, were screened for polymorphisms by direct sequencing of polymerase chain reaction (PCR) products, from 20 unrelated bipolar samples. Primers used for PCR amplification are listed in Table 1. Polymerase chain reaction was performed with initial denaturation at 94°C for 1 min, followed by 35 cycles at 94°C for 15 sec, 50°C–70°C (optimized for each primer pair) for 30 sec, 72°C for 45 sec, and final extension at 72°C for 2 min, with TaKaRa Taq polymerase (Takara Bio, Shiga, Japan) or the Expand Long Template PCR System (Roche Diagnotics, Mannheim, Germany). Detailed information on amplification conditions is available upon request. Direct sequencing of PCR products was performed with the BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, Foster City, California) and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). All the polymorphisms were genotyped by direct sequencing with the help of the SEQUENCHER program (Gene Codes Corporation, Ann Arbor, Michigan), followed by visual inspection by two researchers. When necessary, both strands were sequenced.

Statistical Analysis

Departure from Hardy-Weinberg equilibrium was examined with the χ^2 test. Differences in genotype and allele frequencies were evaluated with Fisher's exact test or the Monte-Carlo method implemented in the CLUMP program (Sham and Curtis 1995) when appropriate. Linkage disequilibrium (LD) statistics were calculated with COCAPHASE (Dudbridge 2002; http://www.hgmp.mrc.ac.uk/~fdudbrid/software/). Estimation and comparison of haplotype frequencies were made with COCAPHASE. Graphic overview of pairwise LD strength between markers was made with GOLD software (Abecasis and Cookson 2000; http://www.well.ox.ac.uk/asthma/GOLD/). Power calculations were performed with Power Calculator (http://calculators.stat.ucla.edu/powercalc/).

Table 1. PCR Primers Used to Search for Nucleotide Variants in the *NCAM1* Gene

Region	Exon Length (bp)	Intron Length (bp)	Primers	Product Size (bp)	3′ End of Primer
Exon 1	75		(F) 5′-AAACTCCACACAAACCTCCTCCC-3′	424	148 bp upstream to exon 1
		1832	(R) 5′-TGCAAAAGGAAGGAAGAGGCCC-3′		159 bp downstream to exon 1
Exon 2	219		(F) 5′-TTCCAGCAGCCATACTCACCCC-3′	498	128 bp upstream to exon 2
		1018	(R) 5′-TTAGGGAGAGAGAATGGGACTG-3′		110 bp downstream to exon 2
Exon 3	144		(F) 5′-TGGAGACTTGCCCAGGACTCA-3′	382	84 bp upstream to exon 3
		376	(R) 5′-AGGACCCAGAAACCACATGAGG-3′		113 bp downstream to exon 3
Exon 4	138		(F) 5′-TCAAAGCCAGGGACGCATTTTC-3′	429	124 bp upstream to exon 4
		1080	(R) 5′-TTACGGTGGGGAGGGGATTTA-3′		126 bp downstream to exon 4
Exon 5	118		(F) 5′-CAATTCCTGACACTAACTCTG-3′	367	124 bp upstream to exon 5
		454	(R) 5′-CCTAAGAAGCCCACATCCATT-3′		85 bp downstream to exon 5
Exon 6	170		(F) 5′-CAGTTGCAGCCCTTGGATAGT-3′	539	182 bp upstream to exon 6
		6366	(R) 5′-ATGATGGTGGCTTGGACTAGG-3′		147 bp downstream to exon 6
Exon 7	143		(F) 5′-ACTAGGGTCTTGTACTTAGCAG-3′	454	94 bp upstream to exon 7
		6784	(R) 5′-TGTGCCTATTCCATTACAAGGG-3′		175 bp downstream to exon 7
VASE	30		(F) 5′-CTAAGGGGGAAAAAAAGCTGGACA-3′	425	181 bp upstream to exon VASE
		10319	(R) 5′-TCATCCACTCCCAACACAGC-3′		173 bp downstream to exon VASE
Exon 8	151		(F) 5′-GATACTCCCAGGTTCTCATGC-3′	583	192 bp upstream to exon 8
		374	(R) 5′-ATGGGAAGAAGACTCAAGGGCA-3′		199 bp downstream to exon 8
Exon 9	185		(F) 5′-TGTTCTGCTTACGTTCCCTGCA-3′	687	256 bp upstream to exon 9
		363	(R) 5′-GAGAAAAGAATAGCAGAGGGGC-3′		204 bp downstream to exon 9
Exon 10	97		(F) 5′-TTGTTTAAGGCTGGGCTGGAG-3′	368	118 bp upstream to exon 10
		332	(R) 5′-AACTCTCTGGCTTTGCTGACC-3′		113 bp downstream to exon 10
Exon 11	171		(F) 5′-ATTGGATCAGCGCATGGGGCA-3′	508	164 bp upstream to exon 11
		1715	(R) 5′-AGGGGCAACAACTCTACAGGCA-3′		132 bp downstream to exon 11
Exon 12	132		(F) 5′-GTCATTTGGGTCTGCCTTTCGG-3′	553	215 bp upstream to exon 12
		8387	(R) 5′-GAAGGGACTGTGTGTTAGCTGTCA-3′		162 bp downstream to exon 12
SEC	239		(F) 5′-GAGGGTGATGCCGAGAAGGAA-3′	661	240 bp upstream to exon SEC
		12086	(R) 5′-CACACGGAGGGAACACCAAGA-3′		142 bp downstream to exon SEC
Exon 13	125		(F) 5′-CTCTCAGTTTGGGCTCAGTC-3′	488	168 bp upstream to exon 13
		4144	(R) 5′-GCTGTAGGGCTGTCTTGGGATT-3′		153 bp downstream to exon 13
Exon 14	178		(F) 5′-GTCCCGTAAGTTTTGCCTATTGTC-3′	434	72 bp upstream to exon 14
		2522	(R) 5′-GCACAGATAGGTACAAGGCAAAAC-3′		138 bp downstream to exon 14
Exon 15	448		(F) 5′-ACCTTCCCTTTCCTTCTGTCCC-3′	746	123 bp upstream to exon 15
		6894	(R) 5′-ATCAGTGCGGTCTGGCTCTTTTAAC-3′		130 bp downstream to exon 15
Exon 16	208		(F) 5′-CTGTTTTCTCAATTCTGGGGCATA-3′	500	148 bp upstream to exon 16
		1364	(R) 5′-CAAATGGAGAACGTGCAATGAAAG-3′		98 bp downstream to exon 16
Exon 17	117		(F) 5′-AAGCTCAAGGTCACACAGCTAG-3′	680	147 bp upstream to exon 17
		1056	(R) 5′-GGTCCCCAGCTTCCCTTATCCTTT-3′		372 bp downstream to exon 17
Exon 18	816		(F) 5′-ATCCTTCTCTCTCTGTGGGCCT-3′	1055	133 bp upstream to exon 18
		1518	(R) 5′-CATCTAACAAGGAGGACACAGCAC-3′		62 bp downstream to exon 18
Exon 19	298		(F) 5′-CTTGGGTGATTTTTAGTGCTCC-3′	1071	146 bp upstream to exon 19
			(R) 5′-GGCAGCTATTTTACACGGACAT-3′		585 bp downstream to exon 19

PCR, polymerase chain reaction; NCAM1, neural cell adhesion molecule 1; F, forward; R, reverse; VASE, variable alternative spliced exon; SEC, secreted exon.

Results

The complete human *NCAM1* spans a region of 314 kilobases (kb) on chromosome 11q23.1, and consists of 19 main exons, exon 0 that encodes the signal peptide, alternatively spliced VASE and SEC exons, and the three-base-pair mini-exon AAG (Figure 1). Two or more alternatively spliced small exons (exons a, b, and c in Figure 1) exist between exons 12 and 13. Although protein isoforms are detected as three major mass classes (180, 140, and 120 kd), combinations of these exons and posttranslational modifications give rise to 20–30 molecular species for NCAM1 (Goridis and Brunet 1992; Kiss and Muller 2001).

Mutation screening allowed us to identify 11 polymorphisms, including five novel variants: IVS0−49delG, IVS0−46_45insT, IVS0−30(GTTTT)$_{4-7}$, IVS6+32T>C, and 2256_2257insATGG (Figure 1). For brevity, the detected single nucleotide polymorphisms (SNPs) were designated as SNP1–11 (Figure 1; Tables 2 and 3). The frequencies (except for that of IVS0−46_45insT, SNP2) are summarized in Tables 2 (SNP3) and 3 (SNPs 1, 4–11). The IVS0−46_45insT genotype could not be accurately determined, owing to the homopolymeric stretch of T nucleotides [(T)$_9$ or (T)$_{10}$]. This polymorphism was thus excluded from subsequent analyses. All polymorphisms were in Hardy-Weinberg equilibrium. Of the 10 polymorphisms, IVS12+21C>A (NCBI dbSNP accession no. rs646558, http://www.ncbi.nlm.nih.gov/SNP/) displayed a nominally significantly different genotypic distribution between patients with bipolar disorder and control subjects (p = .01; Table 3). IVS6+32T>C (novel) and IVS7+11G>C (rs686050) displayed trends toward genotypic association with bipolar disease.

Table 2. Genotypic and Allelic Distributions of the *NCAM1* Gene Polymorphism, IVS0–30 $(GTTTT)_{4-7}$

IVS0–30$(GTTTT)_{4-7}$ (SNP3)	Bipolar Disorder (n = 151)	Unipolar Disorder (n = 78)	Control Subjects (n = 357)
Genotype Counts (Frequency)			
4/4	64 (.42)	25 (.32)	122 (.34)
4/5	24 (.16)	12 (.15)	68 (.19)
4/6	35 (.23)	23 (.29)	92 (.26)
4/7	10 (.07)	2 (.03)	18 (.05)
5/5	3 (.02)	1 (.01)	4 (.01)
5/6	4 (.03)	5 (.06)	18 (.05)
5/7	3 (.02)	3 (.04)	8 (.02)
6/6	4 (.03)	7 (.09)	14 (.04)
6/7	3 (.02)	0 (0)	13 (.04)
7/7	1 (.01)	0 (0)	0 (0)
p^a	.44	.36	
Allele Counts (Frequency)			
4	197 (.65)	87 (.56)	422 (.59)
5	37 (.12)	22 (.14)	102 (.14)
6	50 (.17)	42 (.27)	151 (.21)
7	18 (.06)	5 (.03)	39 (.05)
p^a	.25	.33	

NCAM1, neural cell adhesion molecule 1.
[a]Differences in genotypic and allelic distributions were evaluated by the Monte Carlo method.

Allelic distributions in the above three polymorphisms all displayed significant deviations in bipolar samples compared with control subjects: IVS6+32T>C, nominal p = .04, odds ratio (OR) = 1.47, 95% confidence interval (CI) = 1.03–2.10; IVS7+11G>C, nominal p = .02, OR = 1.37, 95% CI = 1.05–1.80; IVS12+21C>A, nominal p = .004, OR = 1.64, 95% CI = 1.18–2.28 (Table 3). After Bonferroni correction for multiple testing of 10 SNPs and two disease classifications, these deviations were not significant. For unipolar disorder, none of these polymorphisms displayed nominally significant genotypic or allelic associations with the disease.

Power calculations were performed on the basis of an arbitrary assumption of relative risk and frequency of risk allele. When a relative risk of 2.0 was assumed, the bipolar sample in the present study displayed ≥93% power to detect significant association ($\alpha < .05$, frequency of risk allele = .3). The unipolar samples had ≥76% power. With a relative risk of 1.5, our bipolar samples had 51% power to detect significant association ($\alpha <$ .05, frequency of risk allele = .3). The unipolar samples retained 34% power.

Common (frequency of minor allele > .03) variants in the gene were selected for pairwise LD testing: IVS4+80G>C (SNP4); IVS6+32T>C (SNP5); IVS7+11G>C (SNP6); IVS7+155G>T (SNP7); 1621G>T (SNP8); IVS12+21C>A (SNP9); and 2208T>G (SNP10) (Figure 1, Table 4). IVS0–49delG (SNP1) and IVS0–30$(GTTTT)_{4-7}$ (SNP3) were also included for LD calculations to examine the 5′ upstream genomic structure of *NCAM1*. D' (normalized D) and r^2 (squared correlation coefficient) values were computed in patients with bipolar disorder and control subjects. Both LD measures take values between 0 (lack of LD) and 1 (complete LD). Abecasis et al (2001) suggested a D' value of >.33 as a useful measure of LD. Nakajima et al (2002) proposed $r^2 > .1$ as a criterion for useful LD. Linkage disequilibrium relationships between markers are shown graphically in Figure 2. Linkage disequilibrium structure was similar in the two measures (also see Table 4). These data revealed that the region spanning SNP1 through SNP9 was in a block of moderate-to-strong LD, and there was an overt LD gap between SNP9 and SNP10. Polymorphisms associated with bipolar disorder displayed relatively strong LD between IVS6+32T>C (SNP5) and IVS7+11G>C (SNP6) (D' = 1.00, r^2 = .181) and between IVS6+32T>C (SNP5) and IVS12+21C>A (SNP9) (D' = .728, r^2 = .397), but not between IVS7+11G>C (SNP6) and IVS12+21C>A (SNP9) (D' = .277, r^2 = .019) (Table 4).

Next, we examined three SNP-based haplotypic associations in a sliding manner in the bipolar group, with the polymorphisms that spanned the LD block (SNP1–9) (Figure 3, Table 5). All the three SNP combinations except for SNP5-6-7 and SNP6-7-8 showed significant association with bipolar disorder in terms of both global p values and p values for individual risk haplotypes. The haplotypes defined by SNP5-6-7 displayed significant individual haplotypic association (p = .034) and a trend of global association (p = .097), whereas those constructed by SNP6-7-8 showed marginal individual haplotypic association (p = .067) (Figure 3). These haplotype analyses demonstrated that the risk haplotype consisting of SNP1–9 for bipolar disorder was Ins-$(GTTTT)_4$-G-C-C-G-G-A (global p = .033, individual haplotype p = .009) (Figure 3, Table 5).

Discussion

Neural cell adhesion molecule 1 is essential for cell adhesion, cell migration, axonal guidance, signal transduction, and synaptic plasticity during brain development. Bouras et al (2001) reported decreased neuron densities in layers III, V, and VI of Brodmann's area 24 (anterior cingulate cortex) in patients with bipolar disorder. Densities of neurons and pyramidal and glial cells were reduced in the prefrontal cortex of bipolar patients (Rajkowaska et al 2001). Animal studies have also suggested that disruption of NCAM1 function might underlie the pathophysiology of affective disorder through dysregulation of the cytoarchitecture (Cremer et al 1994; Tomasiewicz et al 1993). *NCAM1* is therefore deemed to possess compelling functional relevance to affective disorders.

Our case–control analysis revealed that the IVS6+32T>C (SNP5), IVS7+11G>C (SNP6), and IVS12+21C>A (SNP9) polymorphisms of *NCAM1* are nominally significantly associated with bipolar disorder, with the IVS12+21A (SNP9) allele displaying the strongest association (allelic p = .08 after correction for 10 SNPs and two disease category examinations). Sixty-six percent of our bipolar subjects suffered from bipolar disorder type I. It might be possible that bipolar I and bipolar II disorders are separate entities; however, there seems to be no difference in genetic association with *NCAM1* between bipolar I and II groups in the present study: allele frequencies of IVS12+21A (SNP9), which showed the strongest p value, were similar in bipolar I (.23, allelic p = .067) and bipolar II (.26, allelic p = .058) cohorts. Linkage disequilibrium analysis revealed that IVS12+21C>A (SNP9) was located at the 3′ edge of the LD block, and a gap existed between SNP9 and the neighboring SNP10. These results suggest that the real disease-causing variant(s), if one exists, might reside in the 3′ portion of the haplotype block spanning SNP1 to SNP10. The association of IVS6+32T>C (SNP5) and IVS7+11G>C (SNP6) polymorphisms with bipolar disorder might reflect tapering but remnant LD between these polymorphisms and the neighboring risk variant(s); however, more thorough genetic analyses are needed to precisely locate the genomic boundaries contributing to the development of bipolar disorder.

Table 3. Genotypic and Allelic Distributions of Nine *NCAM1* Gene Polymorphisms

Polymorphism	*n*	Genotype Counts (Frequency)			p^a	Allele Counts (Frequency)		p^a
IVS0-49de IG (SNP1)		I/I	I/D	D/D		I	D	
Bipolar disorder	151	128 (.85)	22 (.15)	1 (.01)	.89	278 (.92)	24 (.08)	.55
Unipolar disorder	78	64 (.82)	13 (.17)	1 (.01)	.95	141 (.90)	15 (.10)	.89
Control subjects	357	295 (.83)	58 (.16)	4 (.01)		648 (.91)	66 (.09)	
IVS4 + 80G > C (SNP4)		G/G	G/C	C/C		G	C	
Bipolar disorder	151	63 (.42)	68 (.45)	20 (.13)	.17	194 (.64)	108 (.36)	.08
Unipolar disorder	78	25 (.32)	38 (.49)	15 (.19)	.85	88 (.56)	68 (.44)	.72
Control subjects	357	118 (.33)	180 (.50)	59 (.17)		416 (.58)	298 (.42)	
IVS6 + 32T > C (SNP5)		T/T	T/C	C/C		T	C	
Bipolar disorder	151	96 (.64)	51 (.34)	4 (.03)	.06	243 (.80)	59 (.20)	.04
Unipolar disorder	78	59 (.76)	17 (.22)	2 (.03)	.37	135 (.87)	21 (.13)	.90
Control subjects	357	260 (.73)	93 (.26)	4 (.01)		613 (.86)	101 (.14)	
IVS7 + 11G > C (SNP6)		G/G	G/C	C/C		G	C	
Bipolar disorder	151	25 (.17)	79 (.52)	47 (.31)	.06	129 (.43)	173 (.57)	.02
Unipolar disorder	78	25 (.32)	34 (.44)	19 (.24)	.45	84 (.54)	72 (.46)	.48
Control subjects	357	91 (.25)	179 (.50)	87 (.24)		361 (.51)	353 (0.49)	
IVS7 + 155G > T (SNP7)		G/G	G/T	T/T		G	T	
Bipolar disorder	151	127 (.84)	23 (.15)	1 (.01)	1.00	277 (.92)	25 (.08)	.90
Unipolar disorder	78	64 (.82)	13 (.17)	1 (.01)	.89	141 (.90)	15 (.10)	.76
Control subjects	357	298 (.83)	55 (.15)	4 (.01)		651 (.91)	63 (.09)	
I621G > T (SNP8)		G/G	G/T	T/T		G	T	
Bipolar disorder	151	61 (.40)	74 (.49)	16 (.11)	.64	196 (.65)	106 (.35)	.38
Unipolar disorder	78	42 (.54)	29 (.37)	7 (.09)	.28	113 (.72)	43 (.28)	.29
Control subjects	357	159 (.45)	166 (.46)	32 (.09)		484 (.68)	230 (.32)	
IVS12 + 21 C > A (SNP9)		C/C	C/A	A/A		C	A	
Bipolar disorder	151	87 (.58)	54 (.36)	10 (.07)	.01	228 (.75)	74 (.25)	.004
Unipolar disorder	78	51 (.65)	26 (.33)	1 (.01)	.51	128 (.82)	28 (.18)	.64
Control subjects	357	249 (.70)	98 (.27)	10 (.03)		596 (.83)	118 (.17)	
2208T > G (SNP10)		T/T	T/G	G/G		T	G	
Bipolar disorder	151	79 (.52)	64 (.42)	8 (.05)	.15	222 (.74)	80 (.26)	.23
Unipolar disorder	78	36 (.46)	36 (.46)	6 (.08)	.54	108 (.69)	48 (.31)	.92
Control subjects	357	178 (.50)	141 (.39)	38 (.11)		497 (.70)	217 (.30)	
2256_2257 insATGG (SNP11)		D/D	D/I	I/I		D	I	
Bipolar disorder	151	143 (.95)	8 (.05)	0 (.00)	.32	294 (.97)	8 (.03)	.33
Unipolar disorder	78	78 (1.00)	0 (.00)	0 (.00)	.14	156 (1.00)	0 (0.00)	.14
Control subjects	357	345 (.97)	12 (.03)	0 (.00)		702 (.98)	12 (.02)	

NCAM1, neural cell adhesion molecule 1; I, insertion; D, deletion; SNP, single nucleotide polymorphism.
[a]Differences in genotypic and allelic distributions were evaluated by Fisher's exact test.

Interestingly, IVS6+32T>C, IVS7+11G>C, and IVS12+21C>A were all located in close proximity to the intron–exon boundaries. Mutations located near splicing donor and acceptor sites were found in patients with frontotemporal dementia, FTDP-17, and affected splicing regulation of τ-protein by causing distortion of the stem-loop structure (Hutton et al 1998). Previous postmortem studies have shown that the VASE- and SEC-NCAM isoforms are increased in the brains of patients with bipolar disorder,

Table 4. Pairwise Linkage Disequilibrium Estimations Between Polymorphisms in the *NCAM1* Gene

Polymorphism	IVS0-49delG (SNP1)	IVS0-30(GTTTT)$_{4-7}$ (SNP3)	IVS4 + 80 G > C (SNP4)	IVS6 + 32 T > C (SNP5)	IVS7 + 11 G > C (SNP6)	IVS7 + 155 G > T (SNP7)	1621 G > T (SNP8)	IVS12 + 21 C > A (SNP9)	2208 T > G (SNP10)
SNP1		1.000 (1.000)	1.000 (1.000)	1.000 (1.000)	1.000 (1.000)	1.000 (1.000)	1.000 (1.000)	.351 (.450)	.277 (.150)
SNP3	.070 (.046)		.944 (1.000)	1.000 (1.000)	.923 (1.000)	1.000 (1.000)	.882 (.792)	.699 (.670)	.112 (.088)
SNP4	.071 (.048)	.838 (.957)		1.000 (1.000)	.885 (.947)	1.000 (1.000)	.896 (.770)	.469 (.601)	.125 (.065)
SNP5	.017 (.021)	.112 (.129)	.114 (.131)		1.000 (1.000)	1.000 (1.000)	1.000 (1.000)	.534 (.728)	.141 (.015)
SNP6	.098 (.116)	.547 (.715)	.548 (.670)	.169 (.181)		.900 (.756)	.847 (.818)	.005 (.277)	.136 (.087)
SNP7	1.000 (1.000)	.067 (.048)	.068 (.050)	.016 (.022)	.076 (.069)		1.000 (.561)	.363 (.483)	.104 (.180)
SNP8	.048 (.047)	.249 (.196)	.273 (.178)	.077 (.126)	.349 (.270)	.046 (.015)		.803 (.862)	.290 (.088)
SNP9	.063 (.054)	.070 (.091)	.031 (.065)	.238 (.397)	.000 (.019)	.065 (.065)	.061 (.130)		.283 (.443)
SNP10	.003 (.005)	.013 (.018)	.009 (.003)	.001 (.000)	.008 (.004)	.005 (.008)	.017 (.001)	.007 (.005)	

The diagonal upper right part shows standardized D′ in control (bipolar) group between two markers calculated by the COCAPHASE program. The lower left part of diagonal shows r^2 (squared correlation coefficient) in control (bipolar) for bi-allelic marker pairs, and squared values of Cramer's coefficient for pairs with the multi-allelic marker, IVS0-30(GTTTT)$_{4-7}$. NCAM1, neural cell adhesion marker 1; SNP, single nucleotide polymorphism.

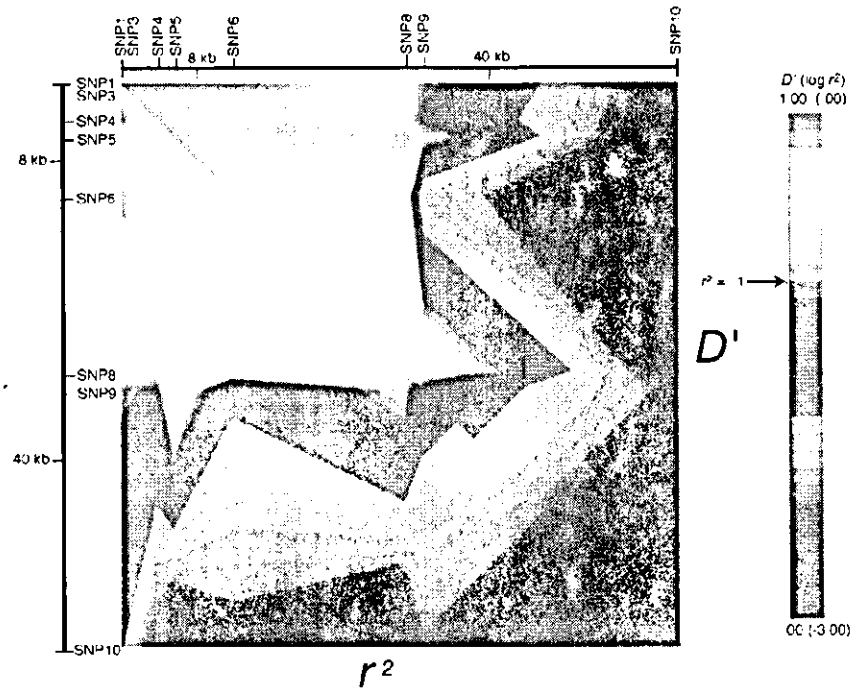

Figure 2. Linkage disequilibrium (LD) map of the *NCAM1*locus. Gold plot of color-coded, pairwise disequilibrium statistics (r^2 in diagonal bottom left and D' in diagonal upper right) is shown. Red and yellow indicate areas of strong LD. For single nucleotide polymorphism (SNP) numbers, see Figure 1.

compared with those of control subjects (Vawter et al 1998, 1999). The VASE exon is thought to play functional roles in the modulation of neurite growth activity (Doherty et al 1992). Use of the SEC exon resulted in premature termination of the coding sequence and production of a truncated NCAM polypeptide in brains (Gower et al 1988). IVS7+11G>C is upstream of the VASE exon, and the IVS12+21C>A variant is upstream of the alternatively spliced small exons and SEC exon. Examination of the correlation between NCAM1 genotypes and the content of alternatively spliced exons in bipolar brains would therefore be intriguing. We recently demonstrated just such a genotype (polymorphic repeats in a gene promoter region)–phenotype (expression level of gene product in postmortem brains) in a study of the *N*-methyl-D-aspartate receptor NR2A subunit gene (Itokawa et al 2003).

NCAM1 displayed a significant association with bipolar disorder but not with unipolar disorder. Power analysis showed that the size of our unipolar sample had adequate power to detect a relative risk of more than 2.0 but might miss small gene effects (relative risk < 1.5). Nevertheless, the failure to discern an

	SNP1	SNP3	SNP4	SNP5	SNP6	SNP7	SNP8	SNP9
SNP1	Ins							
SNP3	$(GTTTT)_4$	$(GTTTT)_5$						
SNP4	G	G	G					
SNP5		C	C	C				
SNP6			C	C	C			
SNP7				G	G	G		
SNP8					G	G	G	
SNP9						A	A	A
SNP10							T	T
SNP11								Del
Global (*P* value)	.014	.034	.038	.097	.136	.006	.015	.024
Individual Haplotype (*P* value)	.010	.031	.029	.034	.067	.005	.005	.018
Risk Allele	Ins	$(GTTTT)_4$	G	C	C	G	G	A
Global (*P* value)	.033							
Individual Haplotype (*P* value)	.009							

Figure 3. Results of three-marker and nine-marker haplotype analyses in bipolar samples. For three-marker analysis, a sliding window of three markers was tested, with two-marker overlaps. Below each over-represented haplotype is the *p* value for that haplotype. "Global (*P* value)" represents the overall significance when the observed versus expected frequencies of all of the haplotypes are considered together. "Individual Haplotype (*P* value)" represents significance of the deviated distribution of the risk haplotype in the bipolar group compared with control subjects. The *p* values were calculated with COCAPHASE (Dudbridge 2002; http://www.hgmp.mrc.ac.uk/~fdudbrid/software/).

Table 5. Estimated Haplotype Frequencies of the *NCAM1* Gene

Haplotype SNP1-3-4-5-6-7-8-9[a]	Frequency[b] Bipolar Subjects (*n* = 151)	Frequency[b] Control Subjects (*n* = 357)	*p*[c]
Del-1-G-T-G-T-G-A	.0501	.0466	.8044
Del-1-G-T-G-T-G-C	.0268	.0456	.1519
Ins-1-G-T-C-G-T-C	.3241	.2916	.3202
Ins-1-G-T-C-G-G-C	.0570	.0547	.8936
Ins-1-G-C-C-G-G-A	.1472	.0883	.0086
Ins-1-G-C-C-G-G-C	.0381	.0493	.4416
Ins-1-C-T-G-G-G-C	.0000	.0146	.0087
Ins-2-C-T-G-G-T-C	.0255	.0240	.8938
Ins-2-C-T-G-G-G-C	.1004	.1136	.5520
Ins-3-C-T-G-G-G-A	.0296	.0158	.2011
Ins-3-C-T-G-G-G-C	.1417	.2043	.0229
Ins-4-C-T-G-G-G-A	.0109	.0112	.9128
Ins-4-C-T-G-G-G-C	.0486	.0406	.6353
Global *p* value[c]			.0326

NCAM1, neural cell adhesion molecule 1; SNP, single nucleotide polymorphism.

[a]SNP1, allele Del = deletion, allele Ins = insertion; SNP3, allele 1 = $(GTTTT)_4$, allele 2 = $(GTTTT)_5$, allele 3 = $(GTTTT)_6$, allele 4 = $(GTTTT)_7$.

[b]Haplotpe frequencies were estimated by COCAPHASE.

[c]Calculated by COCAPHASE

association with unipolar disorder might not be due to the smaller statistical power of the analysis compared with bipolar disorder, because the genotypic and allelic frequencies in unipolar disorder resembled those of control subjects, not subjects with bipolar disorder. The present genetic findings suggest that the role of NCAM1 is pathophysiologically more relevant to bipolar disorder than to unipolar disorder. Such a result is in line with the aforementioned reports on NCAM1 perturbation in bipolar disorder (Vawter et al 1998, 1999) and might be in line with reports on disturbed brain histopathology in bipolar disorder (Bouras et al 2001; Rajkowska et al 2001). Other recent studies have also demonstrated pathophysiologic distinctions between bipolar and unipolar depression (Beyer and Krishnan 2002; Cotter et al 2001; Ongur et al 1998; Vawter et al 2000b).

In conclusion, our data suggest the possible involvement of human *NCAM1* or a nearby gene in vulnerability to bipolar affective disorder.

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Forum Minireview

New Perspectives in the Studies on Endocannabinoid and Cannabis: Cannabinoid Receptors and Schizophrenia

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Abstract. Cannabis consumption may induce psychotic states in normal individuals, worsen psychotic symptoms of schizophrenic patients, and may facilitate precipitation of schizophrenia in vulnerable individuals. Recent studies provide additional biological and genetic evidence for the cannabinoid hypothesis of schizophrenia. Examinations using [^{3}H]CP-55940 or [^{3}H]SR141716A revealed that the density of CB_1 receptors, a central type of cannabinoid receptor, is increased in subregions of the prefrontal cortex in schizophrenia. Anandamide, an endogenous cannabinoid, is also increased in the CSF in schizophrenia. A genetic study revealed that the CNR1 gene, which encodes CB_1 receptors, is associated with schizophrenia, especially the hebephrenic type. Individuals with a 9-repeat allele of an AAT-repeat polymorphism of the gene may have a 2.3-fold higher susceptibility to schizophrenia. Recent findings consistently indicate that hyperactivity of the central cannabinoid system is involved in the pathogenesis of schizophrenia or the neural mechanisms of negative symptoms.

Keywords: cannbinoid, CB_1 receptor, anandamide, CNR1 gene, schizophrenia

Introduction

Schizophrenia is the second most common mental illness after depression. It typically begins in late adolescence or early adulthood with characteristic psychiatric symptoms, for example, delusions and/or hallucinations, loose association, blunted or inappropriate affect, and distortions of perception. The disorder is a chronic and severe mental illness with a lifetime prevalence of about 1% worldwide. Behavior may be seriously impaired, leading to adverse social consequences. Recent development of typical and atypical neuroleptics has produced great improvement in the clinical symptoms of patients, but it is still inadequate, and the overall prognosis for schizophrenia is still far from satisfactory. Such unsuccessful treatment must result, at least partly, from insufficient understanding of the pathogenesis of schizophrenia. To date, various hypotheses for the etiology of schizophrenia have been proposed, for example, the classical dopamine hypothesis, the NMDA-receptor hypothesis, and the current neurodevelopmental hypothesis. Among them, the "cannabinoid hypothesis", which was originally based on clinical findings in marijuana abusers, has been developed as one of the pharmacological etiologies for schizophrenia.

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Cannabis consumption and schizophrenia

There have been a number of case reports indicating that consumption of a relatively large amount of cannabis could precipitate a psychotic state called "cannabinoid psychosis", with hallucinations, delusions, and emotional liabilty, resembling schizophrenia (1 – 4). An Indian study showed that the most potent cannabis preparations produced psychotic symptoms after the shortest period of consumption in cannabis abusers (5). Administration of Δ^9-tetrahydrocannbinol (Δ^9-THC), a major ingredient of cannabis, to normal volunteers induced cognitive impairment in three-dimensions closely resembling that of schizophrenia patients (6). These clinical studies indicate that cannabis may have psychotomimetic effects in previously non-psychotic subjects.

In schizophrenic patients, abuse of cannabis has worsened positive symptoms of schizophrenia (7, 8), even under a regular regimen of antipsychotics (9). Cannabinoid consumption by schizophrenics results in a poor outcome and liability to relapse (10, 11). A prospective study over one year showed that psychotic patients who used cannabis relapsed to a psychotic state sooner and more frequently than patients who did not use cannabis. A dose-response relationship in relapse was also observed (12).

It is possible that cannabis use does not merely precipitate cannabis psychosis or exacerabte schizophrenic symptoms. It may also precipitate endogenous psychosis of schizophrenia in individuals who are vulnerable to the psychosis. Several clinical studies showed high rates of cannabis consumption in schizophrenic patients (13, 14). Schizophrenic patients with a positive urine test for cannabis at admission had a family history of psychosis more frequently than patients with a negative urine test (15). The Edinburgh high-risk study group showed that frequent cannabis use was associated with a six-fold increase in the risk of psychosis in high risk individuals who have a strong family history of psychosis compared with patients without a family history (16). The strongest epidemiological evidence was provided by a Swedish cohort study. Andreasson et al. found that cannabis use before the age 18 years was associated with increased risk of schizophrenia 15 years later (Fig. 1). The relative risk of precipitation of schizophrenia was 2.4 times higher than that of someone who did not use cannabis before the age of 18 years (17). A dose-response relationship was also demonstrated because heavy consumption (more than 50 times by age 18) was associated with a sixfold increase in the incidence of schizophrenia. These findings indicate that enhancement of the cannabinoid system by cannabis consumption produces de novo psychosis in normal individuals, worsens psychotic symptoms previously seen in schizophrenic patients, and may facilitate precipitation of endogenous psychosis of schizophrenia, especially in individuals who have vulnerability to psychosis.

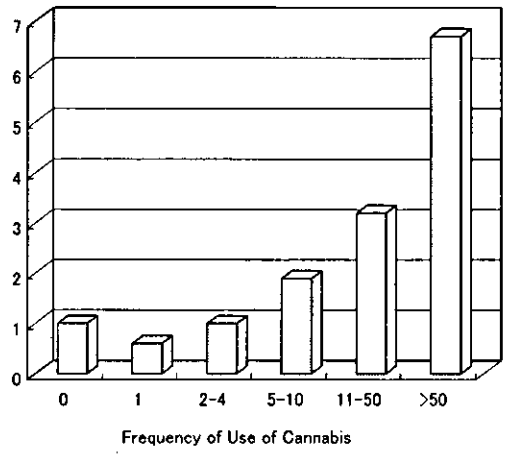

Fig. 1. Epidemiologic study of morbidity risk of cannabis use before 18 year for development of schizophrenia. Bar graph is based on the data of Andreasson et al. (1987) (Ref. 17). The vertical axis indicates the odds ratio of schizophrenia.

Postmortem study of brain cannabinoid receptors

In 1988, the existence of specific binding sites for cannabinoids in the brain were discovered and designated as the central cannabinoid receptors or CB_1 receptors, which are coupled to G protein (18) (Table 1). A second type of cannabinoid receptor, designated peripheral cannabinoid receptors or CB_2 receptors, was subsequently found to exist in the spleen and immune system. Therefore, the psychotomimetic effects of cannabis and marijuana must be mediated via CB_1 receptors. Two independent groups have measured CB_1-receptor densities in schizophrenic brains postmortem.

Table 1. Subtypes of cannabinoid receptors

	CB_1 receptor	CB_2 receptor
Amino acids	472 AA	360 AA
Locus	6q14-q15	1p36.11
Gene name	CNR1	CNR2
Endogenous ligand	2-Arachidonoylglycerol, Anadamide	*N*-Palmitylethanolamine
Distribution		
CNS	Substantia nigra, Putamen, Hippocampus, Cerebellum, Cerebral cortex	none
Peripheral	Testis, Uterus, Lung	Spleen, Tonsile, Lymph Node
Physiology	Spatial cognition, Mood elevation, Short-term memory, Substance dependence	Inflammation, Immune function?