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Supportive evidence for reduced expression of *GNB1L* in schizophrenia

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***Background*: Chromosome 22q11 deletion syndrome (22q11DS) increases the risk of development of schizophrenia more than 10 times compared with that of the general population, indicating that haploinsufficiency of a subset of the more than 20 genes contained in the 22q11DS region could increase the risk of schizophrenia. In the present study, we screened for genes located in the 22q11DS region that are expressed at lower levels in postmortem prefrontal cortex of patients with schizophrenia than in those of controls. *Methods*: Gene expression was screened by Illumina Human-6 Expression BeadChip arrays and confirmed by real-time reverse transcription-polymerase chain reaction assays and Western blot analysis. *Results*: Expression of *GNB1L* was lower in patients with schizophrenia than in control subjects in both Australian (10 schizophrenia cases and 10 controls) and Japanese (43 schizophrenia cases and 11 controls) brain samples. *TBX1* could not be evaluated due to its low expression levels. Expression levels of the other genes were not significantly lower in patients with schizophrenia than in control subjects. Association analysis of tag single-nucleotide polymorphisms in the *GNB1L* gene region did not confirm excess homozygosity in 1918 Japanese schizophrenia cases and 1909 Japanese controls. Haloperidol treatment for 50 weeks increased *Gnb1l* gene expression in prefrontal cortex of mice. *Conclusions*: Taken together with the impaired prepulse inhibition observed in heterozygous *Gnb1l* knockout mice reported by the previous study, the present findings support assertions that *GNB1L* is one of the genes in the 22q11DS region responsible for increasing the risk of schizophrenia.**

Key words: 22q11DS/haloperidol/prefrontal cortex/postmortem brain

Introduction

Schizophrenia, a devastating mental disorder that affects approximately 1% of the world's population, is a genetically complex disorder. The multifactorial polygenic model has received the most support as the mode of inheritance that underlies the familial distribution of schizophrenia; therefore, a variety of genetic, environmental, and stochastic factors are likely involved in the etiology. However, it is also possible that specific genes play major roles in susceptibility to schizophrenia. Genes involved in 22q11.2 deletion syndrome (22q11DS) substantially increases susceptibility to schizophrenia. 22q11DS is associated with several diagnostic labels including DiGeorge syndrome, velocardiofacial (or Shprintzen) syndrome (VCFS), conotruncal anomaly face, Cayler syndrome, and Opitz GBBB syndrome. Schizophrenia is a late manifestation in approximately 30% of 22q11DS cases, which is comparable to the risk to offspring of 2 parents with schizophrenia. The 22q11 deletion is detected relatively frequently in patients with schizophrenia;

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a number of studies have shown that 22q11DS schizophrenia is a true genetic subtype of schizophrenia[1,2].

Although the deleted region is approximately 3 Mbp in most patients with 22q11DS, the critical region is approximately 1.5 Mbp.[3,4] Less than 30 genes are located in the 22q11DS region. Studies of 22q11DS patients without the common chromosomal deletion suggested that the *TBX1* is a major contributor to the conotruncal malformations of 22q11DS.[5] One of the mutations in the *TBX1* was found to be a loss-of-function mutation.[6] Mice heterozygous for a null mutation in *Tbx1* develop conotruncal defects.[7] Deletion of one copy of the *Tbx1* affects the development of the fourth pharyngeal arch arteries, whereas the homozygous mutation severely disrupts the pharyngeal arch artery system.[8] The contribution of the *TBX1* haploinsufficiency to psychiatric disease was suggested by the identification of a family with VCFS in a mother and her 2 sons. These 3 patients all had a null mutation of the *TBX1*, and one of the sons was diagnosed with Asperger syndrome after psychiatric assessment.[9]

Contribution of genes in the 22q11DS region to susceptibility to schizophrenia has been examined mainly by genetic association studies. Associations between schizophrenia and nucleotide variations in the *ZNF74*,[10] *DGCR*, [11] *DGCR14*,[12] *PRODH*,[13] *ZDHHC8*,[14] *COMT*,[15–18] and *CLDN5*[19,20] genes have been reported. These associations, however, have not been confirmed in other populations[19–22] or by meta-analyses.[19–24]

Studies of genetically engineered mice have provided supporting evidence for roles of the genes located in the human 22q11DS region in schizophrenia. *Prodh* knockout mice exhibited deficits in learning and responses to psychomimetic drugs.[25] Observation of overlapping loci across 5 heterozygous mice strains with different deletion sites revealed that a 300-kb locus, which contains the *Gnb1l*, *Tbx1*, *Gp1bb*, and *Sept5* genes, is crucial for impaired sensorimotor gating measured by prepulse inhibition test (PPI).[9] In that study, the authors speculated that the *GP1BB* was unlikely to be related to schizophrenia because it is expressed only in platelets. The *GP1BB* causes Bernard-Soulier disease, which has no associated psychiatric disorders. The *Sept5* heterozygous knockout mice did not show impaired PPI. *Gnb1l* or *Tbx1* heterozygous knockout mice showed reduced PPI.[9] Therefore, the authors concluded that the *Tbx1* and *Gnb1l* are strong candidates for psychiatric disease in patients with 22q11DS.[9] In another study, however, *Tbx1* heterozygous knockout mice showed normal locomotor activity, habituation, nesting, and locomotor responses to amphetamine.[25]

Recently, Williams et al[26] reported associations between polymorphisms in the *GNB1L* gene region and schizophrenia in the United Kingdom, German, and Bulgarian population. They found excess homozygosity at rs5746832 and rs2269726 in male schizophrenia subjects and that the markers associated with male schizophrenia were related with cis-acting changes in *GNB1L* expression. These mouse and human studies indicated a correlation between *GNB1L* gene expression and psychosis.

The working hypothesis of the present study was that genes in the 22q11DS region involved in the susceptibility to schizophrenia were likely to be expressed at lower levels in patients with schizophrenia than in control subjects. We performed a scan of expressional changes of the genes in the 22q11DS region in schizophrenic and control prefrontal cortex and found that the *GNB1L* gene was compatible with our hypothesis.

Materials and methods

Human Postmortem Brains

Brain specimens were from individuals of European descent Australian and Japanese. Australian sample comprised 10 schizophrenic patients and 10 age- and gender-matched controls (Supplementary Table S1). The diagnosis of schizophrenia was made according to the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV criteria (American Psychiatric Association 1994) by a psychiatrist and a senior psychologist. Control subjects had no known history of psychiatric illness. Tissue blocks were cut from gray matter in an area of the prefrontal cortex referred to as Brodmann's area 9 (BA9). Japanese samples of BA9 gray matter from Japanese brain specimens consisted of 6 schizophrenic patients and 11 age- and gender-matched controls (Supplementary Table S1). In addition, postmortem brains of 37 deceased Japanese patients with schizophrenia were also analyzed (Supplementary Table S1). The Japanese subjects met the DSM-III-R criteria for schizophrenia. The study was approved by the Ethics Committees of Central Sydney Area Health Service, University of Sydney, Niigata University, University of Tsukuba, Tokyo Metropolitan Matsuzawa Hospital, and Tokyo Institute of Psychiatry.

RNA Isolation and Gene Expression Microarray

Total RNA was extracted from brain tissues with ISOGEN Reagent (Nippon Gene Co, Tokyo, Japan). The RNA quality was checked using a Nanodrop ND-1000 spectrophotometer (LMS, Tokyo, Japan) to have an OD 260/280 ratio of 1.8–2 and an OD 260/230 of 1.8 or greater. Microarrays were used to screen for differential gene expression between Australian schizophrenic patients and controls. In brief, 500 ng of total RNAs were reverse transcribed to synthesize first- and second-strand complementary DNA (cDNA), purified with spin columns, then in vitro transcripted to synthesize biotin-labeled complementary RNA (cRNA). A total of 1500 ng of biotin-labeled cRNA was hybridized on Sentrix® Human-6 Expression BeadChip (Illumina Inc., San Diego, CA) at 55°C for 18 h. The hybridized BeadChip was washed and labeled with streptavidin-Cy3, then scanned with an Illumina BeadStation 500 System

(Illumina Inc). Scanned image was imported into BeadStudio (Illumina Inc) for analysis. Forty-six thousand transcripts can be analyzed by a single BeadChip.

Real-time Quantitative RT-polymerase chain reaction

Expression of the *GSCL*, *HIRA*, *SEPT5*, *GNB1L*, *TBX1*, and *CDC45L* genes was analyzed by TaqMan Real-time polymerase chain reaction (PCR) system (Applied Biosystems, Foster City, CA). From RNA, cDNA was synthesized with Revertra Ace (Toyobo, Tokyo, Japan) and oligo dT primer. Expression of these 6 genes was analyzed with an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems), with the TaqMan gene expression assays for *GSCL* (Hs00232019_m1), *HIRA* (Hs00983699_m1), *SEPT5* (Hs00160237_m1), *GNB1L* (Hs00223722_m1), *TBX1* (Hs00271949_m1), and *CDC45L* genes (Hs00185895_m1) and normalized to expression of Human *GAPDH* Control Reagents (Applied Biosystems). *GNB1L* expression was analyzed in Australian samples and replicated the analysis in Japanese subjects.

Protein Isolation and GNB1L Protein Levels in Brain

Protein was extracted from prefrontal cortex tissues with Laemmli Buffer. Western blotting method was used to compare GNB1L protein levels between schizophrenics and controls. Each of 2 μg protein was run on Pro-Pure™ SPRINT NEXT GEL (Amresco, Solon, OH) and transferrd to BioTrace™ PVDF (Nihon Pall Ltd, Tokyo, Japan). Polyclonal antibodies against the human GNB1L protein (OTTHUMP00000028644) were generated by injecting rabbits with the following peptide: CAGSKDQRISLWSLYPRA (MBL, Nagoya, Japan). Mouse polyclonal antibody against beta-actin (Sigma Aldrich Japan, Tokyo, Japan) was also used for normalization purpose. The bound primary antibodies were detected with goat anti-rabbit or anti-mouse IgG antibody HRP conjugate (MBL) and Immobilon™ Western, Chemiluminescent HRP Substrate (Millipore, Billerica, MA) on X-film (Fujifilm Medical, Tokyo, Japan). The signals of GNB1L or beta-actin of each subject on X-films were quantitated by computer software, ImageJ 1.40g (http://rsb.info.nih.gov/ij/), and GNB1L protein levels were normalized to beta-actin and compared.

Peripheral Blood and Brain DNA Sample and Genotyping

The subjects comprised 1918 unrelated Japanese patients with schizophrenia (1055 men, 863 women; mean age ± standard deviation [SD], 48.9 ± 14.5 years) diagnosed according to DSM-IV with consensus from at least 2 experienced psychiatrists and 1909 mentally healthy unrelated Japanese control subjects (1012 men, 893 women; mean age ± SD, 49.0 ± 14.3 years) of whom the first- and second-degree relatives were free of psychosis as self-reported by the subjects. The association analysis was approved by the Ethics Committees of the University of Tsukuba, Niigata University, Fujita Health University, Nagoya University, Okayama University, and Teikyo University, National Center of Neurology and Psychiatry, University of Tokyo, and all participants provided written informed consent. DNAs were extracted from these blood samples and the same brain tissues used for gene expression analysis. The tag single-nucleotide polymorphisms (SNPs) comprising rs5746832, rs5746834, rs2269726, rs748806, rs29807124, rs5993835, rs13057609, rs4819523, rs2073765, rs7286924, rs10372, rs3788304, and rs11704083 at the *GNB1L* gene region were selected by Haploview program using HapMap Project Japanese data set (http://www.hapmap.org/), as the previously reported schizophrenia-associated SNPs, rs5746832 and rs2269726, were forced included. The TaqMan reaction was performed in a final volume of 3 μl consisting of 2.5 ng genomic DNA and Universal Master Mix (EUROGENTEC, Seraing, Belgium), and genotying was performed with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).

Genotyping quality control consisted of ≥98% successful calls. We confirmed concordance among repeat genotyping in ≈10% of genotypes.

Brain GNB1L Expression and Genotyping

The correlations between *GNB1L* expression and 13 SNPs, rs5746832, rs5746834, rs2269726, rs748806, rs29807124, rs5993835, rs13057609, rs4819523, rs2073765, rs7286924, rs10372, rs3788304, and rs11704083, were examined in Australian and Japanese brain tissues, respectively.

Mice Experiments

Mice treated with haloperidol were studied to examine the effects of antipsychotic treatments on *Gnb1l* gene expression. Thirty-nine C57/BJ6 male mice (age, 8 weeks; weight, 20–25 g) were housed under 10 h/14 h light/dark conditions with normal food and water ad libitum, where groups of 5 or 6 mice were housed separately, and 0.5 mg/kg haloperidol or saline was injected intraperitoneally once each day for 4 weeks or for 50 weeks. The dosage of haloperidol was at maximum clinically used, and 4 or 50 weeks for treatment term correspond to several years or half a lifetime in human terms, respectively. We used extreme but likely condition to clear up the effect of the medication. We determined the dosage of haloperidol according to the previous studies.[27–31] Mice were sacrificed 4 h after the last injection to obtain brain tissues.

The prefrontal cortex was taken, and RNA was extracted with RNeasy kit (Qiagen, K.K., Tokyo, Japan). A cDNA was synthesized with Revertra Ace (Toyobo) and oligo dT primer. Expression of *Gnb1l* was analyzed by TaqMan real-time polymerase chain reaction (PCR) with an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems), with the TaqMan gene expression assay for *Gnb1l* (Mm00499153_m1). Expression of

Gnb1l was normalized to that of rodent *Gapdh* with Rodent *Gapdh* Control Reagents (Applied Biosystems).

All animal procedures were performed according to protocols approved by the Animal Care and Use committee of University of Tsukuba.

Statistics

Microarray analysis was performed with GeneSpring software version 7.3.1 (Silicon Genetics, Redwood, CA). The mean background noise level was first corrected in each sample, and then per-chip normalization was applied to eliminate systematic differences between chips. Two-tailed Student's *t*-test was used to examine the difference between schizophrenic patients and controls. In real-time PCR experiments, *GAPDH* or *Gapdh* was used as an internal control, and measurement of threshold cycle (Ct) was performed in triplicate. Data were collected and analyzed with Sequence Detector Software version 2.1 (Applied Biosystems) and the standard curve method. Relative gene expression was calculated as the ratio of expression of the target gene to the internal control (*GAPDH* or *Gapdh*). Correlations of *GNB1L* gene expressions and 2 quality parameters, postmortem interval (PMI) and pH, of brain samples were analyzed with analysis of variance (ANOVA) one-way tests by JMP computer software version 5.1. The density of images reflecting GNB1L protein levels was also compared between schizophrenics and controls with the Wilcoxon test implemented in JMP computer software version 5.1. Deviation from Hardy-Weinberg equilibrium (HWE), allelic associations, and linkage disequilibrium (LD) between SNPs were evaluated with Haploview software version 3.11. A nominal association was defined when the given P value for allelic or genotypic tests was less than 5% (uncorrected $P < .05$). If a nominal significant association was found in the analysis, permutation test was also performed with Haploview software version 3.11. Correlations of *GNB1L* gene expressions and either protein expression or genotypes of the tag SNPs were analyzed with ANOVA one-way tests by JMP computer software version 5.1.

Results

Human-6 Expression BeadChip demonstrated that *GSCL* (GI_48885362-S) and *TBX1* (GI_18104949-I) of 28 genes located in the 22q11DS region were expressed at lower levels in schizophrenic brains than in the control brains in the Australian samples ($P < .05$) (Supplementary Table S2). However, the signals of these transcripts were low, and reliable confidence was not obtained from any subject. Expression of *CDC45L* (GI_34335230-S) tended to be lower in schizophrenic brains than in control brains ($P = .07$). Data of *GNB1L* were not available in this platform (Supplementary Table S2).

We used real-time PCR experiments to evaluate expression of the 3 genes that were potentially underexpressed in schizophrenia prefrontal cortex by microarray and *GNB1L*, which was not assessed by the microarray in the Australian and Japanese brain samples. The difference in gene expression between the schizophrenia and control groups was not confirmed for *CDC45L*. In addition, because the reliability of *HIRA* and *SEPT5* was not sufficient due to weakly expressed sequences in the array screening, we reexamined expression levels of these genes by real-time PCR method and did not find significant differences in gene expression between the schizophrenia and control groups. Expressions of *TBX1* and *GSCL* were too low to obtain reliable signals with the TaqMan gene expression assay (Hs00271949_m1 and Hs00232019_m1, respectively). Relative expression of *GNB1L* was significantly lower in Australian schizophrenic prefrontal brains than in Australian control brains (average ratio = 0.57, $P < .001$) and in Japanese patients with schizophrenia than in control subjects (average ratio = 0.53, $P < .0001$) (figure 1A). No difference in *GNB1L* expression was observed between the Japanese and Australian schizophrenic patient groups (data not shown). *GNB1L* expression was not significantly correlated with pH of the brain tissue samples overall (figure 2), neither with gender ($P = .62$) nor PMI ($F = 0.61$, $P = .44$). Western blotting analysis also demonstrated the lower levels of GNB1L protein in brains of the schizophrenia sample than in those of the control sample from each ethnic group (approximate average ratio = 0.75, $P = .027$ in Australian sample and approximate average ratio = 0.69, $P = .033$ in Japanese sample) (figure 1B). There is a significant correlation between gene and protein expression observed in our samples ($F = 4.7$, $P = .037$).

There were no significant associations of tag SNPs at the *GNB1L* gene studied in the present study with schizophrenia in our Japanese case-control sample (table 1). Also no significant differences were found in distributions of homozygotes and heterozygotes between schizophrenics and controls (table 1). Williams et al[26] reported male-specific associations of rs5746832 and rs2269726 with schizophrenia and correlation between those markers and the gene expression. However, such male-specific associations of rs5746832 and rs2269726 were not observed in our sample (table 1).

There was a nominally significant correlation between rs5748832 and *GNB1L* expressions in whole subjects ($P = .014$) and in Japanese ($P = .028$), but not in Australian ($P = .66$) (table 2). An allele of rs5748832 is correlated with high *GNB1L* expression in this study, while the previous study showed the opposite direction of correlation.[26]

Significant deviation from HWE in the genotypic distributions was observed at rs4819523 in the control group. Lower proportions of heterozygotes than those expected by HWE seemed to cause these deviations. Although genotype errors, chance findings, or actual

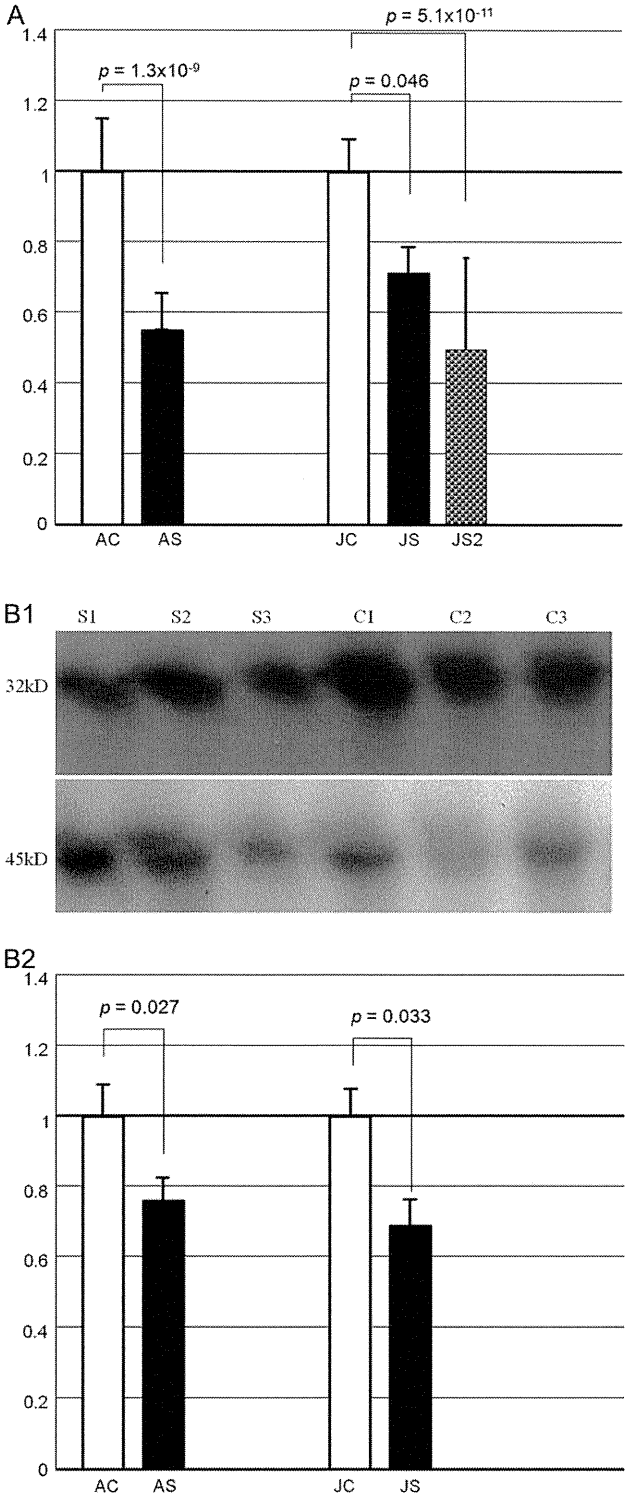

Fig. 1. GNB1L expression in schizophrenic brain (A). Relative expression of the *GNB1L* gene in prefrontal cortex from Australian control subjects (AC, n = 10), Australian schizophrenics (AS, n = 10), Japanese controls (JC, n = 11), Japanese schizophrenics (JS, n = 6), and additional Japanese schizophrenics (JS2, n = 37). The vertical scores show average of relative expression and ±1 SD in comparison with control subjects in each ethnic population, respectively. (B-1) A partial result of Western blotting was shown. Upper: GNB1L (Although expected size would be 35 kD, bands are expressed at 32 kD according to the antibody protocol) Lower: beta actin (45 kD). Samples S1–S3 are from schizophrenic patients and C1–C3 are from controls. (B-2) Relative expression of the GNB1L protein in prefrontal cortex from Australian control subjects (AC, n = 10), Australian schizophrenics (AS, n = 10), Japanese controls (JC, n = 11), and Japanese schizophrenics (JS, n = 6). The vertical scores show average of relative expression and ±1 SD in comparison with control subjects in each ethnic population, respectively.

structural variations in some subjects might have potentially caused these deviations, we could not determine which was most likely to cause these HWE deviations.

Gnb1l expression in mice was examined to exclude the possibility that reduced *GNB1L* expression was the effects of chronic treatment with antipsychotic drugs. The patients whose brains were examined in the present study had received long-term medication of typical antipsychotic drugs; therefore, we chose haloperidol as a representative antipsychotic drug. As a result, while *Gnb1l* gene expression in prefrontal cortex of mice treated with haloperidol for 4 weeks was not changed, the expression was higher in those treated with haloperidol for 50 weeks than in those with saline injected (P = .02) as shown in figure 3.

Discussion

In the present study, we hypothesized that haploinsufficiency of some genes in the 22q11DS region might increase the susceptibility to schizophrenia not only in patients with 22q11DS but also in the those without 22q11DS and that such genes would be expressed at lower levels in the brains of schizophrenic patients than in control subjects. *GNB1L* appears to meet this hypothesis. Reduced *GNB1L* gene expression was detected in both mRNA and protein levels in Australian and Japanese subjects, suggesting that lower *GNB1L* gene expression produces lower GNB1L protein levels which underlie schizophrenia across ethnicities. Treatment of mice with haloperidol indicated that the reduction of *GNB1L* expression is not likely a consequence of antipsychotic medication treatment, though the possibility of reduction of *GNB1L* expression by other antipsychotic drugs remains. The present study did not provide evidence of whether *TBX1* expression is altered significantly in schizophrenic brains because the signals detected by Illumina's Sentrix® Human-6 Expression BeadChip or TaqMan assay were very weak. Paylor et al[9] mapped PPI deficits in a panel of mouse mutants and found that PPI was impaired by either haploinsufficiency of *Tbx1* or *Gnb1l*. The present study of human brains confirms that *GNB1L* is an important candidate for susceptibility to schizophrenia.

There is little information about the function of GNB1L. *GNB1L* expression is relatively low in adult brain but is high in fetal brain. *GNB1L* encodes a guanine

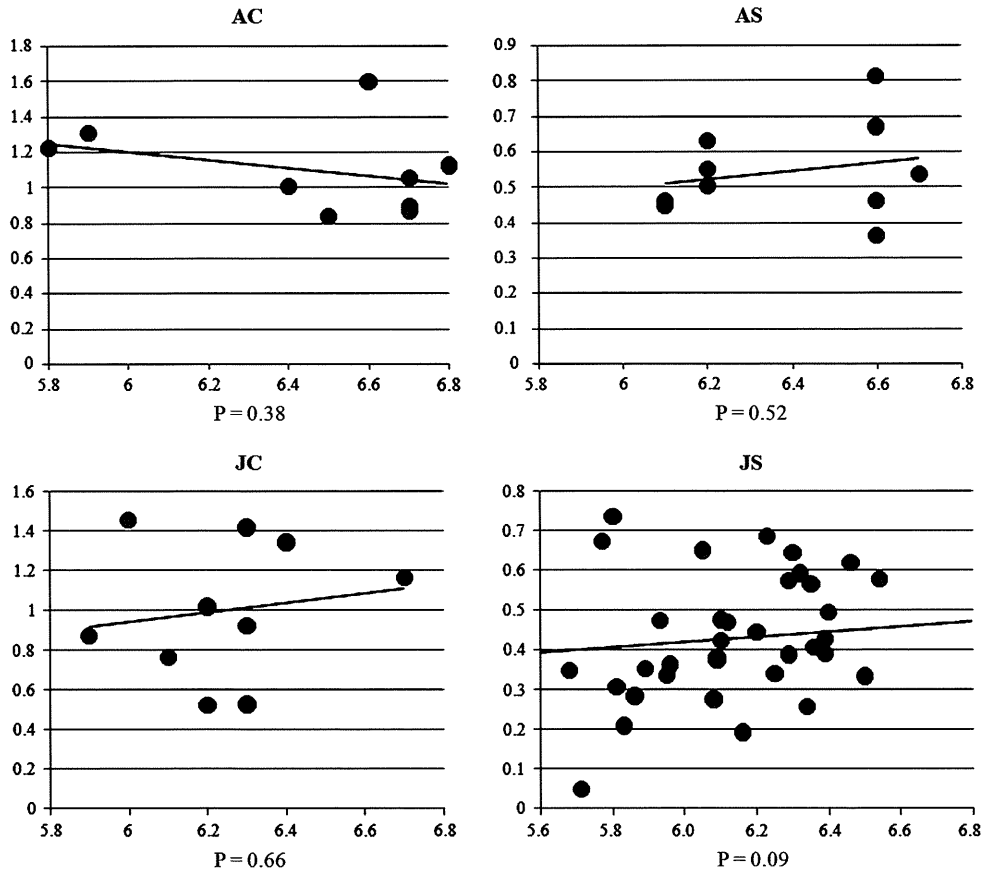

Fig. 2. *GNB1L* expression and pH in human postmortem brain Correlation of *GNB1L* expression and pH of the human postmortem brain subjects used in the same experiments shown in figure 1; AC, Australian controls; AS, Australian schizophrenics; JC, Japanese controls; JS, Japanese schizophrenics; JS2, additional Japanese schizophrenic samples. The vertical scale shows relative *GNB1L* expression and horizontal scale shows pH. Statistical *P* values are calculated below each graph.

nucleotide–binding protein (G protein), beta polypeptide 1–like, which is a member of the WD repeat protein family. WD repeats are minimally conserved regions of approximately 40 amino acids typically bracketed by Gly-His and Trp-Asp (GH-WD) that may facilitate formation of heterotrimeric or multiprotein complexes. Members of this family are involved in a variety of cellular processes, including cell cycle progression, signal transduction, apoptosis, and gene regulation. GNB1L contains 6 WD repeats.[32] GNB1L shows homology to the human guanine nucleotide–binding protein β subunit (GNB1). GNB1 functions in G-protein–coupled receptor protein signaling pathways and intracellular signaling cascade.

Williams et al[26] reported excess homozygosity at rs5746832 and rs2269726 in male schizophrenia subjects and that the markers associated with male schizophrenia were related with cis-acting changes in *GNB1L* expression. Firstly in the present study, we failed to confirm the association in our Japanese case-control population. Secondly, we found a nominally significant correlation between rs5746832 and *GNB1L* expression in the Japanese brain samples, but failed to find it in our limited number of the Australian samples. Further, the association between allele and gene expression in our Japanese samples was in the opposite direction from that reported in the Caucasian samples. It might be due to possible differences in LD block between haplotype phases across rs5746832 and harboring potential cis-acting variations of the gene between 2 ethnic populations. Even if such cis-acting variations are present, diagnosis has tremendous effect on the gene expression, in comparison to that of the SNP. The power of the present study to replicate the findings of excess homozygosity in male subjects is greater than 90% assuming the odd ratio of greater than 1.5 found in UK populations by Williams et al.[26] However, if the odd ratio assumes 1.3 observed in a German population by them, the power drops to 0.65. Although the gene frequencies of rs5746832 and rs2269726 were significantly different between Caucasian and Japanese populations, the frequencies of homozygotes were almost the same between 2 populations. Because of small sample size, we did not attempt allele-specific expression analysis in our brain sample. Therefore, we could not conclude whether lower GNB1L gene/protein expression in schizophrenia was

Table 1. . Analysis of Tag Single-Nucleotide Polymorphisms at the *GNB1L* Gene in the Japanese Case-Control Population

Population		Genotype count (frequency)			HWE *P*	*P*	Allele count (frequency)		*P*	Homozygote	Heterozygote	*P*
rs5746832		AA	AG	GG			A	G				
Affected	*n* = 1889	501 (0.27)	958 (0.51)	430 (0.23)	.49		1960 (0.52)	1818 (0.48)		931 (0.49)	958 (0.51)	
Male only	*n* = 1008	256 (0.25)	531 (0.53)	221 (0.22)	.08					477 (0.47)	531 (0.53)	
Controls	*n* = 1876	484 (0.26)	916 (0.49)	476 (0.25)	.31	.17	1884 (0.50)	1868 (0.50)	.14	960 (0.51)	916 (0.49)	.24
Male only	*n* = 1047	260 (0.25)	524 (0.50)	263 (0.25)	.97	.23				523 (0.50)	524 (0.50)	.23
rs5746834		GG	GT	TT			G	T				
Affected	*n* = 1898	1652 (0.87)	234 (0.12)	12 (0.01)	.24		3538 (0.93)	258 (0.07)		1664 (0.88)	234 (0.12)	
Controls	*n* = 1893	1653 (0.87)	228 (0.12)	12 (0.01)	.18	.96	3534 (0.93)	252 (0.07)	.81	1665 (0.88)	228 (0.12)	.77
rs2269726		TT	TC	CC			T	C				
Affected	*n* = 1905	338 (0.18)	896 (0.47)	671 (0.35)	.20		1572 (0.41)	2238 (0.59)		1009 (0.53)	896 (0.47)	
Male only	*n* = 1050	176 (0.17)	51 (0.49)	363 (0.35)	.87					539 (0.51)	511 (0.49)	
Controls	*n* = 1906	309 (0.16)	911 (0.48)	686 (0.36)	.82	.45	1529 (0.40)	2283 (0.60)	.31	995 (0.52)	911 (0.48)	.65
Male only	*n* = 1042	174 (0.17)	49 (0.48)	370 (0.36)	.77	.90				544 (0.52)	498 (0.48)	.69
rs748806		TT	TC	CC			T	C				
Affected	*n* = 1888	537 (0.28)	919 (0.49)	432 (0.23)	.31		1993 (0.53)	1783 (0.47)		969 (0.52)	919 (0.48)	
Controls	*n* = 1895	526 (0.28)	911 (0.48)	458 (0.24)	.10	.64	1963 (0.52)	1827 (0.48)	.39	984 (0.51)	911 (0.49)	.72
rs29807124		CC	CT	TT			A	G				
Affected	*n* = 1872	1688 (0.90)	177 (0.09)	7 (0.00)	.31		3553 (0.95)	191 (0.05)		1695 (0.91)	177 (0.09)	
Controls	*n* = 1873	1693 (0.90)	174 (0.09)	6 (0.00)	.50	.95	3560 (0.95)	186 (0.05)	.79	1699 (0.90)	174 (0.10)	.82
rs5993835		AA	AG	GG			A	G				
Affected	*n* = 1881	1474 (0.78)	374 (0.20)	33 (0.02)	.10		3322 (0.88)	440 (0.12)		1507 (0.80)	374 (0.20)	
Controls	*n* = 1871	1484 (0.79)	365 (0.20)	22 (0.01)	.93	.31	3333 (0.89)	409 (0.11)	.30	1506 (0.80)	365 (0.20)	.77
rs13057609		AA	AG	GG			A	G				
Affected	*n* = 1888	13 (0.01)	271 (0.14)	1604 (0.85)	.68		297 (0.08)	3479 (0.92)		1617 (0.86)	271 (0.14)	
Controls	*n* = 1883	10 (0.01)	272 (0.14)	1601 (0.85)	.67	.82	292 (0.08)	3474 (0.92)	.86	1611 (0.86)	272 (0.14)	.93
rs4819523		GG	GC	CC			G	C				
Affected	*n* = 1886	536 (0.28)	932 (0.49)	418 (0.22)	.74		2004 (0.53)	1768 (0.47)		954 (0.51)	932 (0.49)	
Controls	*n* = 1896	562 (0.30)	887 (0.47)	447 (0.24)	.008	.26	2011 (0.53)	1781 (0.47)	.90	1009 (0.53)	887 (0.47)	.10
rs2073765		CC	CT	TT			C	T				
Affected	*n* = 1887	90 (0.05)	586 (0.31)	1211 (0.64)	.08		766 (0.20)	3008 (0.80)		1301 (0.69)	586 (0.31)	
Controls	*n* = 1895	76 (0.04)	590 (0.31)	1229 (0.65)	.62	.52	742 (0.20)	3048 (0.80)	.47	1305 (0.69)	590 (0.31)	.97
rs7286924		AA	AT	TT			A	T				
Affected	*n* = 1892	808 (0.43)	848 (0.45)	236 (0.12)	.56		2464 (0.65)	1320 (0.35)		1044 (0.55)	848 (0.45)	
Controls	*n* = 1898	800 (0.42)	872 (0.46)	226 (0.12)	.62	.75	2472 (0.65)	1324 (0.35)	.98	1026 (0.54)	872 (0.46)	.47
rs10372		AA	AG	GG			A	G				
Affected	*n* = 1902	10 (0.01)	244 (0.13)	1648 (0.87)	.77		264 (0.07)	3540 (0.93)		1658 (0.87)	244 (0.13)	
Controls	*n* = 1904	6 (0.00)	244 (0.13)	1654 (0.87)	.34	.60	256 (0.07)	3552 (0.93)	.65	1660 (0.87)	244 (0.13)	.96
rs3788304		CC	CG	GG			C	G				
Affected	*n* = 1907	1056 (0.55)	720 (0.38)	131 (0.07)	.58		2832 (0.74)	982 (0.26)		1187 (0.62)	720 (0.38)	
Controls	*n* = 1906	1058 (0.56)	720 (0.38)	128 (0.07)	.71	.98	2836 (0.74)	976 (0.26)	.81	1186 (0.62)	720 (0.38)	.97
rs11704083		AA	AG	GG			A	G				
Affected	*n* = 1909	660 (0.35)	915 (0.48)	334 (0.17)	.58		2235 (0.59)	1583 (0.41)		994 (0.52)	915 (0.48)	
Controls	*n* = 1897	646 (0.34)	931 (0.49)	320 (0.17)	.62	.76	2223 (0.59)	1571 (0.41)	.92	966 (0.51)	931 (0.49)	.50

Table 2. Correlation Between Genotype and *GNBIL* Gene Expression in Brain

SNP	Genotype	*n*	Expression	Genotype	*n*	Expression	Genotype	*n*	Expression	*P* value
rs5746832	AA	19	0.82	AG	16	0.63	GG	21	0.56	.014
Australian	AA	8	0.90	AG	7	0.75	GG	4	0.84	.660
Japanese	AA	11	0.77	AG	9	0.54	GG	17	0.50	.028
rs5746834	GG	46	0.68	GT	11	0.52	TT	2	0.71	.391
rs2269726	TT	22	0.58	TC	20	0.72	CC	15	0.72	.224
rs748806	TT	15	0.84	TC	15	0.55	CC	32	0.63	.105
rs29807124	CC	47	0.68	CT	7	0.85	TT	3	0.29	.063
rs5993835	AA	53	0.61	AG	6	0.62	GG	0	NA	.794
rs 13057609	AA	0	NA	AG	7	0.57	GG	54	0.67	.479
rs4819523	GG	14	0.66	GC	26	0.61	CC	19	0.72	.601
rs2073765	CC	4	0.89	CT	17	0.48	TT	38	0.70	.520
rs7286924	AA	33	0.64	AT	21	0.77	TT	9	0.49	.112
rslO372	AA	0	NA	AG	8	0.67	GG	52	0.52	.261
rs3788304	CC	37	0.65	CG	21	0.64	GG	5	0.73	.731
rs11704083	AA	22	0.75	AG	23	0.56	GG	17	0.67	.412

due to cis-acting differences by genetic polymorphisms in this locus or not in this study.

The present study showed that reduced expression of *GNB1L* may be involved in the pathophysiology of schizophrenia; however, it does not exclude the possibility that other genes in the 22q11DS region contribute to the susceptibility to schizophrenia. The array used in the present study did not examine all isoforms of the genes in the 22q11DS region. In addition, the reliability of weakly expressed sequences in the array screening is not sufficient. Therefore, we reexamined expression levels of the genes, which reliable data (greater than 0.96 confidence) was produced by the array in no subjects, by real-time PCR method. The study is also limited by the areas and ages of the brains examined. We examined only adult postmortem prefrontal cortex. Differential gene expression in other brain regions or during other developmental stages may also influence the susceptibility to schizophrenia.

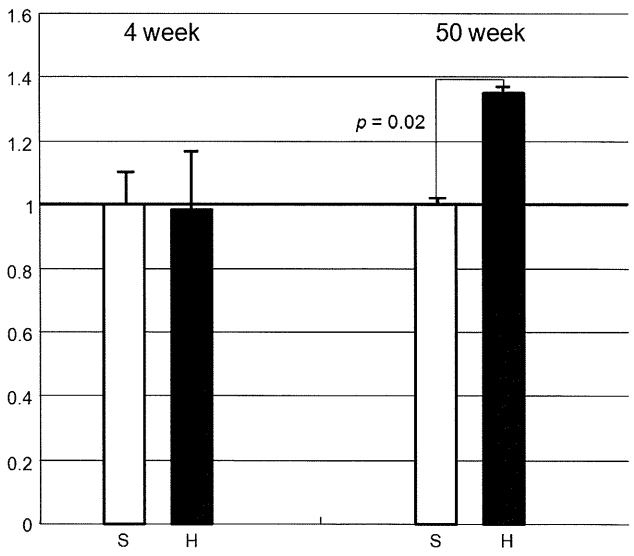

Fig. 3. Effect of haloperidol treatment on *Gnb1l* expression. Relative expression of the *Gnb1l* gene in mouse prefrontal cortex in saline treated (S) or haloperidol treated (H) mice during 4 or 50 weeks. The vertical scale shows relative *Gnb1l* expression compared with that in saline-treated mice, with bars for ±1 SD calculated in each group, respectively.

The consortium data of the Stanley Medical Research Institute showed no significant differences ($P > .05$) in the following gene expression levels in postmortem prefrontal cortex between patients with schizophrenia and controls: *DGCR6*, *PRODH*, *DGCR2*, *STK22B*, *DGCR14*, *CLTCL1*, *CLTCL1*, *HIRA*, *UFD1L*, *CDC45L*, *CLDN5*, *TBX1*, *FLJ21125*, *TXNRD2*, *COMT*, *ARVCF*, *DKFZp761P1121*, *DGCR8*, *HTF9C*, *RANBP1*, and *ZDHHC8*. The expression of *RTN4R* might be potentially reduced ($P = .02$). No data were available for *GSCL*, *MRPL40*, *SEPT5*, *GP1BB*, and *GNB1L* (http://www.stanleyresearch.org/brain/menu.asp).

A trans-acting effect on expression of the disease gene may also be expected to modulate disease susceptibility. Large-scale studies in humans have indicated that a significant proportion of the heritable variance in gene expression is attributable to trans-acting polymorphism.[33,34] As one of the examples, recent study reported that microRNAs regulate gene expression posttranscriptionally.[35] Even for schizophrenia, Bray et al[36] indicated that the reduction in *DTNBP1* expression in schizophrenia is likely to result in part from trans-acting risk factors. Such

trans-acting factors that regulate *GNB1L* gene expression, however, have not been identified.

In conclusion, the present study further supports the role of *GNB1L* in the pathophysiology of schizophrenia.

Supplementary Material

Supplementary tables are available at http://schizophreniabulletin.oxfordjournals.org/.

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Brain Cannabinoid CB2 Receptor in Schizophrenia

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Background: Neural endocannabinoid function appears to be involved in schizophrenia. Two endocannabinoid receptors, CB1 and CB2, are found in the brain and elsewhere in the body. We investigated roles of CB2 in schizophrenia.

Materials and Methods: An association study was performed between tag single nucleotide polymorphisms (SNPs) in the *CNR2* gene encoding the CB2 receptor and schizophrenia in two independent case-control populations. Allelic differences of associated SNPs were analyzed in human postmortem brain tissues and in cultured cells. Prepulse inhibition and locomotor activity in C57BL/6JJmsSlc mice with CB2 receptor antagonist AM630 administration was examined.

Results: The analysis in the first population revealed nominally significant associations between schizophrenia and two SNPs, and the associations were replicated in the second population. The R63 allele of rs2501432 (R63Q) ($p = .001$), the C allele of rs12744386 ($p = .005$) and the haplotype of the R63-C allele ($p = 5 \times 10^{-6}$) were significantly increased among 1920 patients with schizophrenia compared with 1920 control subjects in the combined population. A significantly lower response to CB2 ligands in cultured CHO cells transfected with the R63 allele compared with those with Q63, and significantly lower CB2 receptor mRNA and protein levels found in human brain with the CC and CT genotypes of rs12744386 compared with TT genotype were observed. AM630 exacerbated MK-801- or methamphetamine-induced disturbance of prepulse inhibition and hyperactivity in C57BL/6JJmsSlc mice.

Conclusions: These findings indicate an increased risk of schizophrenia for people with low CB2 receptor function.

Key Words: Association, cAMP, cannabinoid, G protein coupled receptor, gene, methamphetamine, MK-801, mouse, postmortem brain, schizophrenia

Tetrahydrocannabinol (THC) in marijuana acts on cannabinoid receptors, and natural endocannabinoids are important regulators of various aspects of psychobehavioral, immunologic, and metabolic functions. Many relationships between marijuana use and schizophrenia have been reported: a dose-response relationship has been found between the amount of cannabis used in adolescence and the subsequent risk of developing schizophrenia (1,2), more psychotic symptoms are experienced by schizophrenic patients who use cannabis (3), schizophrenia-like symptoms can occur in nonschizophrenic people after cannabis use (4), and there is a decrease in gray matter density in the right posterior cingulate cortex in first-episode schizophrenics who use cannabis compared with those who do not use cannabis (5). Further, biological phenomena related to endogenous cannabinoids such as significantly higher amounts of the endocannabinoid anandamide in the blood occur more frequently in patients with acute schizophrenia than in healthy volunteers (6), and significantly higher levels of anandamide are detected in the cerebrospinal fluid (CSF) of first-episode schizophrenic patients than that of healthy volunteers (7,8).

Endogenous cannabinoids bind to and activate two G protein-coupled receptors, the predominantly central cannabinoid receptor type 1 (CB1) and predominantly peripherally expressing cannabinoid receptor type 2 (CB2). CB1 receptor has mainly been investigated in the endogenous cannabinoid system in the central nervous system (CNS) (9–11). In schizophrenic patients, the cannabinoid CB1 receptor agonist CP-55940 disrupts sensory gating and neuronal oscillation (12). Some studies have also suggested an association between microsatellite and single nucleotide polymorphism (SNP) genetic markers in the *CNR1* gene (encoding the CB1 receptor) and the incidence of schizophrenia (13–16). However, neither nonsynonymous polymorphisms nor polymorphisms in the *CNR1* gene that influence the full-length *CNR1* transcript expression have been reported.

In addition to human studies, prepulse inhibition (PPI) is frequently used in pharmacobehavioral studies of animal models. Prepulse inhibition refers to the reduction in amplitude of the

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startle reflex that occurs when a brief, subthreshold stimulus immediately precedes a startle stimulus (17). Deficits in PPI are observed in several psychiatric disorders, particularly in schizophrenia (18), and it has been postulated that this impairment of sensorimotor gating reflects at least some portion of the cognitive dysfunction observed in patients with schizophrenia (19,20). In relation to the cannabinoid system, Long *et al.* (21) reported that cannabidiol reverses MK-801-induced disruption of PPI in mice.

Recently, attention has been drawn to the expression of the CB2 receptor in the CNS (22–24). This receptor has been reported to be involved in alcohol preference in mice and in human alcoholism (25). Clinical remission of schizophrenia has been reported to be accompanied by significant decreases in anandamide and *CNR2* mRNA levels, which encode the CB2 receptor, in peripheral blood mononuclear cells (6). Thus, CB2 receptors may play a role in psychiatric disorders.

In this study, we investigated genetic associations between *CNR2* gene polymorphisms and schizophrenia and functions of potentially associated SNPs in cultured cells and human postmortem brain. The effects of CB2 receptor inverse agonist on mouse behavior were also investigated.

Materials and Methods

Participants in the Association Study

Consensual diagnosis of schizophrenia was made according to the DSM-IV. Control subjects had no history of mental illness, and second-degree relatives were free of psychosis in a brief psychiatric interview. The first screening group of subjects (East Japan) comprised 1152 unrelated Japanese patients with schizophrenia and 1194 control subjects. The second group of subjects (West Japan) for replication analysis comprised 768 unrelated Japanese patients with schizophrenia and 726 control subjects (see details in Methods and Materials section of Supplement 1). HapMap Yoruba subjects were also genotyped because the allele frequency of rs2501432 was not known.

SNP Analysis

Five tag SNPs [rs9424339, rs2502959, rs2501432 (R63Q), rs2229579 (H316T), and rs12744386] were selected using the Haploview version 3.32 software (http://www.broad.mit.edu/mpg/haploview/) from the Japanese subject data in the in the HapMap database (Figure 1). These SNPs contain two common nonsynonymous polymorphisms, and rs12744386, which was associated with different *CNR2* gene expression by *cis*-acting fashion (C allele, effect = −.490, H^2 = 11.64, logarithm of odds (LOD) = 8.819) in lymphoblast cells, as listed in the SNP browser 1.01 database (26) (Figure 2A).

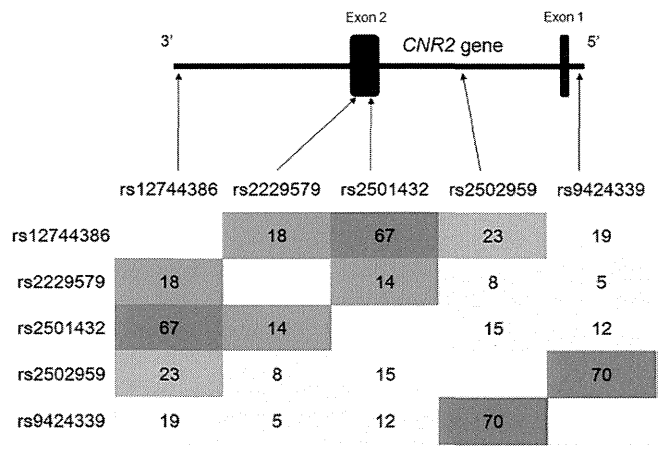

Figure 1. *CNR2* tag single nucleotide polymorphisms and the genetic structure. The genetic structure of *CNR2* is shown at top; the gene consists of two exons, and only the second exon encodes amino acids. Location and linkage disequilibrium (r^2 values) of five single nucleotide polymorphisms, including two functional polymorphisms, are shown.

Genotypes were determined using TaqMan SNP genotyping assay (Applied Biosystems, Foster City, California) or by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method (see details in Methods and Materials of Supplement 1).

Human Postmortem Brains

Brain tissue of gray matter in an area of the prefrontal cortex (Brodmann's area 9 [BA9]) was received from two tissue banks and one research institute (Table S1 and Methods and Materials in Supplement 1).

CNR2 Gene and Protein Expression Analysis

Expression of the *CNR2* gene was analyzed using the TaqMan Realtime PCR system with the TaqMan gene expression assays Hs00361490-m1. TaqMan *GAPDH* Control Reagent and TaqMan β-actin Control Reagents were used to normalize the data, respectively (Applied Biosystems). Expression of the CNR2 protein was analyzed with the Western blot method. Human CB2 Receptor Polyclonal Antibody (1:1000 dilution) (#101550, Lot. 165113, Cayman Chemical, Ann Arbor, Michigan) was used as the primary antibody, or polyclonal antibody to beta-Actin (1: 500 dilution; IMG-5142A, Lot. 03,231,897B-04, Imgenex, San Diego, California), for normalization (see details in Methods and Materials of Supplement 1).

Functional Analysis of the CNR2 Gene in Comparison Between R63 and Q63

CB2 receptors with either the *CNR2* R63 or Q63 allele were expressed in cultured CHO cells (see details in Methods and Materials of Supplement 1). One millimolar forskolin as final concentration was added to the culture medium for 15 min, and then the endocannabinoid 2-arachidonoylglycerol (2-AG; 1 μmol/L, final concentration; Cayman Chemical), CB2 selective agonist JWH-015 (50 μmol/L, final concentration; Cayman Chemical); or CB2 selective inverse agonist AM630 (100 μmol/L, final concentration) (Tocris Bioscience, Ellisville, Missouri) was administered to the cells. cAMP level was measured 15 min after the administration using the camp-Screen System (Applied Biosystems, Bedford, MA). Effects of those ligands on the cyclic adenosine monophosphate (cAMP) levels were examined in each allele type of CB2 receptor expressed CHO cells (see details in Methods and Materials of Supplement 1). Because the CB2 receptor is a Gi-type G protein-coupled receptor, decreased or increased levels of cAMP were considered the proper response to the agonists or inverse agonists, respectively.

Prepulse Inhibition and Locomotor Tests in Mice

Effects of pretreatment with CB2 ligand AM630 (3 mg/kg or 30 mg/kg intraperitoneally) on drug (either MK-801 or methamphetamine)-induced prepulse inhibition (PPI) and locomotor activity were evaluated in C57BL/6JJmsSlc male mice (Japan SLC, Shizuoka, Japan). Experimental procedures are described in Methods and Materials of Supplement 1.

All animal procedures were performed according to protocols approved by the Animal Care and Use Committee of the University of Tsukuba.

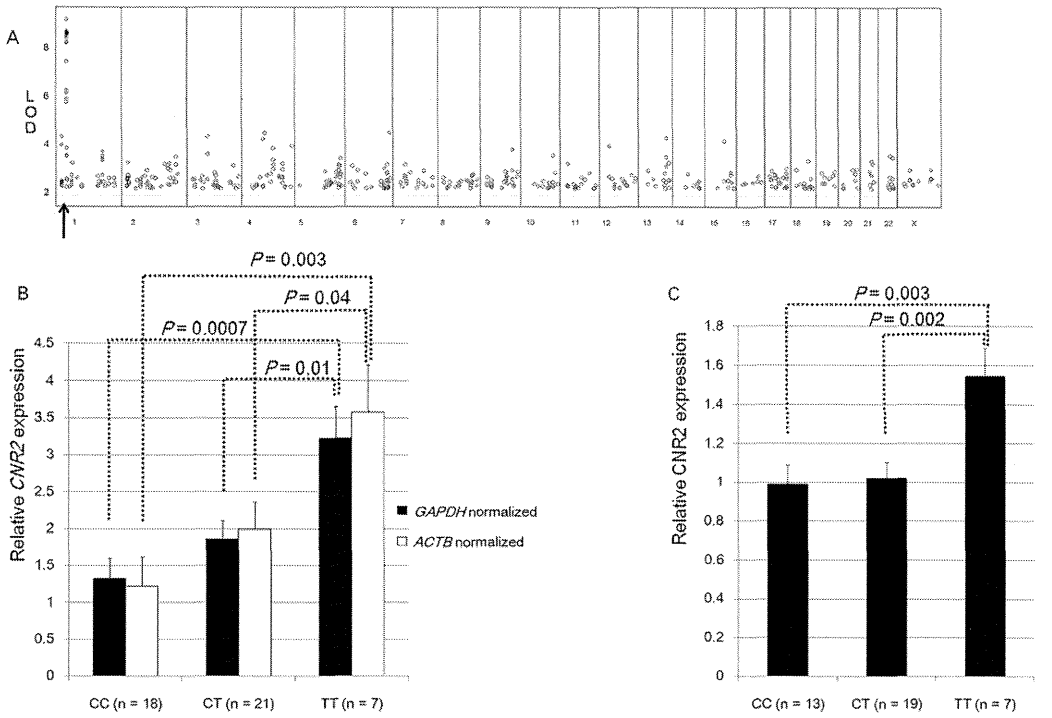

Figure 2. Analysis of cannabinoid CB2 receptor expression. **(A)** A *cis*-acting gene expression change of *CNR2* in lymphoblasts. Results of the search for polymorphisms in the *CNR2* gene locus from the mRNA by single nucleotide polymorphism browser (http://www.sph.umich.edu/csg/liang/asthma/). The genetic markers indicated by the red arrow are correlated with *CNR2* expression in lymphoblasts. One of the strongest effects on *CNR2* gene expression was induced by rs12744386. **(B)** Correlation between *CNR2* gene expression and rs12744386 genotype in the human postmortem brain. The vertical axis shows the relative *CNR2* expression level in each of the three genotype groups, compared with mean gene expression in the total samples. **(C)** Correlation between CNR2 protein expression and rs12744386 genotype in human postmortem brain. The vertical axis shows the relative CNR2 protein expression in each of the three genotype groups, compared with mean gene expression in the total samples. Five subjects with the CC genotype and two subjects with the CT genotype were not available for the analysis. LOD, logarithm of odds.

Statistics

Deviation from predicted Hardy-Weinberg frequencies, allelic associations, and linkage disequilibrium (LD) between SNPs were evaluated with Haploview software, version 3.11. Because two-stage analyses were performed for genetic associations, SNPs showing nominally significant allelic association ($p < .05$) were subsequently analyzed in an independent population. A significant association was defined when the given p value for allelic tests was less than 5% after permutation analysis in the second and combined populations. Haplotype associations were also evaluated by permutation analysis using Haploview software version 3.11. The global haplotype association was tested with COCAPHASE from the UNPHASED software 2.403 (27).

The relationship between *CNR2* gene expression levels and diagnosis of schizophrenia was analyzed by t test. Relationships between *CNR2* gene expression or protein expression levels and rs12744386 genotypes were analyzed using one-way analysis of variance (ANOVA), followed by post hoc analysis using a Student's t test. The correlation between *CNR2* gene expression and CNR2 protein expression levels was analyzed by linear regression analysis. The correlation between *CNR2* expression levels and either postmortem interval (PMI) or pH in brain tissues was assessed by linear regression analysis. The cAMP levels detected by enzyme-linked immunosorbent assay after treatment with CB2 ligands were analyzed between CHO cells transfected with each allelic type of CB2 receptor by ANOVA, followed by post hoc analysis using a Student's t test. Effects of drug treatment, prepulse, and the interaction on percent PPI were analyzed by ANOVA, followed by post hoc analysis using a Student's t test to evaluate effect of AM630 pretreatments with two doses on either MK-801 or methamphetamine induced PPI, respectively (Bonferroni correction for two comparisons). The effect was then analyzed at each prepulse intensity. Effects of drug treatment, AM630 pretreatments, and the interaction on locomotor activity were analyzed by ANOVA, followed by post hoc analysis using a Student's t test to evaluate effect of two doses of AM630 pretreatments (Bonferroni correction for two comparisons). ANOVA and Student's t tests were carried out using JMP software version 5.1 (SAS Institute, Japan).

Results

Table 1 shows the distribution of the genotypes of the tag SNPs in the *CNR2* gene region, and Figure 1 shows the LD pattern within those tag SNPs. None of the SNPs deviated significantly from the expected Hardy-Weinberg equilibrium. Nominally, significant differences were found in allele frequencies of rs12744386 ($p = .05$, odds ratio [OR] = 1.13 with 95% confidence interval [CI]: 1.00–1.27) and rs2501432 (R63Q) ($p = .003$, OR = 1.19, 95% CI: 1.06–1.34) between schizophrenic and control subjects from east Japan (Table 1). The significant differences were replicated in subjects from west Japan; rs12744386 (OR = 1.19, 95% CI: 1.03–1.38, permutation $p = .04$) and rs2501432 (R63Q) (OR = 1.19, 95% CI: 1.02–1.37, permutation $p = .04$) between schizophrenic and control subjects from west Japan (Table 1).

In the combined populations, the distributions of allele frequencies between schizophrenic and control subjects were

Table 1. Genotype and Allele Distributions of tag Single Nucleotide Polymorphisms (SNPs) in the *CNR2* in the Case Control Subjects

SNP	Samples (*n*)	Genotype Count (Frequency)						*p*	Allele Count (Frequency)				*p*
First Screening													
rs12744386	East Japan	CC		CT		TT			C		T		
Schizophrenic	1146	448	39%	537	47%	161	14%		1433	63%	859	37%	
Control	1185	420	35%	575	49%	190	16%	.14	1415	60%	955	40%	.049
rs2229579	East Japan	AA		AG		GG			A		G		
Schizophrenic	1147	34	3%	292	25%	821	72%		360	16%	1934	84%	
Control	1171	27	2%	274	23%	870	74%	.28	328	14%	2014	86%	.11
rs2501432	East Japan	GG/GG (R/R)		GG/AA (R/Q)		AA/AA (Q/Q)			GG (Arg)		AA (Gln)		
Schizophrenic	1111	432	39%	482	43%	197	18%		1346	61%	876	39%	
Control	1170	374	32%	569	49%	227	19%	.0025	1317	56%	1023	44%	.003
rs2502959	East Japan	AA		AC		CC			A		C		
Schizophrenic	1149	119	10%	536	47%	494	43%		774	34%	1524	66%	
Control	1183	108	9%	535	45%	540	46%	.35	751	32%	1615	68%	.16
rs9424339	East Japan	AA		AT		TT			A		T		
Schizophrenic	1147	131	11%	537	47%	479	42%		799	35%	1495	65%	
Control	1181	129	11%	525	44%	527	45%	.38	783	33%	1579	67%	.23
Replication Study													
rs12744386	West Japan	CC		CT		TT			C		T		
Schizophrenic	761	304	40%	355	47%	102	13%		963	63%	559	37%	
Control	722	252	35%	349	48%	121	17%	.064	853	59%	591	41%	.019
rs2501432	West Japan	GG/GG (R/R)		GG/AA (R/Q)		AA/AA (Q/Q)			GG (Arg)		AA (Gln)		
Schizophrenic	745	248	33%	370	50%	127	17%		866	58%	624	42%	
Control	698	217	31%	319	46%	162	23%	.014	753	54%	643	46%	.024
Combined													
rs12744386	Total	CC		CT		TT			C		T		
Schizophrenic	1907	752	39%	892	47%	263	13%		2396	63%	1418	37%	
Control	1908	673	35%	924	48%	311	16%	.013	2270	59%	1546	41%	.0028
rs2501432	Total	GG/GG (R/R)		GG/AA (R/Q)		AA/AA (Q/Q)			GG (Arg)		AA (Gln)		
Schizophrenic	1859	681	37%	852	46%	326	18%		2214	60%	1504	40%	
Control	1876	591	32%	890	47%	395	21%	.0011	2072	55%	1680	45%	.00015

significantly different for rs12744386 (OR = 1.15, 95% CI: 1.05–1.26, permutation $p = .005$) and rs2501432 (R63Q) (OR = 1.19, 95% CI: 1.09–1.31, permutation $p = .001$) (Table 1). Because of the relatively strong LD between rs12744386 and rs2501432 ($r^2 = .67$), the haplotype was constructed with these two SNPs to examine the association between the haplotype and schizophrenia. As a result, the haplotype of the C allele of rs12744386 and R63 allele showed a strong association with schizophrenia in our Japanese subjects (OR = 1.26, 95% CI: 1.15–1.37, permutation $P = 5 \times 10^{-6}$), which was also observed independently in two groups of subjects (Table 2). The global haplotype association p value was 5.1×10^{-9}.

rs12744386 was suggested to be associated with *CNR2* mRNA levels in lymphoblastoid cell lines by SNP Browser (allele *C*: Effect = −.49, H2 = 11.64, LOD = 8.819; Figure 2A). This association was confirmed in postmortem prefrontal brain samples. The rs12744386 genotypes were associated with the *CNR2* mRNA levels in the brain [*GAPDH*-normalized, $F(2,45) = 6.7$, $p = .003$; *ACTB*-normalized, $F(2,45) = 4.9$, $p = .01$; Figure 2B], and with the CNR2 protein levels in the brain [$F(2,38) = 6.3$, $p = .005$; Figure 2C] with the lowest expression in the CC genotype and the highest expression in the TT genotype. Post hoc analysis showed a significant difference between TT and other genotypes (Figure 2B). There was a significant correlation between results for gene expression normalized by two internal controls, *GAPDH* and *ACTB* [$r^2 = .67$, $F(1,45) = 87.83$, $p < .0001$]. There was also significant correlation between results for gene expression normalized by *ACTB* and protein expression normalized by ACTB [$r^2 = .12$, $F(1,33) = 4.2$, $p = .05$]. There were no significant correlations between PMI and *CNR2* expression [*GAPDH*-normalized, $F(1,44) = .6$, NS; β-*actin*-normalized, $F(1,44) = .7$, ns]; pH and *CNR2* expression [*GAPDH*-normalized, $F(1,43) = .4$, NS, *ACTB*-normalized, F(1.43) = .05, ns]; or diagnosis of schizophrenia and *CNR2* expression [*GAPDH*-normalized, $F(1,45) = .4$, ns, *ACTB*-normalized, $F(1,45) = 1.4$, NS].

Regarding the rs2501432 (R63Q) polymorphism, residue 63 of the CB2 receptor protein of the mouse (*Mus musculus*), rat (*Rattus norvegicus*), rhesus monkey (macaca mulatta), and chimpanzee (*Pan troglodytes*) is arginine (R63), which is also the major allele of humans (Figure S1 in Supplement 1). A similar allelic frequency for Q63 (of rs2502959) was observed both in Japanese and Caucasian populations (approximately 45%). A

Table 2. Haplotype rs12744386-R63Q Association Between *CNR2* and Schizophrenia

Haplotype	East Japan				West				Total				
	Frequency Patients	Frequency Control	χ^2	p	Frequency Patients	Frequency Control	χ^2	p	Frequency Patients	Frequency Control	χ^2	p	Permutation p
C-Arg	.578	.522	10	1.50×10^{-3}	.574	.504	15	1.00×10^{-4}	.577	.522	25	7.51×10^{-7}	5×10^{-6}
T-Gln	.346	.373	3.9	.049	.361	.373	.4	.508	.352	.373	4	4.48×10^{-2}	.1625
C-Gln	.047	.064	6.8	9.10×10^{-3}	.058	.086	8.6	3.40×10^{-3}	.052	.073	15	9.04×10^{-5}	.0005
T-Arg	.029	.030	0	.836	.007	.037	33	1.14×10^{-9}	.02	.033	12	6.0×10^{-4}	.0031

lower frequency was observed in an African population (17.3%; Figure S1 in Supplement 1). Analysis using the GENETYX software (Genetyx Corporation, Tokyo, Japan; http://www.sdc.co.jp/genetyx/) predicted a structural change of the receptor by the amino acid substitution and by Chou-Fasman, Robson, or hydrophilic/hydrophobic structure analysis (Figure S2 in Supplement 1).

In this study, responses of relative cAMP levels to saline and 2-AG, AM630, or JWH-015 in Q63- or R63-allele CNR2-transfected CHO cells were measured. CB2 receptor ligands did not change cAMP levels in the untransfected CHO cells. ANOVA revealed significant main effects of allele [$F(1,17) = 7.5$, $p = .01$] and of the interaction between 2-AG and allele [$F(1,17) = 7.5$, $p = .01$], but not of 2-AG [$F(1,17) = .4$, ns]. Further, ANOVA revealed significant main effects of AM630 administration [$F(1,29) = 14.8$, $p = .0006$], of allele [$F(1,29) = 4.2$, $p = .05$], and of the interaction between AM630 administration and allele [$F(1,29) = 4.2$, $p = .05$]. ANOVA showed trends of main effects of allele [$F(1,61) = 3.0$, $p = .09$], JWH-015 administration [$F(1,61) = 3.1$, $p = .08$], and the interaction between JWH-015 administration and allele [$F(1,61) = 3.0$, $p = .09$]. Post hoc analysis demonstrated that relative cAMP levels were reduced in CHO cells with the Q63 type of CB2 receptor expressed, in response to both agonists, 2-AG (corrected $p = .09$) and JWH-015 (corrected $p = .03$). No significant reduction of cAMP levels was observed in those with R63 type of CB2 receptor in response to both ligands (Figure 3A,B). Levels of cAMP in CHO cells with the Q63 type of CB2 receptor were significantly less than in those with the R63 type CB2, in response to both agonists, 2-AG (corrected $p = .002$) and JWH-015 (corrected $p = .03$), respectively. The inverse agonist AM630 increased cAMP levels significantly more in CHO cells with the Q63 allelic CB2 receptor (corrected $p = .01$), but it was not significant in those with the R63 allelic CB2 receptor. Levels of cAMP were increased more significantly in CHO cells with the Q63 than in those with the R63 allelic CB2 receptor (corrected $p = .06$; Figure 3C). Unless the CB2 receptor was expressed in CHO cells, cAMP levels did not change in response to JWH-015 and to AM630 (Figure 3B and 3C).

We evaluated the effect of pretreatment with the CB2 receptor inverse agonist AM630 on PPI, combined with MK-801 or methamphetamine treatment separately, in mice. Alone, the CB2 receptor inverse agonist AM630 did not affect PPI in mice. ANOVA revealed significant main effects of prepulse intensity [$F(3,303) = 42.4$, $p < .0001$] and MK-801 [$F(4,304) = 25.5$, $p < .0001$], but not of the interaction [$F(12,312) = 1.4$, ns]. Post hoc analysis revealed pretreatment with AM630 at a dose of 30 mg/kg produced a significantly greater reduction in % PPI than MK-801 treatment alone ($p = .002$), but pretreatment with AM630 at the dose of 3 mg/kg did not significantly reduce % PPI ($p = .2$) compared with saline pretreatment. When analyzed at each prepulse intensity, the reduction of % PPI by pretreatment with AM630 at the dose of 30 mg/kg was observed at 85 dB prepulse intensity ($p = .03$; Figure 4A).

ANOVA revealed significant main effects of prepulse intensity [$F(3,207) = 38.1$, $p < .0001$] and of methamphetamine [$F(4,208) = 3.3$, $p = .01$], but not of the interaction [$F(12,216) = .3$, ns]. Post hoc analysis revealed pretreatment with AM630 at a dose of 3 mg/kg produced a significantly greater reduction in % PPI than methamphetamine treatment alone ($p = .04$), and pretreatment with AM630 at a dose of 30 mg/kg produced a significantly greater reduction in % PPI than methamphetamine treatment

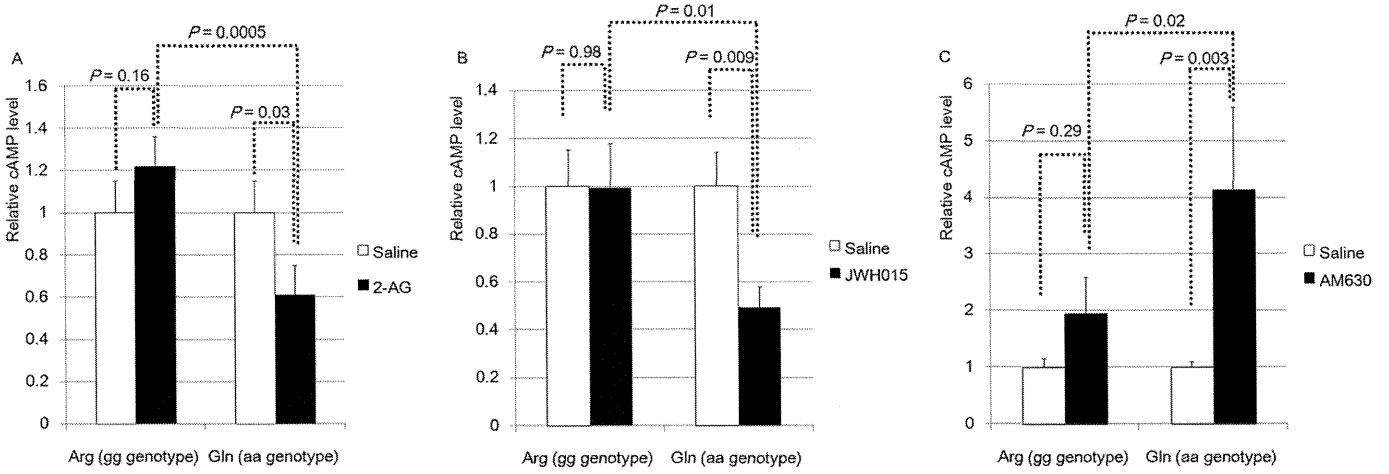

Figure 3. Functional analysis of the cannabinoid CB2 receptor with R63Q polymorphism. In cultured CHO cells expressed cloned human CB2 receptor, forskolin-evoked cyclic adenosine monophosphate (cAMP) level was regulated by three kinds of CB2 receptor ligands in this cell signaling system. CB2 receptor function in response to the ligands between Gln63 and Arg63 in the *CNR2* was analyzed. Black bars present cAMP levels in saline-treated CHO cells, and white bars show those in CHO cells treated with the ligand. Nominal *p* values are shown. **(A)** When endocannabinoid 2-AG binds to CB2 receptor, the evoked cAMP level is downregulated in CHO cells with Gln63 type CB2 receptor but not regulated in those with Arg63 type receptor. **(B)** When CB2 selective agonist JWH-015 binds to CB2 receptor, the evoked cAMP level is downregulated in CHO cells with Gln63 type CB2 receptor but not regulated in those with Arg63 type receptor or in naive CHO cells. **(C)** When CB2 selective inverse agonist AM630 binds to CB2 receptor, the evoked cAMP level is upregulated in CHO cells with Gln63 type CB2 receptor but not regulated in those with Arg63 type receptor or in naive CHO cells.

alone ($p = .03$). Although the overall effect was significant, post hoc tests did not reveal significant differences at individual prepulse intensities (Figure 5A).

ANOVA revealed significant effects of either MK-801 or methamphetamine administration on the locomotor activity of mice in home cages [$F(5,399) = 69.2$, $p < .0001$, $F(5,339) = 94.2$, $p < .001$, respectively, Figures 4B and 5B]. Both drugs produce significant hyperlocomotion. Post hoc analysis revealed that although AM630 alone did not produce significant hyperlocomotion, AM630 pretreatments at doses of both 3 mg/kg and 30 mg/kg significantly increased methamphetamine-induced locomotion (both $p < .0001$). Also, AM630 pretreatments at a dose of 30 mg/kg significantly increased MK-801-induced locomotion ($p < .0001$) than saline pretreatments.

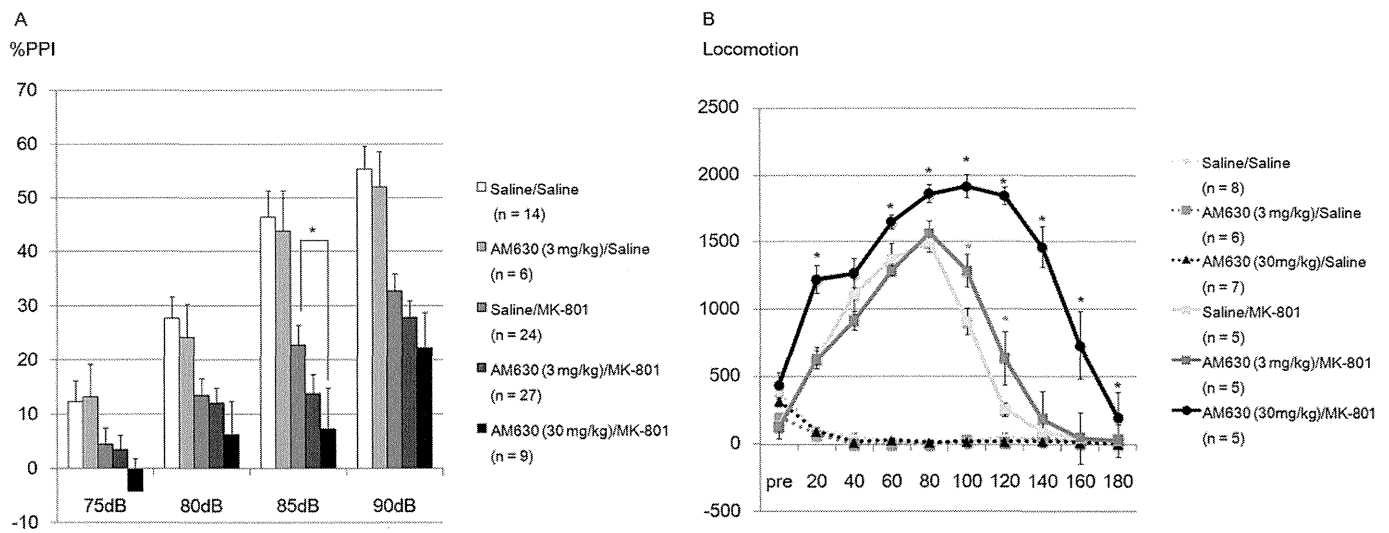

Figure 4. MK-801 induced alteration of prepulse inhibition (PPI) and locomotion influenced by AM630. **(A)** The vertical axis shows % PPI. Saline pretreated and treated before PPI test (Saline/Saline, $n = 14$), AM630 (3 mg/kg intraperitoneal [IP]) pretreated and saline treated before PPI test (AM630 [3 mg/kg]/ saline, $n = 6$), saline pretreated and MK-801 (.5 mg/kg IP) treated before PPI test (saline/MK-801, $n = 24$), AM630 (3 mg/kg IP) pretreated and MK-801 (.5 mg/kg IP) treated before PPI test (AM630 [3 mg/kg]/ MK-801, $n = 27$), AM630 (30 mg/kg IP) pretreated and MK-801 (.5 mg/kg IP) treated before the PPI test (AM630 [30 mg/kg]/ MK-801, $n = 9$). *Nominal $p < .05$ is shown. **(B)** The vertical axis shows locomotion activity in the home cage for every 20-min time period. The horizontal axis shows as follows: pre, 10 min after pretreatment and 20 min before treatment; 20, 40, 60, 80, 100, 120, 140, 160, and 180, minutes from MK-801 injection (.5 mg/kg IP) 30 min after pretreatment. Saline pretreated and treated (Saline/Saline, $n = 8$); 3 mg/kg AM630 pretreated and saline treated (AM630 [3 mg/kg]/saline, $n = 6$); 30 mg/kg AM630 pretreated and saline treated (AM630 [30 mg/kg]/MK-801, $n = 7$); saline pretreated and MK-801 treated (saline/MK-801, $n = 8$); 3 mg/kg (AM630 pretreated and MK-801 treatedAM630 [3 mg/kg]/MK-801, $n = 8$); 30 mg/kg AM630 pretreated and MK-801 treated ($n = 5$). *Nominal $p < .05$ is shown for difference of activity between saline/MK-801 and either AM630 (3 mg/kg)/MK-801 in dark gray or AM630 (30 mg/kg)/MK-801 in black at each time period.

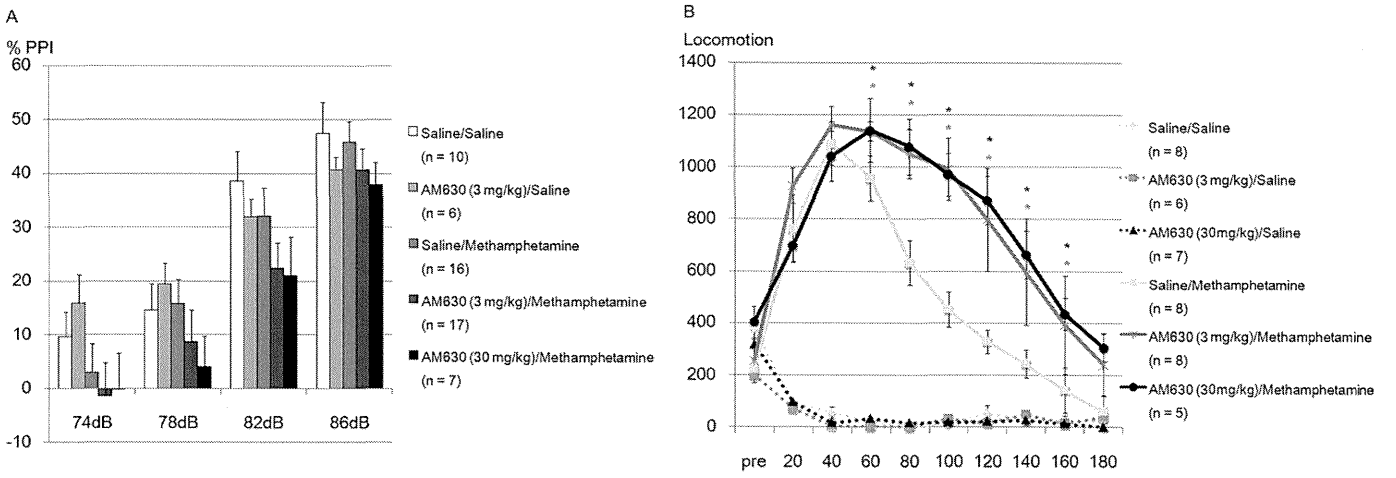

Figure 5. Methamphetamine-induced alteration of prepulse inhibition (PPI) and locomotion influenced by AM630. **(A)** The vertical axis shows % PPI. Saline pretreated and treated before PPI test (Saline/Saline, $n = 10$); AM630 (3 mg/kg intraperitoneal [IP]) pretreated and saline treated before PPI test (AM630 [3 mg/kg]/saline, $n = 6$); saline pretreated and methamphetamine treated (2 mg/kg IP) before PPI test (saline/methamphetamine, $n = 16$); AM630 (3 mg/kg IP) pretreated and methamphetamine treated before PPI test (AM630 [3 mg/kg]/methamphetamine, $n = 17$); AM630 (30 mg/kg IP) pretreated and methamphetamine treated before PPI test (AM630 [30 mg/kg]/methamphetamine, $n = 7$). **(B)** The vertical axis shows locomotion activity in the home cage for every 20 min. The horizontal axis shows as follows: pre, 10 min after pretreatment and 20 min before treatment; 20, 40, 60, 80, 100, 120, 140, 160, and 180, minutes from methamphetamine injection (2.0 mg/kg IP) 30 min after pretreatment. Saline pretreated and treated (Saline/Saline, $n = 8$), 3 mg/kg AM630 pretreated and saline treated (AM630 [3 mg/kg]/saline, $n = 6$), 30 mg/kg AM630 pretreated and saline treated (AM630 [30 mg/kg]/saline, $n = 7$), saline pretreated and methamphetamine treated (saline/methamphetamine, $n = 5$), 3 mg/kg AM630 pretreated and methamphetamine treated (AM630 [3 mg/kg]/methamphetamine, $n = 5$), 30 mg/kg AM630 pretreated and methamphetamine treated (AM630 [30 mg/kg]/methamphetamine, $n = 5$). *Nominal $p < .05$ is shown for difference of activity between saline/methamphetamine and either AM630 (3 mg/kg)/methamphetamine in dark gray or AM630 (30 mg/kg)/methamphetamine in black at each time period.

Discussion

We found two SNPs in and near the *CNR2* gene associated with schizophrenia in Japanese populations. One of these two SNPs, rs12744386, was found to be associated with gene expression levels (the risk allele was associated with low levels of *CNR2*) and another, rs2501432, was a missense R63Q. By the transfection experiments, we showed that, compared with the protective allele, the risk allele of the missense polymorphism had a poor response to CB2 ligands. Therefore, it was thought that both risk alleles of the two SNPs associated with schizophrenia were related to the direction of lower functioning of *CNR2*. Furthermore, these two SNPs were in LD with each other ($r^2 = .67$), and a haplotype composed of these two alleles associated with a lower functioning of *CNR2* had a greater association with schizophrenia than with the allelic association of either SNP alone. These findings provide evidence for the association between the *CNR2* gene and schizophrenia, although, because of the haplotype structure in the Japanese population, there is a possibility that the strength of association of either SNP might be inflated by the other SNP.

This study confirms that rs2501432 (R63Q) of *CNR2* gene is functional. The CB2 receptor is a guanine nucleotide–binding protein (G protein) coupled receptor, which is a member of the Gi-type receptor family. Codon 63 is located at the first intracellular domain immediately after the first transmembrane domain. Alteration of hydrophilic/hydrophobic structure at this point may alter signal transduction efficiency in cells after ligand stimulation. Our finding of a less efficient response to endocannabinoid 2-AG in the common R63 type than in the minor Q63 type is consistent with the findings of endocannabinoid-induced inhibition of lymphocyte proliferation in human peripheral tissue (25,28,29). Our study identifies that rs12744386 is a marker associated with changes in *CNR2* mRNA expression (in *cis*-acting fashion) both in lymphoblasts and brain tissue. Because the haplotype of the R63 and the C allele was more significantly associated with schizophrenia than each allele alone, it is possible that genetic variations in the *CNR2* genome region related to the reduced function of CB2 receptors may synergistically confer susceptibility to schizophrenia.

The genetic findings of lower functioning of *CNB2* associated with schizophrenia may be supported by the findings of the pharmacologic experiments using the animal model and CB2 antagonists in this study. MK-801- or methamphetamine-induced disturbance of PPI has been used as an animal model of schizophrenia (30–32). MK-801 reduced PPI, and when MK-801 was used in combination with the CB2 inverse-agonist AM630, PPI was further reduced. However, AM630 alone did not have any effect on PPI in any prepulse-pulse sessions. Methamphetamine alone at the dose of 2.0 mg/kg did not show a significant change in PPI; however, when methamphetamine was used in combination with AM630, PPI was significantly reduced. AM630 also enhanced MK-801- and methamphetamine-induced hyperlocomotion in mice, although there was no effect of AM630 alone. Therefore, reduced CB2 functioning itself is not likely to cause schizophrenia, but it is hypothesized that, when combined with other risk factors, it could be harmful for schizophrenia-susceptible individuals. Furthermore, because these pharmacologic models are related with the glutamatergic and dopaminergic neural systems underlying schizophrenia, our study indicates that CB2 function is related to these neural systems.

CB2 receptors perform many functions. An animal model for the neurodevelopmental hypothesis of schizophrenia was demonstrated using neonatal lesions in hippocampus, when pregnant mother rats were exposed to the bacterial endotoxin lipopolysaccharide (33,34). Although the CB2 receptor has been known to be linked to the immune system in the peripheral

body, its function was also involved in neural progenitor proliferation in the hippocampus (35,36). De la Fuente *et al.* (37) proposed an effect of maternal deprivation in neuroimmunoendocrine interactions. Maternal deprivation has also been shown to lead to a disruption of PPI and startle habituation, as well as reduced latent inhibition (38–41). The effect of amphetamine was enhanced in early maternally deprived rats (42). At the same time, early maternal deprivation induced an increase in the number of degenerating hippocampal neurons and astrocytes and increased corticosterone and 2-AG hippocampal levels (43,44). Therefore, this animal model of a specific aspect of schizophrenia also has implications on the endocannabinoid system. Another recent study showed an analysis of the effects of early maternal deprivation on CB1 and CB2 receptors in the hippocampus, indicating a decrease of CB1 receptor expression and an increase of CB2 receptor expression (45). The present study provided further evidence for the involvement of CB2 in schizophrenia.

The present study had some limitations. The possible effect of AM630 on CB1 and interaction between CB1 and CB2 receptors could not be excluded. A further study using *Cnr1* knockout mice is needed to explore this pharmacologic possibility and clarify the functions of *CNR2* in brain. Many genetic association studies have not been replicated and confirmed in other populations, particularly in the case of psychiatric disease. Replication studies in other ethnic populations are needed to confirm whether the CB2 receptor plays a role in susceptibility to schizophrenia. Several genomewide Association studies (GWAS) that have been reported or are being conducted may enable us to compare the allele frequencies of many SNPs in other populations. However, GWAS data sets with Affymetrix 500K or Illumina HumanHap550 platforms did not include the SNPs of rs12744386 or rs2501432, which were found to be associated with schizophrenia in this study. Particularly, the allele frequencies of rs2501432 are not recorded in the DbSNP database.

In conclusion, this study indicates that a genetically predetermined lower functioning of CB2 receptors increases susceptibility to schizophrenia when combined with other risk factors. However, this simple understanding needs to be further elucidated because CB2 receptors perform many functions, and the incidence of the R63 allele is found more commonly in patients who have other psychiatric or physiologic disorders, such as alcoholism, major depression, autoimmune disease, or osteoporosis (25,28,29).

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Competing Interests: The authors reported no biomedical financial interests or potential conflicts of interest.

Supplementary material cited in this article is available online.

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Immunohistochemical study of vesicle monoamine transporter 2 in the hippocampal formation of PCP-treated mice

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ABSTRACT

The exact pathophysiology of schizophrenia is unknown despite intensive scientific studies using molecular biology, psychopharmacology, neuropathology, etc. It is thought that neurodevelopmental failures such as neuronal network incompetence and the inappropriate formation of neurons affect the neurotransmitters. Several animal models have been created to investigate the etiology of this disease. In this study, we investigated the expression of vesicle monoamine transporter 2 (VMAT2), which has a significant role in neurotransmission, in the hippocampal formation in 1-phenylcyclohexylpiperazine (PCP)-treated mice using immunohistochemical staining technique to clarify neuronal abnormalities. PCP-treated mice are thought to be one of novel animal models for schizophrenia. The expression of VMAT2 in the hippocampal formation was significantly reduced overall in the PCP-treated mice compared to that in control (saline-treated) mice, also these reductions were observed throughout the brain. These facts implied that the pathophysiology of this disease involves abnormal monoaminergic transmission through VMAT2, despite PCP was the *N*-methyl-D-aspartate (NMDA) receptor antagonist that might induce glutamatergic abnormality. Since insufficient or excess release of neurotransmitter might alter neurochemical function and neurotransmission, VMAT2 might be an important target for biological research in psychiatric disease including schizophrenia.

1. Introduction

Schizophrenia is a major mental disease affecting approximately 1% of the general population. Impaired regulation of monoaminergic neurotransmission, including dopaminergic, serotonergic, and catecholaminergic transmission, is thought to be one aspect of the pathophysiology of this disease (Howes and Kapur, 2009; Seeman, 2009). Although studies indicate an impairment of neuronal transmission, whether this disruption is a causal, additional, or secondary change remains unclear. The VMAT2 protein packages neurotransmitters such as dopamine, serotonin, and norepinephrine that enter synaptic vesicles for release into the synaptic clefts. Within the synaptic vesicles, VMAT2 regulates vesicle loading and consequently defines quantal size, receptor sensitivity, and synapic plasticity (Pothos et al., 2000; Pothos, 2002). Therefore, this protein may have a major role in the pathophysiology of neuropsychiatric diseases including schizophrenia (Taylor et al., 2000), mood disorders (Zubieta et al., 2001), methamphetamine (MAP) neurotoxicity (Volz et al., 2009), and Parkinson's disease (Mooslehner et al., 2001). It was suggested that dopamine-related psychostimulants like methylphenidate and MAP alter the expression and/or the function of VMAT2 in animals (Fleckenstein et al., 2009).

Animals treated with psychostimulants including MAP and cocaine (COC) and genetically modified animals have been used widely as models of schizophrenia (Bickel and Javitt, 2009; Desbonnet et al., 2009). One such model is the PCP-treated mouse (Mouri et al., 2007). PCP acts as a noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, and repeated PCP treatment impaired NMDA receptor function and decreased levels of extracellular glutamate (Nabeshima et al., 2006). PCP is thought to be a psychostimulant capable of producing both the

Abbreviations: CA, cornu ammonis; COC, cocaine; DG, dentate gyrus granule cell layer; MAP, methamphetamine; NMDA, *N*-methyl-D-aspartate; PC, personal computer; PCP, 1-phenylcyclohexylpiperazine; Pyrl, pyramidal layer; Sra, stratum radiatum; VMAT2, vesicle monoamine transporter 2.

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