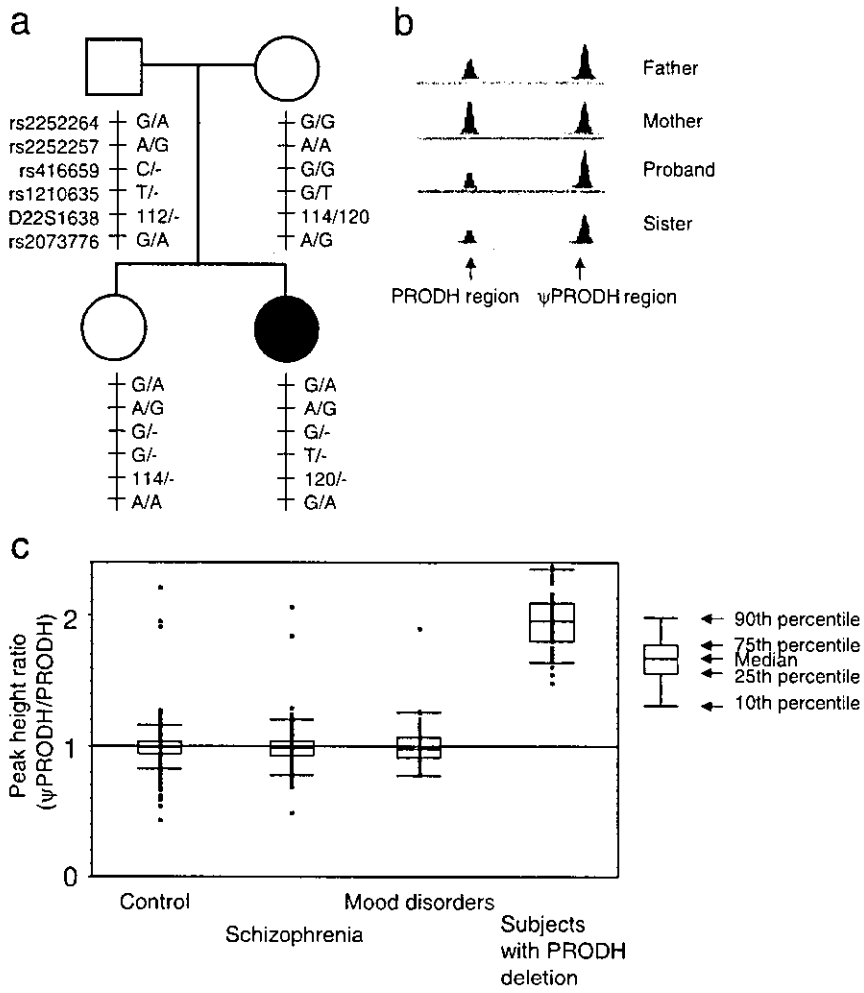

Fig. 1. A family with the PRODH deletion and detection of the deletion by a homologous gene quantitative amplification. (a) A family with the PRODH deletion and genotypes of the markers within and around the deletion. Closed circle indicates the proband with schizophrenia. Other family members are psychosis-free. 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Japanese SNP database, http://snp.ims.u-tokyo.ac.jp/index.html), and heterozygosity of D22S1638 was 0.8123 (our unpublished data).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.

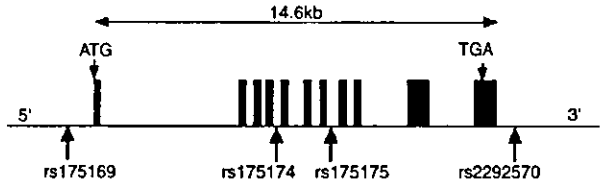

Fig. 1. Genomic structure of ZDHHC8 and SNPs used in association analysis and LD mapping. Vertical bars represent exons of ZDHHC8, and each number under arrows represents SNP ID.

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+32T>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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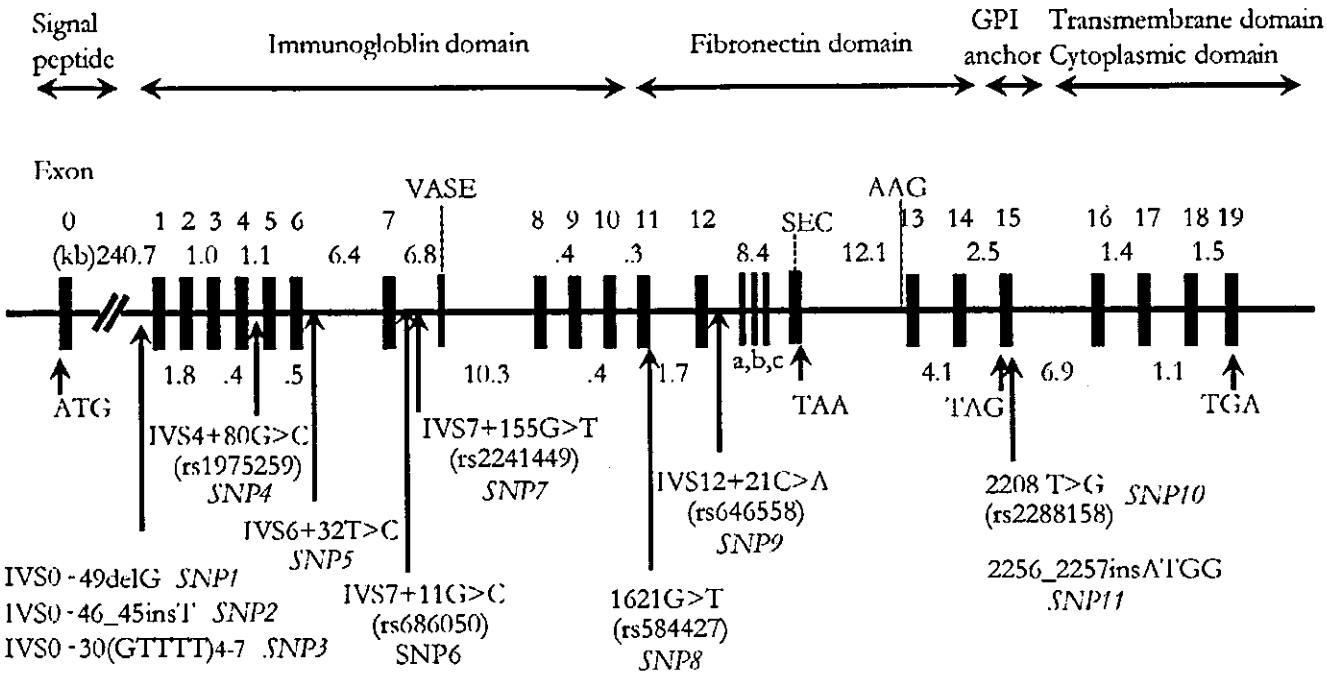

Figure 1. Genomic structure and locations of polymorphic sites for human NCAM1. Exons 0 through 14 are common in all neural cell adhesion molecule (NCAM) isoforms. In addition to the common exons, NCAM 180 uses exons 16, 17, 18, and 19; NCAM 140 uses exons 16, 17, and 19; and NCAM 120 uses exon 15. Locations 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 (α < .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 (α < .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.

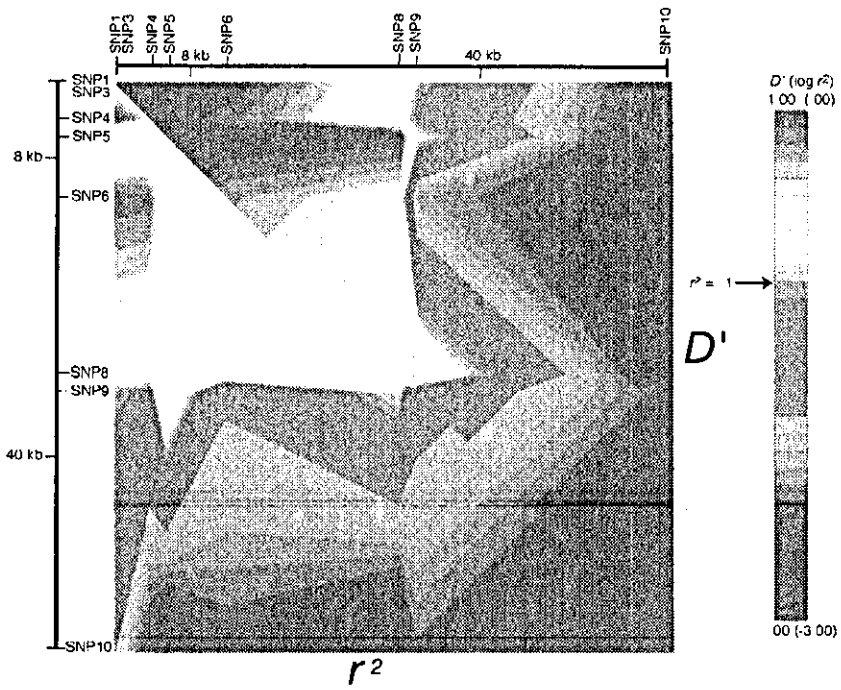

Figure 2. Linkage disequilibrium (LD) map of the *NCAM1*locus. Gold plot of color-coded, pairwise disequilibrium statistics (r^2 in diagonal bottom left and D' in diagonal upper right) is shown. Red and yellow indicate areas of strong LD. For single nucleotide polymorphism (SNP) numbers, see Figure 1.

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	.033	.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.

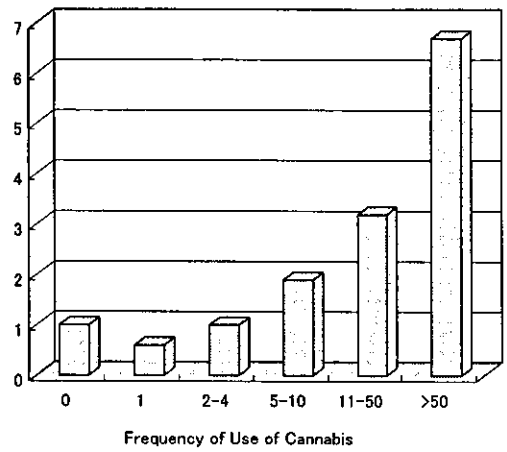

Fig. 1. Epidemiologic study of morbidity risk of cannabis use before 18 year for development of schizophrenia. Bar graph is based on the data of Andreasson et al. (1987) (Ref. 17). The vertical axis indicates the odds ratio of schizophrenia.

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?

Dean et al. (19) used in situ radioligand binding and autoradiography to measure [^{3}H]CP-55940, a non-selective cannabinoid agonist, in the dorsolateral prefrontal cortex (Brodmann's area 9), caudate-putamen, and areas of the temporal lobe and found a significant increase in [^{3}H]CP-55940 binding in the dorsolateral prefrontal cortex in subjects with schizophrenia that was independent of recent cannabis ingestion. They also found an increase in the density of CB_1 receptors in the caudate-putamen that was independent of diagnosis in subjects who had recently ingested cannabis. They speculated that changes in CB_1 receptors in the dorsolateral prefrontal cortex may be associated with the pathology of schizophrenia. Zavitsanou et al. (20) used [^{3}H]SR141716A as a radioligand for quantitative autoradiography of CB_1 receptors because SR141716A is a more potent and selective ligand for CB_1 receptors than CP-55940. They examined the anterior cingulate cortex, which plays an important role in normal cognition, particularly in relation to motivation and attention, and found a statistically significant 64% increase in [^{3}H]SR141716A-specific binding in the schizophrenia group as compared to the control group. These two independent postmortem studies showed an increase of CB_1 receptors in subregions of the prefrontal cortex, dorsolateral and anterior cingulate regions in schizophrenia. The major clinical symptoms observed in schizophrenia are classified into three categories: positive, negative, and disorganized symptoms. Negative symptoms consist of blunting of affect, poverty of speech and thought, apathy, anhedonia, reduced social drive, and loss of motivation. Such negative symptoms, including cognitive impairment, may result from dysfunction of the prefrontal cortex. Because cannabinoids disarrange cognition, attention, and motivation, it is possible that the increased numbers of CB_1 receptors in the prefrontal cortex may be involved in the pathology of schizophrenia particularly in relation to negative symptoms.

Endocannabinoids in cerebrospinal fluid (CSF)

Following the discovery of cannabinoid receptors in the brain, endogenous ligands for cannabinoid receptors were found in mammalian tissues. These are arachidonic acid derivatives. To date, two molecules have been identified as endocannabinoids acting on CB_1 receptors: anandamide (*N*-arachidonyl-ethanolamine) (21) and 2-AG (2-arachidonylglycerol) (22). They are able to reproduce the most typical behavioral effects of Δ^9-THC, such as inhibition of locomotor activity, analgesia on a hot plate, and hypothermia, in rodents. Therefore, they act as agonists in vivo for the cannabinoid systems. Leweke et al. (23) examined CSF concentrations of endocannabinoids in schizophrenia. They found that CSF levels of anandamide and palmitylethanolamide, another endocannabinoid acting mainly on CB_2 receptors, were increased by twofold in schizophrenic patients compared to non-schizophrenic controls, and 2-AG in the CSF was below detection in both groups. The increase of the two endocannabinoids in the CSF of schizophrenics was not affected by medication. Because increases in the CSF levels of anandamide and palmitylethanolamide should reflect increases in the brain, hyperactivity of the endocannabinoid system in the central nervous system may be involved in the pathogenesis of schizophrenia.

Genetic studies

CB_1 receptors are encoded by the CNR1 gene (MIM114610), which was cloned by Matsuda et al. in 1992 (24). CB_1 is located at 6q14 – q15, a site including a schizophrenia susceptibility locus, 6q13 – q26, which was designated as the Schizophrenia 5 locus (SCZ5, OMIM 603175), as revealed by Cao et al. (25) using two independent series of pedigrees. Two polymorphisms, an AAT-repeat microsatellite in the 3' flanking region and a 1359 G/A polymorphism at codon 453, a silent mutation, in the coding exon of the CNR1 gene, were reported previously. Additional screening of the promoter and entire coding region of the CNR1 gene by direct sequencing revealed two novel polymorphisms, 843G/C and L352S (26); however, their heterozygosities were as low as 1%, and the estimated genetic significance may be small. Therefore, the association with schizophrenia was studied using the AAT repeat and 1359 G/A polymorphisms of the CNR1 gene (27). Although the genotypic and allelic distribution of the 1359 G/A polymorphism was not different in schizophrenic and control subjects in a Japanese population, which is consistent with a Caucasian study (28), allelic distributions of the AAT-repeat polymorphism differed significantly between them (Fig. 2). Schizophrenia is usually divided into three subtypes by clinical features: paranoid, hebephrenic, and catatonic types. The hebephrenic subtype of schizophrenia, but not others, showed a strong association with the CNR1 gene ($P = 0.0028$). The 9-repeat allele of the AAT-repeat polymorphism was a genetic risk factor for susceptibility to hebephrenic schizophrenia (odds ratio = 2.3), and the 17-repeat allele of it was a strong negative risk factor, thus protecting against susceptibility to hebephrenic schizophrenia (odds ratio = 0.21). Namely, an individual with a 9- or 17-repeat allele should have a risk of hebephrenic schizophrenia increased by about twice

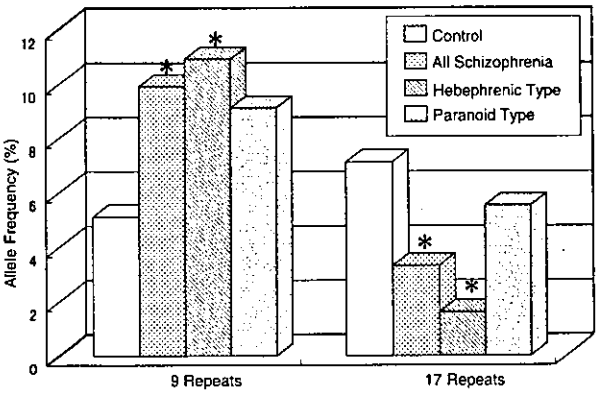

Fig. 2. Association of AAT-repeat polymorphism of the CNR1 gene with schizophrenia. *P<0.05. Data from Ref. 27.

and decreased by one fifth, respectively, compared to an individual without either allele. This finding was not supported by a Chinese study (29), in which schizophrenia was examined only in total rather than by subtypes. Although further investigation in different populations is needed to confirm the significance of the CNR1 gene as a genetic risk factor for hebephrenic schizophrenia, the relationship between hebephrenic schizophrenia and the CNR1 gene is very intriguing. Clinical studies have reported that long-term cannabis use causes a combination of symptoms including cognitive impairment, lack of motivation, and impaired attention, the so-called "amotivational syndrome," which resembles the core negative symptoms of schizophrenia (30). Hebephrenic schizophrenia is characterized by disorganized symptoms and also progressive deterioration of negative symptoms such as blunted affect and abulia. We have also consistently found that the CNR1 gene is not associated with methamphetamine psychosis, which shows resemblance to the paranoid type but not the hebephrenic type of schizophrenia, because it has few negative symptoms (unpublished data). The genetic evidence indicates that enhanced endocannabinoid activity may be involved in the negative symptoms of schizophrenia.

Fatty acid amide hydrolase (FAAH, MIM 602935) serves as a primary and rapid catabolic regulator of anandamide, 2-AG, and related fatty acid amide-signaling molecules in vivo (31). FAAH knock-out mice exhibit a 15-fold augmentation of endogenous brain levels of anandamide and an array of intense CB_1-receptor-dependent behavioral responses, including hypomotility, analgesia, catalepsy, and hypothermia (32). Recently, the Pro129Thr missense and functional mutation was identified in the FAAH gene. Sipe et al. (33) found that its homozygous form is strongly associated with both street-drug use and problem drug/alcohol use. However, we have found that there is no association between the Pro129Thr mutation of the FAAH gene and susceptibility to schizophrenia in a Japanese population (34). Possible involvement of the gene in negative symptoms of schizophrenia including cognitive function should be elucidated.

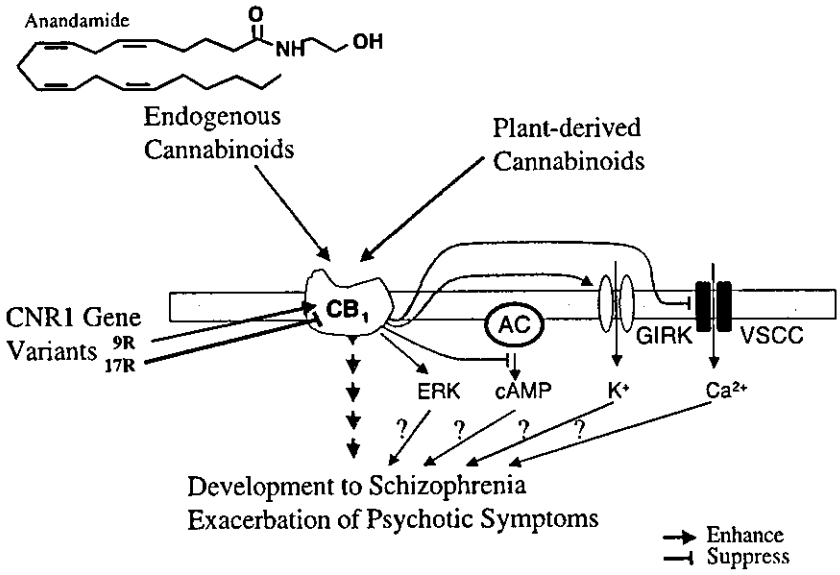

Fig. 3. Cannabinoid system and schizophrenia.

Conclusion

Several lines of biological and genetic evidence that support the cannabinoid hypothesis for schizophrenia are summarized in Fig. 3. Enhanced signaling of the cannabinoid system due to heavy consumption of exogenous cannabis, for example, marijuana, or increased endocannabinoid anandamide, which is mediated by CB_1 receptors in the brain, could precipitate schizophrenia. Increased CB_1 receptor density in the prefrontal cortex of schizophrenic patients should facilitate neural transmission of cannabinoids. In addition, genetic variants of the CNR1 gene, which encodes CB_1 receptors, could alter the efficiency of the receptor function or expression rate of the receptor molecules. All of the various findings observed in schizophrenia could converge into hyperactivity of the cannabinoid systems in the brain, which should play an important role in the pathogenesis of schizophrenia. Particularly, it is most significant clinically that the possible involvement of the cannabinoid system in the neural basis for the negative symptoms of schizophrenia. The recent development of atypical neuroleptics, for example, clozapine, olanzapine, and risperidone, is greatly beneficial to schizophrenic patients because they have been reported to improve not only positive but also negative symptoms with fewer adverse effects than conventional neuroleptics. However, the degree of improvement is not sufficient, especially in negative symptoms. More effective therapy for negative symptoms should be sought. The cannabinoid hypothesis of schizophrenia could be a key to development of an innovative therapy and comprehensive understanding of the etiology of schizophrenia.

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A nonsynonymous polymorphism in the human fatty acid amide hydrolase gene did not associate with either methamphetamine dependence or schizophrenia

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Abstract

Genetic contributions to the etiology of substance abuse and dependence are topics of major interest. Acute and chronic cannabis use can produce drug-induced psychosis resembling schizophrenia and worsen positive symptoms of schizophrenia. The endocannabinoid system is one of the most important neural signaling pathways implicated in substance abuse and dependence. The fatty acid amide hydrolase (FAAH) is a primary catabolic enzyme of endocannabinoids. To clarify a possible involvement of FAAH in the etiology of methamphetamine dependence/psychosis or schizophrenia, we examined the genetic association of a nonsynonymous polymorphism of the FAAH gene (Pro129Thr) by a case-control study. We found no significant association in allele and genotype frequencies of the polymorphism with either disorder. Because the Pro129Thr polymorphism reduces enzyme instability, it is unlikely that dysfunction of FAAH and enhanced endocannabinoid system induce susceptibility to either methamphetamine dependence/psychosis or schizophrenia.

Keywords: The fatty acid amide hydrolase (FAAH); Methamphetamine dependence/psychosis; Schizophrenia; Nonsynonymous polymorphism; The Pro 129Thr polymorphism

In 1988, the existence of a cannabinoid receptor in the brain was found, and its gene was cloned two years later [7,19]. To date, at least two different cannabinoid receptors, CB1, CB2, and putative endogenous agonists, including anandamide and 2-arachidonylglycerol, have been identified [8]. CB1 receptors are the only cannabinoid receptors that have been found in the central nervous system (CNS). A number of studies

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