of copy number variations with functional implication at *USP46* locus. Although we could not find copy number variations in this locus, it cannot be ruled out that unknown copy number variations located in this locus have an important role in the etiology of psychiatric disorders.

In conclusion, our data argue against the presence of any strong genetic susceptibility factors for bipolar disorder or schizophrenia in the region *USP46*. However, considering the limitations of this genetic association study and supportive evidence from various datasets, expansion of samples or resequencing strategy would be required for a more conclusive result.

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Diagnostic classification of schizophrenia by neural network analysis of blood-based gene expression signatures

Makoto Takahashi [a,1], Hiroshi Hayashi [b,1], Yuichiro Watanabe [a], Kazushi Sawamura [a], Naoki Fukui [a], Junzo Watanabe [a], Tsuyoshi Kitajima [c,d], Yoshio Yamanouchi [c,d], Nakao Iwata [c,d], Katsuyoshi Mizukami [e], Takafumi Hori [e], Kazutaka Shimoda [f], Hiroshi Ujike [g], Norio Ozaki [d,h], Kentarou Iijima [b], Kazuo Takemura [b], Hideyuki Aoshima [b], Toshiyuki Someya [a,*]

[a] *Department of Psychiatry, Niigata University Graduate School of Medical and Dental Sciences, Asahimachi-dori 1-757, Niigata 951-8510, Japan*
[b] *R&D Department, SRL Inc., Tokyo 191-0031, Japan*
[c] *Department of Psychiatry, Fujita Health University School of Medicine, Aichi 470-1192, Japan*
[d] *CREST, Japan Science and Technology Agency, Saitama 332-0012, Japan*
[e] *Department of Psychiatry, Institute of Clinical Medicine, University of Tsukuba, Ibaraki 305-8575, Japan*
[f] *Department of Psychiatry, Dokkyo Medical University School of Medicine, Tochigi 321-0293, Japan*
[g] *Department of Neuropsychiatry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan*
[h] *Department of Psychiatry, Nagoya University Graduate School of Medicine, Aichi 466-8550, Japan*

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ABSTRACT

Gene expression profiling with microarray technology suggests that peripheral blood cells might be a surrogate for postmortem brain tissue in studies of schizophrenia. The development of an accessible peripheral biomarker would substantially help in the diagnosis of this disease. We used a bioinformatics approach to examine whether the gene expression signature in whole blood contains enough information to make a specific diagnosis of schizophrenia. Unpaired *t*-tests of gene expression datasets from 52 antipsychotics-free schizophrenia patients and 49 normal controls identified 792 differentially expressed probes. Functional profiling with DAVID revealed that eleven of these genes were previously reported to be associated with schizophrenia, and 73 of them were expressed in the brain tissue. We analyzed the datasets with one of the supervised classifiers, artificial neural networks (ANNs). The samples were subdivided into training and testing sets. Quality filtering and stepwise forward selection identified 14 probes as predictors of the diagnosis. ANNs were then trained with the selected probes as the input and the training set for known diagnosis as the output. The constructed model achieved 91.2% diagnostic accuracy in the training set and 87.9% accuracy in the hold-out testing set. On the other hand, hierarchical clustering, a standard but unsupervised classifier, failed to separate patients and controls. These results suggest analysis of a blood-based gene expression signature with the supervised classifier, ANNs, might be a diagnostic tool for schizophrenia.

1. Introduction

Genome-wide gene expression analysis by DNA microarray and bioinformatics procedures has been conducted to elucidate common gene pathways that underpin the biological mechanisms of schizophrenia (Aston et al., 2004; Hakak et al., 2001; Hemby et al., 2002; Iwamoto et al., 2005, 2004; Mirnics et al., 2000, 2001; Sugai et al., 2004; Tkachev et al., 2003). However, the clinical use of microarray technology is not so widespread in schizophrenia research as compared with cancer research (Rhodes et al., 2004), due to the difficulty in interpreting results obtained from postmortem

* Corresponding author. Tel.: +81 25 227 2210; fax: +81 25 227 0777.
E-mail address: psy@med.niigata-u.ac.jp (T. Someya).
[1] These authors contributed equally to this work.

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brain tissue that are complicated by agonal factors, anatomical inconsistency, and cellular heterogeneity of the cortical and subcortical regions. Postmortem brain studies use less accessible materials and therefore are limited by small sample size and repeated use of the same cohorts (Iwamoto and Kato, 2006). As a more accessible tissue, several researchers have undertaken expression profiling of peripheral blood cells (Sullivan et al., 2006; Tsuang et al., 2005; Vawter et al., 2004; Zvara et al., 2005). On a transcriptional expression level, peripheral blood cells were reported to share significant similarities with tissues from multiple brain regions (Sullivan et al., 2006). Interestingly, Tsuang et al. (2005) and Middleton et al. (2005) have shown that a set of genes extracted from gene expression signature of isolated peripheral blood cells can discriminate between schizophrenia and control groups.

These studies suggest that analysis of high dimensional data is useful to generate a biomarker of schizophrenia since it can combine data from several molecules, each of which shows small difference but is not exclusively associated with this disease (Schwarz and Bahn, 2008). In cancer research, classification by gene expression signature is widely used to predict tumor classes, drug responses, and prognosis of individual subjects (Khan et al., 2001; Lin et al., 2007; O'Neill and Song, 2003). Development of such classifier will greatly help our diagnosis of schizophrenia that is solely dependent on clinical symptoms so far. There are two approaches in classification: supervised and unsupervised methods. In contrast to unsupervised clustering, supervised classifiers learn a function from training data that consist of pairs of input objects (e.g., gene expression signatures) and desired outputs (e.g., diagnoses) (De Bruyne et al., 2007). The artificial neural network (ANN) is one of those classifiers that works very well, at identifying patterns or trends in a large amount of data with little theory.

Purpose of the present study is to examine whether microarray date obtained from whole blood cells contain enough information to classify schizophrenia. We present here that ANN model can correctly predict the diagnosis with sufficient accuracy.

2. Materials and methods

2.1. Subjects

Samples from 52 patients with schizophrenia and 49 normal controls were analyzed. Patients with schizophrenia or schizophreniform disorder were recruited from outpatients or inpatients of psychiatry unit at 6 centers across Japan. Those who were antipsychotics-free and had no comorbidity were included in the study. Control subjects were recruited from hospital staff and student volunteers who showed no evidence of present or past mental illness. All subjects were evaluated using the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I). At least two trained psychiatrists confirmed diagnosis of schizophrenia and specified its subtypes. In patients with schizophreniform disorder, the diagnosis of schizophrenia was reconfirmed 6 months after the onset of the first psychotic episode. The study protocol was approved by the ethics committee of each institution, and written informed consent was obtained from all subjects. If patients were floridly psychotic, informed consent was provided first by their parents or husband/wife, and then the consent was provided again by patients themselves after the psychotic symptoms were ameliorated.

We assessed 611 patients to recruit 52 antipsychotic-free patients without commorbidity. The patients and controls were of similar age (31.8 vs. 31.2 years; $p=0.776$) but significantly different in gender due to our sampling bias (21 males, 31 females vs. 35 males, 14 females; $p=0.0017$) (Table 1). In both patients and controls, there was no abnormal finding in standard laboratory workup including blood chemistries, complete blood count, urinalysis and electrocardiogram. Among 52 patients, 34 patients were drug-naïve, 8 patients were antipsychotics-naïve, and 10 patients were drug-free. All of the patients manifested active-phase symptoms. Current episode was the first psychotic episode for 23 of 34 drug-naïve patients. It was also the first episode for 8 neuroleptic-naïve patients, but they were taking antidepressants, benzodiazepines, or mood stabilizers for prodromal symptoms. Drug-free patients were those who stopped taking medication and relapsed due to non-adherence. They were drug-free for more than 8 weeks.

2.2. RNA isolation and microarray procedures

We extracted total RNA from whole blood because in vitro handling for cell isolation could alter gene expression (Ohmori et al., 2005). A total of 5 ml venous blood was collected in PAXgene Blood RNA Tubes (Qiagen, Valencia, CA) and frozen at □ 80 °C within 2 h after blood withdrawal. Total RNA was isolated from each of the frozen samples with PAXgene Blood RNA Kit (Qiagen) according to the manufacturer's instructions. Quantity and quality of total RNA were checked by A260/280 readings of spectrophotometry and an

Table 1
Demographic details of patients and controls.

	Control	Schizophrenia	Subtypes			
			Paranoid	Disorganized	Catatonic	Undifferentiated
N	49	52	21	9	1	21
Age	31.2±9.5	31.8±11.4	38.1±13.0	26.1±6.5	30	27.8±8.5
Sex (M/F)	35/14	21/31*	9/12	4/5	1/0	7/14
Age at onset	–	24.5±9.4	30.9±11.5	19.8±3.2	30	20.8±6.0
Medication history						
Drug-naïve	–	34	18	5	1	10
Drug-free	–	10	2	3	0	5
Neuroleptics-naïve	–	8	1	1	0	6

* $p<0.01$ compared to controls by chi-square test.

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Agilent BioAnalyzer (Agilent, Santa Clara, CA). Mean ratio of 28S/18S rRNA was 1.8 ± 0.3 and 1.9 ± 0.3 (mean ± SD) for patient and control groups, respectively.

Gene expression profiles were determined using CodeLink Human Whole Genome Bioarray (GE Healthcare Bio-Sciences, Chandler, AZ) according to the manufacturer's protocol. Briefly, cDNA was synthesized with 0.5 μg of total RNA and transcribed into biotinylated cRNA using iExpress Assay Reagent Kit (GE Healthcare Bio-Sciences, Chandler, AZ). Ten micrograms of the biotinylated cRNA was fragmented at 94 °C for 20 min and hybridized to CodeLink Human Whole Genome Bioarray, which contains probes for 54,847 transcripts. The hybridized cRNA probes to oligonucleotide arrays were stained with Cy5-streptavidin and scanned with an Agilent DNA Microarray Scanner (Agilent, Santa Clara, CA).

2.3. Data processing and normalization

The TIFF image from the microarray scanner was quantified using CodeLink Expression Analysis ver4.2 (GE Healthcare Bio-Sciences, Chandler, AZ). The mean intensity was taken for each spot and background corrected by subtracting the surrounding median local background intensity. Raw intensities were global median normalized for each bioarray. Probes were filtered to include only those present in more than two thirds of samples and those with average signal intensities between the 30th and

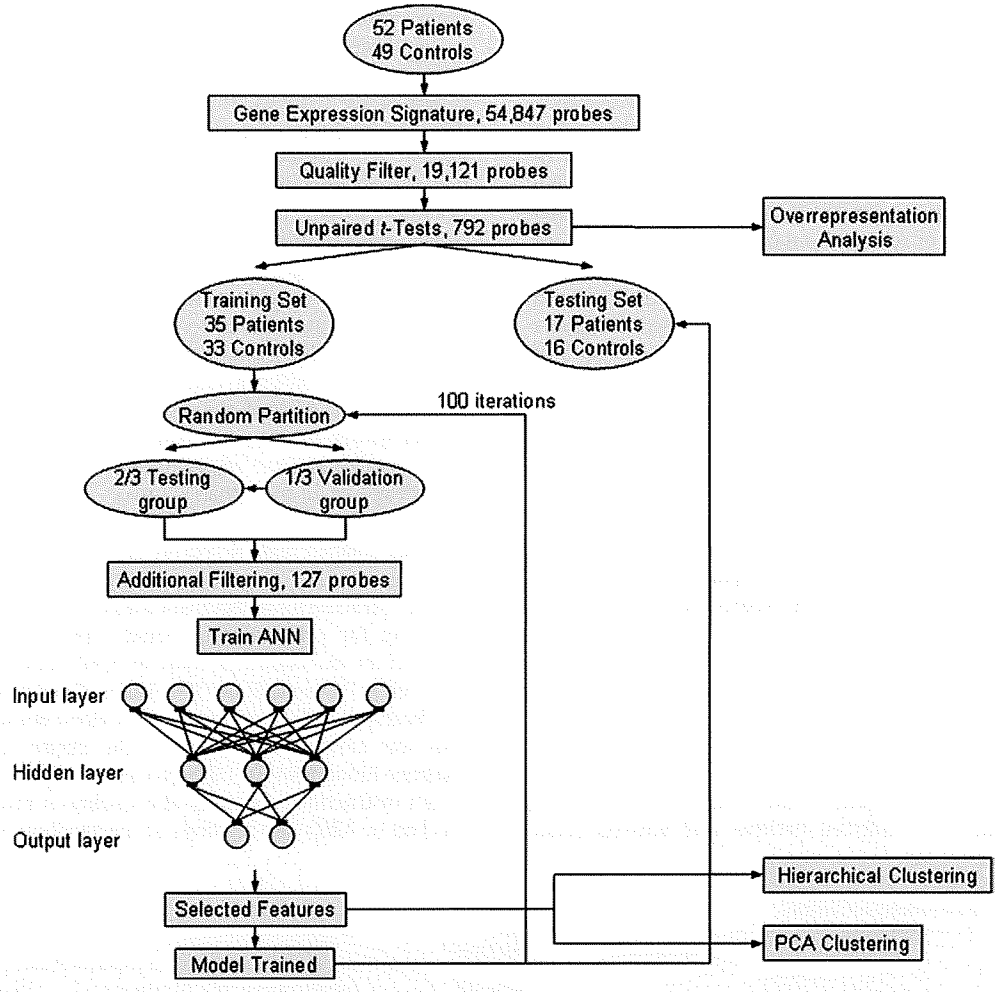

Fig. 1. Workflow for a 3-fold cross-validation artificial neural network (ANN) analysis. A gene expression signature was obtained from whole blood cDNA from 52 patients with schizophrenia and 49 normal controls using CodeLink Human Whole Genome Bioarray, containing about 55,000 probes. After a quality filter, 19,121 probes were used as a dataset for further analysis. To reduce dimensionality and skewness of the data, we selected 127 probes with moderate signal intensity and small coefficient of variation (CV) from 792 differentially expressed probes and subjected them to stepwise forward selection. Subjects were subdivided into training and testing sets to perform hold-out cross-validation. Training sets were randomly partitioned into three groups. One group was selected as a validation set, whereas the remaining two groups were used to train the network. The output produced by the first data passage through the ANN was compared with the ideal output, a known diagnosis, and an error is generated. The error was backpropagated through the ANN, and the weights of various connections between the neural units were adjusted. Then, a different validation set was selected from the same partitioning, and the remaining groups were used for training. The same step was repeated again to use each of the three groups as a validation set. Stepwise forward selection identified the best feature at each round of 3-fold cross-validation cycled with the repartitioning of the groups until an optimal feature size was determined. ANNs were then trained 100 times with selected predictors as the input, and the constructed model was used to classify testing set samples.

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98th percentile of all the probes. Probes with no NCBI accession number were excluded. Probes under significant influence of gender ($p<0.05$, unpaired t-test) were also excluded. This procedure reduced the number of probes included in the analysis from 54,847 to 19,121.

2.4. Overrepresentation analysis

The gene expression data were imported into ArrayAssist ver5.5.1 (Stratagene, Cedar Creek, TX). Differentially regulated probes between the two groups were analyzed by unpaired t-tests. Probes with $p<0.01$ were considered to be differentially expressed. A correction for multiple testing was tried by calculating the false discovery rate (FDR), the rate at which significant features are truly null, using the QVALUE program (Storey and Tibshirani, 2003). For estimating q values, π_0 was determined with automated choice of λ based on the distribution of 19,121 p values using a smoothing method. FDR 0.05 was considered as significant.

Functional overrepresentation of differentially expressed genes was analyzed with the gene annotation tool DAVID, which integrates almost all major bioinformatics resources (Huang da et al., 2007). DAVID calculates the p value for the extent of overrepresentation of a given biological meaning by performing Fisher's exact test.

2.5. Artificial neural network construction and testing

For the purpose of hold-out cross-validation, subjects were subdivided into training and testing sets. The training set included 35 patients with schizophrenia (29.9 years: 14 males, 21 females) and 33 normal controls (30.9 years: 23 males, 10 females). The remaining 17 patients (32.8 years: 7 males, 10 females) and 16 controls (33.5 years: 12 males, 4 females) were held out for the testing. Mean age was similar but gender ratio was significantly different within each set ($p=0.014$ and $p=0.049$ in the training and testing sets, respectively).

We constructed artificial neural networks (ANNs) using the ArrayAssist program. Our ANNs were three-layer back propagation networks trained with a learning rate of 0.5, a momentum rate of 0.3, and 100 iterations (Fig. 1). The program was implemented with algorithms of stepwise forward selection to enable the determination of optimal gene features for the ANN model (Lancashire et al., 2008). Selected 127 probe datasets were used as an input for forward selection. The ideal outputs were set at 0 for controls and 1 for schizophrenia. Beginning from an empty set of features, the forward selection algorithm added a single probe that produced the best performance as a predictor at each step of 3-fold cross-validations. After determining an optimal feature size, ANNs trained 100 times to adjust weights of links. The constructed ANN model was then applied to testing samples.

2.6. Clustering analysis

To compare supervised and unsupervised methods, clustering analysis was performed on training set samples that were used for ANN construction. The ArrayAssist program was used to perform principal component analysis (PCA) clustering and agglomerative hierarchical clustering. The method employed for hierarchical clustering was complete linkage and Euclidean distance.

3. Results

3.1. Characteristics and annotation of a blood-based gene expression signature

Among 19,121 quality filtered probes, 792 probes were identified as differentially expressed; 256 probes were down-regulated and 536 probes were up-regulated in patients with schizophrenia compared with controls. Since the gender ratio was different between the two groups, our filtering process excluded probes with significant gender differences. Down-regulated and up-regulated probes were separately profiled by DAVID. Among 256 down-regulated probes, 167 genes were annotated. Top-ranked Gene Ontology terms included cell adhesion, nervous system development, and neurotransmitter binding and receptor activity (Table 2, Table S1, S2). Unigene EST annotated 73 of these genes as expressed in brain tissue (Table S3). On the other hand, among 536 up-regulated probes, 381 genes were annotated but their association with central nervous system was not clear. The annotated genes contained 11 genes that were reported to be associated with schizophrenia (*NRG1*, *NTF3*, *CHRNB2*, *CCKAR*, *DAOA*, *DAO*, *L1CAM*, *NEUROG1*, *ZNF74*, *PRODH*, and *CHGA*).

As the main purpose of the study was to find the usefulness of microarray data in a clinical practice, probes suitable for laboratory testing were required. Calculation of the q value revealed that among 792 differentially expressed probes, only 17 passed a q value threshold of 0.05, which was too conservative to

Table 2
Top 10 biological process and molecular function Gene Ontology terms overrepresented by down-regulated genes.

Term	Count	%	p value
Biological process			
Biological adhesion	16	9.58%	0.0011
Cell adhesion	16	9.58%	0.0011
Nervous system development	16	9.58%	0.0012
Multicellular organismal process	45	26.95%	0.0023
Ion transport	16	9.58%	0.0024
Generation of neurons	8	4.79%	0.0079
Neurogenesis	8	4.79%	0.0118
Neuron differentiation	7	4.19%	0.0142
Sodium ion transport	5	2.99%	0.0167
Developmental process	37	22.16%	0.0169
Molecular function			
Photoreceptor activity	3	1.80%	0.0061
Alkali metal ion binding	7	4.19%	0.0063
Neurotransmitter receptor activity	5	2.99%	0.0079
Neurotransmitter binding	5	2.99%	0.0090
Calcium ion binding	16	9.58%	0.0112
Cation binding	44	26.35%	0.0154
Ion binding	47	28.14%	0.0185
Metal ion binding	46	27.54%	0.0209
Lipid binding	9	5.39%	0.0323
Gated channel activity	7	4.19%	0.0365

Count of genes involved in the term is listed, together with the percentage of involved genes in total genes. The smaller the Fisher's exact p value, the more enriched the term.

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be used as a criterion for ANN input data. We therefore conducted additional filtering based on coefficient of variation (CV) and average signal intensity. CV, defined as the ratio of the standard deviation to the mean, is a measure of dispersion of probability distribution. As smaller CV is indispensable for the precise laboratory testing, we considered probes with CV less than 30% are acceptable. This resulted in an exclusion of probes with large fold change (FC) in the mean expression level, since larger FC accompanied larger CV (Fig. 2A, C). On the other hand, in spite of normal distribution of FCs (Fig. 2A), the distribution of average signal intensities was largely skewed (Fig. 2B). The large skewness of our data might lead to the construction of a less reproducible ANN model. We performed a pilot study by constructing ANNs with shifting the threshold window of average signal intensities around a median of differentially expressed probes (4.0). ANNs were most reproducible with the threshold window of 1.0 to 3.0, and therefore it was introduced as the second criterion. This procedure of additional filtering reduced the number of probes included in the further analysis from 792 to 127 (Fig. 2D).

3.2. Feature selection through ANN training and model construction

We used ANNs to examine whether a blood-based gene expression signature can distinguish patients with schizophrenia from normal controls. Forward selection (Lancashire et al., 2008) was performed using the training set of samples to identify a subset of differentially expressed probes that were important for the distinction. Among 127 probes, maximal classification accuracy was obtained with 14 probes (Fig. 3A) for 8 known genes and 6 expressed sequence tags (ESTs). Eight genes and two ESTs were expressed in the brain (UniGene), and only *DAOA* (*G72*) was associated with schizophrenia (OMIM) (Fig. 3B).

Since FC values in 14 predictors were relatively small, we carried out unsupervised standard methods to see to what extent these predictors separate patients from controls. Hierarchical clustering grouped one patient into controls and grouped 20 controls together with 34 patients (Fig. 4A), suggesting that one-dimensional Euclidean distance by 14 predictors was too close among samples to separate patients

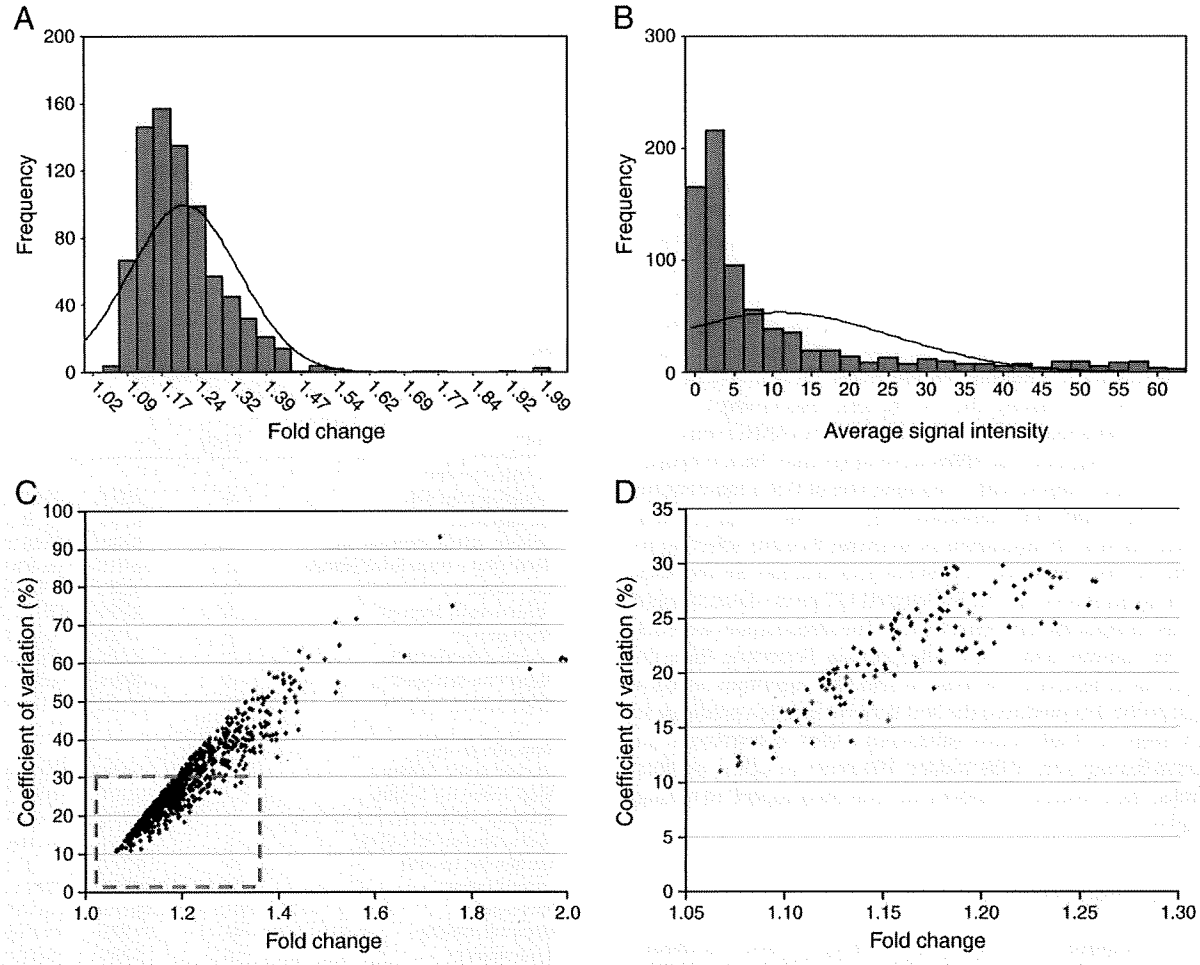

Fig. 2. Descriptive statistics of 792 differentially expressed probes identified by unpaired *t*-tests with a *p* value cut-off of 0.01. Shown are the frequencies of absolute fold change (A) and average signal intensity (B). The normal distribution curve was overlayed. (C) Values of the coefficient of variation were plotted against absolute fold change values. Probes with less than 30% CV (red dashed square) were filtered with an average signal intensity threshold of 1.0–3.0 (D). Probes selected as predictors are highlighted in red.

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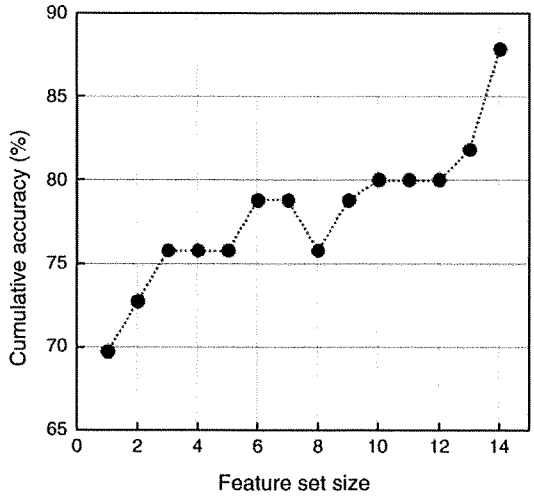
A



B

	Acc. no.	Symbol	FoldΔ	NCCV	SZCV
1	NM_032630*	*CINP*	1.18	19.2	24.5
2	AL833915*	*TDRD9*	1.13	10.7	22.9
3	NM_172370*	*DAOA*	1.09	16.5	10.1
4	BF056784*		1.18	7.2	12.6
5	NM_006667*	*PGRMC1*	1.20	28.4	25.2
6	BC033250		1.21	17.6	26.3
7	BM789800		1.16	16.4	18.2
8	NM_138386*	*NAF1*	-1.10	15.9	11.9
9	BX538315*		1.15	15.4	17.9
10	NM_139248*	*LIPH*	-1.07	14.1	11.3
11	NM_199227*	*MAP1D*	-1.10	25.3	13.0
12	BQ051075		1.11	12.0	14.8
13	NM_005543*	*INSL3*	1.09	31.8	20.4
14	BU621125		-1.18	22.9	19.6

Fig. 3. Identification of predictor genes by a stepwise forward selection ANN model. A. Each round of 3-fold cross-validation finds one probe that performs best as a predictor for classification. The cycle was repeated until selected features achieved maximal accuracy. B. Predictors in order of their appearance in the selection algorithm. Positive and negative values in fold change (Fold Δ) represents up- and down-regulation in schizophrenia patients, respectively. *Presence of expression in the brain (UniGene). *CINP*, cyclin-dependent kinase 2-integrating protein; *TDRD9*, Tudor domain containing 9; *DAOA* (*G72*), D-amino acid oxidase activator; *PGRMC1*, progesterone receptor membrane component 1; *NAF1*, nuclear assembly factor 1 homolog; *LIPH*, lipaze member H; *MAP1D*, methionine aminopeptidase 1D; *INSL3*, insuline-like 3 (Leidig cell); NCCV, CV in normal controls; and SZCV, CV in patients with schizophrenia.

from controls by this type of method. PCA clustering was also done to generate a three-dimensional plot from 14 dimensions (predictors). Visualization of 68 training samples demonstrated some segregation according to the clinical diagnosis (Fig. 4B), but it was difficult to find linearly separable space between the two groups.

ANNs were then trained with the training set of samples. The constructed ANN model correctly predicted 31 of 35 patients and 31 of 33 controls (Fig. 4C). Finally, performance of the model was tested using hold-out samples. Our ANN modeling was validated by correctly predicting 14 of 17 patients and 15 of 16 controls. This corresponded to 87.9% accuracy with 82.4% sensitivity and 93.8% specificity. In addition, we subdivided all the samples again into four subsets and classified them by the ANN, respectively. Diagnosis of each set was predicted with 80.0 to 96.2% accuracy, indicating robustness of the ANN to different groupings (Table 3).

4. Discussion

Using supervised method, ANN, we found that schizophrenia can be classified by blood-based gene expression signature. Accuracy of the ANN model was 91.2%, which was not so high as similar reports in cancer research (Khan et al., 2001; Lin et al., 2007; O'Neill and Song, 2003). However, in contrast to the use of tumor samples in those studies, we are not able to obtain tissues from the brain. Considering the use of peripheral whole blood as a material, we feel that the performance of our ANN model is acceptable. In schizophrenia, Tsuang et al. (2005) presented a diagnostic equation obtained from logistic regression of eight gene expression levels in isolated peripheral blood cells. They adopted ANN to verify their finding, but their report did not describe the accuracy of it. Middleton et al. (2005) provided a list of genes expressed in peripheral blood cells that predicted diagnosis of schizophrenia with 95% or greater accuracy using two types of supervised classifiers. However, they did not cross-validated their list. The larger sample size of the present study allowed us to conduct hold-out cross-validation, which revealed that the testing set accuracy was comparable to the training set one.

Hold-out method is one of the standard cross-validation methods that are widely used to evaluate prediction by computer-based learning (Li, 2007). In ANN modeling of microarray datasets, the method is utilized by partitioning samples into training and testing sets after the identification of differentially expressed probes (Khan et al., 2001; Xu et al., 2002). Purpose of the method is to confirm that constructed ANN model does not come from overfitting of model parameters to features in the training set, which will result in poor predictive accuracy in the testing set. Therefore, validation of our ANN model does not mean to show replicated data in an independent cohort. It simply suggests that the model can be generalized to other subset of samples that are not included for the model construction.

Predictors in our ANN model were chosen by forward selection to minimize error in supervised learning. As a result, FCs in selected predictors were so small that hierarchical or PCA clustering could not discriminate between patients and controls. In postmortem brain studies of schizophrenia, top-ranked genes with 1.3-fold or greater change were shown to separate patients well from controls (Hakak et al., 2001; Iwamoto et al., 2004). We were not sure, however, whether top-ranked genes expressed in whole blood represent important biological pathways of the brain that are involved in schizophrenia. Previous research has demonstrated that the selection of top-ranked genes might lose essential information for the purpose of classification, since they are usually highly correlated (Jaeger et al., 2003; Liu et al., 2004). Low-ranked genes are sometimes necessary for classifiers to achieve greater accuracy (Li et al., 2003). We therefore consider that it is reasonable for the forward selection algorithms to identify probes with relatively small FC as predictors for classification.

In peripheral blood, variations in transcription level among individuals are attributed to genetic or epigenetic components

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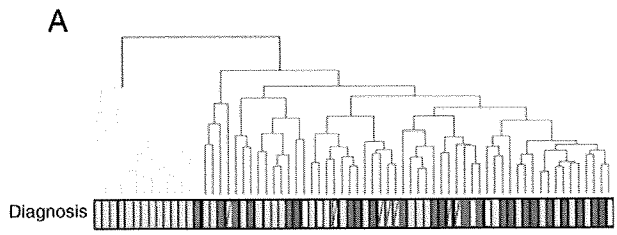

C

ANN prediction	Sensitivity (%)	Specificity (%)	Accuracy (%)
Training set (n = 68)	88.6	93.9	91.2
Testing set (n = 33)	82.4	93.8	87.9

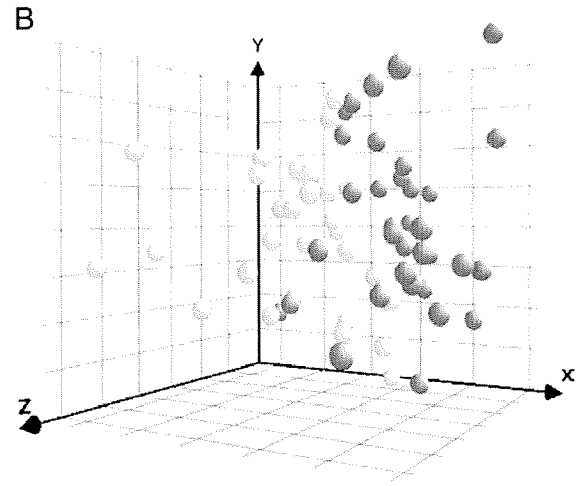

Fig. 4. Classification by 14 predictors identified with stepwise forward selection. A. Hierarchical clustering resulted in two ambiguous clusters. One patient was grouped into controls, and 20 controls were grouped together with patients. Closed green square, normal controls; closed red square, drug-naïve patients with schizophrenia; open red square, drug-free patients with schizophrenia; and open red square with a slash, antipsychotic-naïve patients with schizophrenia. Note that past exposure to antipsychotics or current medication status did not affect the expression pattern of the predictors. B. Plot of the three principal components generated from 14 selected predictors revealed segregation by diagnosis but with some overlap. Green sphere, normal controls; red sphere, patients with schizophrenia. C. Performance of ANN prediction in training set and testing set samples.

and cellular composition (Cheung et al., 2003; Eady et al., 2005; Radich et al., 2004; Whitney et al., 2003). For convenience as a diagnostic tool and to minimize technical fluctuations (Debey et al., 2004; Ohmori et al., 2005), we stabilized RNA as blood was withdrawn into PAXgene Blood RNA Tubes instead of isolating a specific type of cells with time delay (Rainen et al., 2002). It has been shown that homeostasis of cellular composition in healthy individuals keeps consistent patterns of global gene expression as compared with individuals with lymphoma or bacterial infection (Whitney et al., 2003). Our forward selection algorithm did not chose genes associated with specific blood cell types. Therefore, so far as excluding samples with physiological illness, we assume that variations by cellular composition are maximally suppressed by supervised training in ANNs.

Among 14 predictors, *DAOA* (*G72*) was the only gene associated with schizophrenia (Chumakov et al., 2002). Selective degradation of D-serine by DAOA and D-amino acid oxidase results in attenuated neurotransmission via NMDA receptor (Mothet et al., 2000), which probably links the up-regulation of *DAOA* (*G72*) in the present study to the earlier finding of decreased D-serine levels in the serum of patients with schizophrenia (Hashimoto et al., 2003). However, we should investigate how 14 genes are expressed in the brain and whether they are associated with schizophrenia before discussing the biological significance of them in peripheral blood.

We demonstrate here that by using the ANN method we can classify schizophrenia based on gene expression signature obtained from whole peripheral blood. Since we constructed the ANN model using antipsychotics-free patients without comorbidity, ANN-based classification may be applied only for a subset of patients, especially with first-episode psychosis. There are several limitations in the present study: small sample size compared to the large scale microarray, gender difference between the two groups, and absence of quantitative reverse transcription-PCR (RT-PCR) results to corroborate microarray data. Statistical power of our sample size (50) was about 22% by computation at web-based bioinformatics service (http://bioinformatics.mdanderson.org/MicroarraySampleSize/). Therefore, our results, in terms of the predictor selection, should be interpreted cautiously. Before replicating the differential expression by RT-PCR, we are currently developing a custom array with less than several hundred probes. This will increase statistical power even with the same sample size. Moreover, high accuracy of our ANN model needs to be replicated by independent cohort with gender-matched groups before applying it to clinical practice.

Table 3
Performance of ANN prediction in regrouped sample sets.

Sample set	Group	Age (mean±SD)	Sex (M/F)	Sensitivity (%)	Specificity (%)	Accuracy (%)
A	NC	31.3±10.6	8/4	76.9	83.3	80.0
	SZ	36.8±9.2	5/8			
B	NC	32.6±11.0	9/3	84.6	100.0	92.0
	SZ	27.0±10.9	5/8			
C	NC	32.6±11.1	10/2	92.3	91.7	92.0
	SZ	28.8±7.7	6/7			
D	NC	28.5±4.6	8/5	92.3	100.0	96.2
	SZ	32.8±14.8	5/8			

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Contributors

Someya, Aoshima and Watanabe Y designed the study and wrote the protocol. Takahashi, Sawamura, Fukui, Watanabe J, Kitajima, Yamanouchi, Iwata, Mizukami, Hori, Shimoda, Ujike and Ozaki recruited subjects. Iijima and Takemura performed gene expression profiling, and Hayashi undertook the statistical analysis. Takahashi wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

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Conflict of interest

Hayashi, Iijima, Takamura and Aoshima work for R&D Department of SRL Inc. All other authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.schres.2009.12.024.

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ARCHIVAL REPORT

Brain Cannabinoid CB2 Receptor in Schizophrenia

Hiroki Ishiguro, Yasue Horiuchi, Maya Ishikawa, Minori Koga, Keiko Imai, Yoshimi Suzuki, Miyuki Morikawa, Toshiya Inada, Yuichiro Watanabe, Makoto Takahashi, Toshiyuki Someya, Hiroshi Ujike, Nakao Iwata, Norio Ozaki, Emmanuel S. Onaivi, Hiroshi Kunugi, Tsukasa Sasaki, Masanari Itokawa, Makoto Arai, Kazuhiro Niizato, Shyuji Iritani, Izumi Naka, Jun Ohashi, Akiyoshi Kakita, Hitoshi Takahashi, Hiroyuki Nawa, and Tadao Arinami

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

From the Department of Medical Genetics (HI, YH, MIs, MK, KI, YS, MM, TA), Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Ibaraki; Core Research for Evolutional Science and Technology (HI, YH, MK, MIt, TA), Japan Science and Technology Agency, Kawaguchi-shi, Saitama; Seiwa Hospital (TI), Institute of Neuropsychiatry, Tokyo; Department of Psychiatry (YW, MT, TSo), Niigata University, Graduate School of Medical and Dental Sciences, Niigata; Department of Neuropsychiatry (HU), Okayama University, Graduate School of Medicine, Dentistry & Pharmaceutical Sciences, Shikata-cho, Okayama; Department of Psychiatry (NI), Fujita Health University School of Medicine, Toyoake, Aichi; and Department of Psychiatry (NO), School of Medicine, Nagoya University, Nagoya, Aichi, Japan; Department of Biology (ESO), William Paterson University, Wayne, New Jersey; National Center of Neurology and Psychiatry (HK), Tokyo; Health Service Center (TSa), University of Tokyo, Tokyo; Department of Schizophrenia Research (MIt, MA), Tokyo Institute of Psychiatry, Tokyo; Tokyo Metropolitan Matsuzawa Hospital (MIt, KN, SI), Department of Psychiatry, Tokyo; Department of Human Genetics (IN), Graduate School of Medicine, University of Tokyo, Tokyo; Department of Genome Epidemiology (JO), Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Ibaraki; and Brain Research Institute (AK, HT, HN), Niigata University, Niigata, Japan.

Address correspondence to Hiroki Ishiguro, M.D., Ph.D., Department of Medical Genetics, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8575, Japan. E-mail: hishigur@md.tsukuba.ac.jp.

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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).

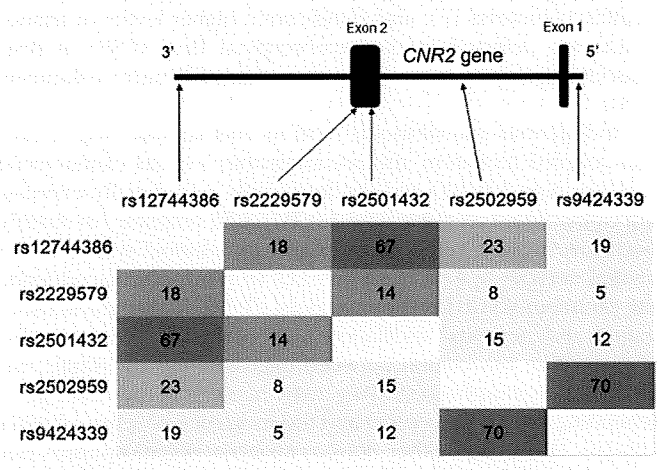

Figure 1. *CNR2* tag single nucleotide polymorphisms and the genetic structure. The genetic structure of *CNR2* is shown at top; the gene consists of two exons, and only the second exon encodes amino acids. Location and linkage disequilibrium (r^2 values) of five single nucleotide polymorphisms, including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

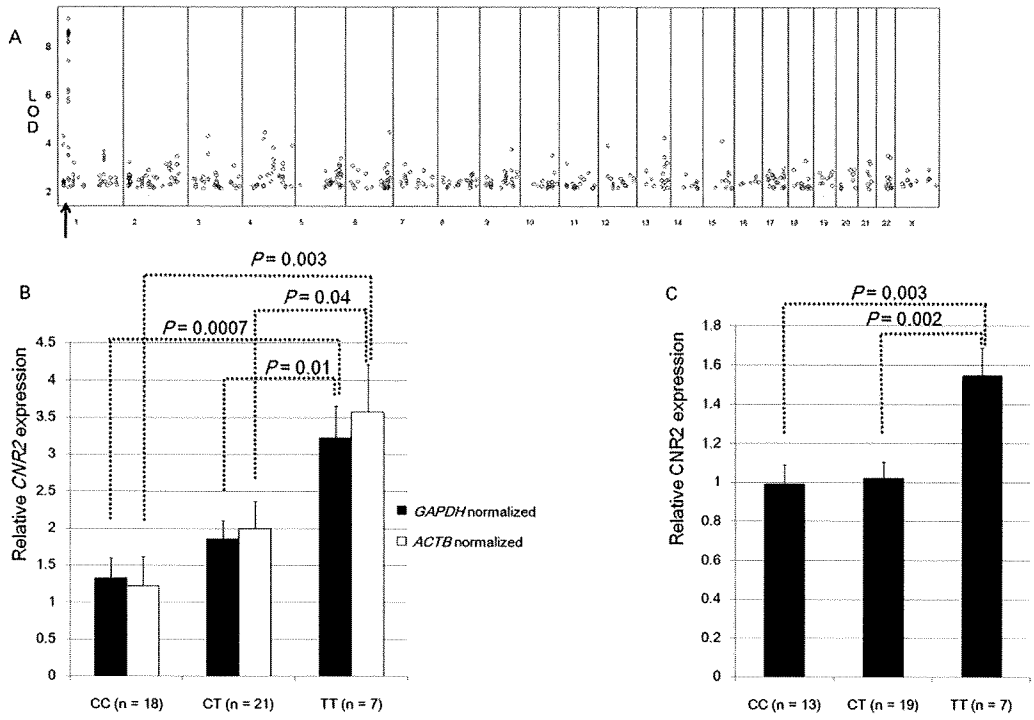

Figure 2. Analysis of cannabinoid CB2 receptor expression. **(A)** A *cis*-acting gene expression change of *CNR2* in lymphoblasts. Results of the search for polymorphisms in the *CNR2* gene locus from the mRNA by single nucleotide polymorphism browser (http://www.sph.umich.edu/csg/liang/asthma/). The 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				Permutation
	Frequency Patients	Frequency Control	χ^2	p	Frequency Patients	Frequency Control	χ^2	p	Frequency Patients	Frequency Control	χ^2	p	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

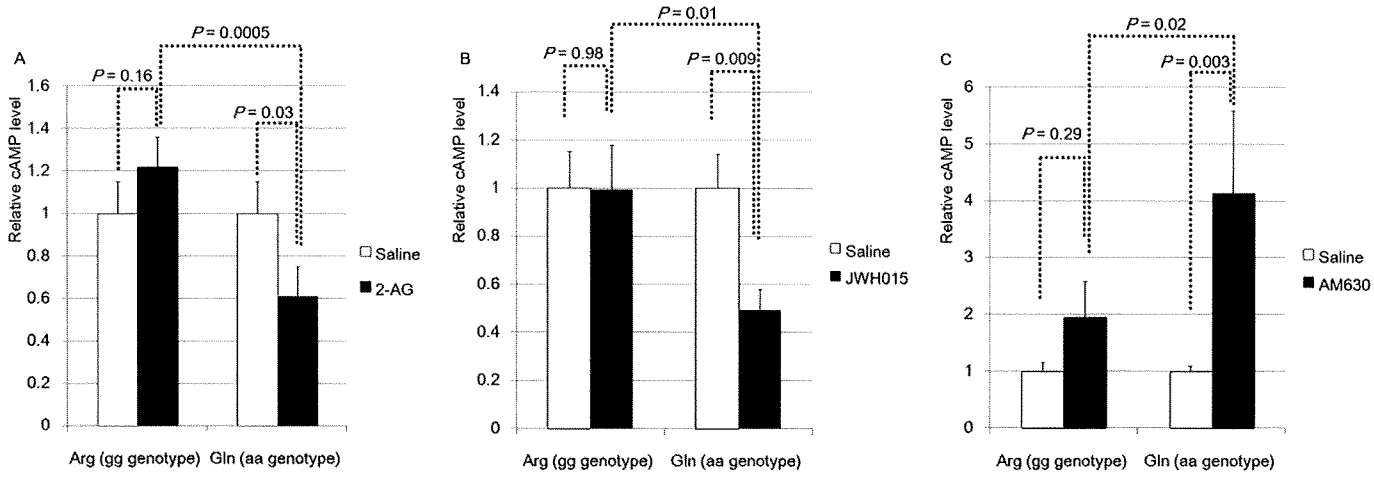

Figure 3. Functional analysis of the cannabinoid CB2 receptor with R63Q polymorphism. In cultured CHO cells expressed cloned human CB2 receptor, forskolin-evoked cyclic adenosine monophosphate (cAMP) level was regulated by three kinds of CB2 receptor ligands in this cell signaling system. CB2 receptor 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.

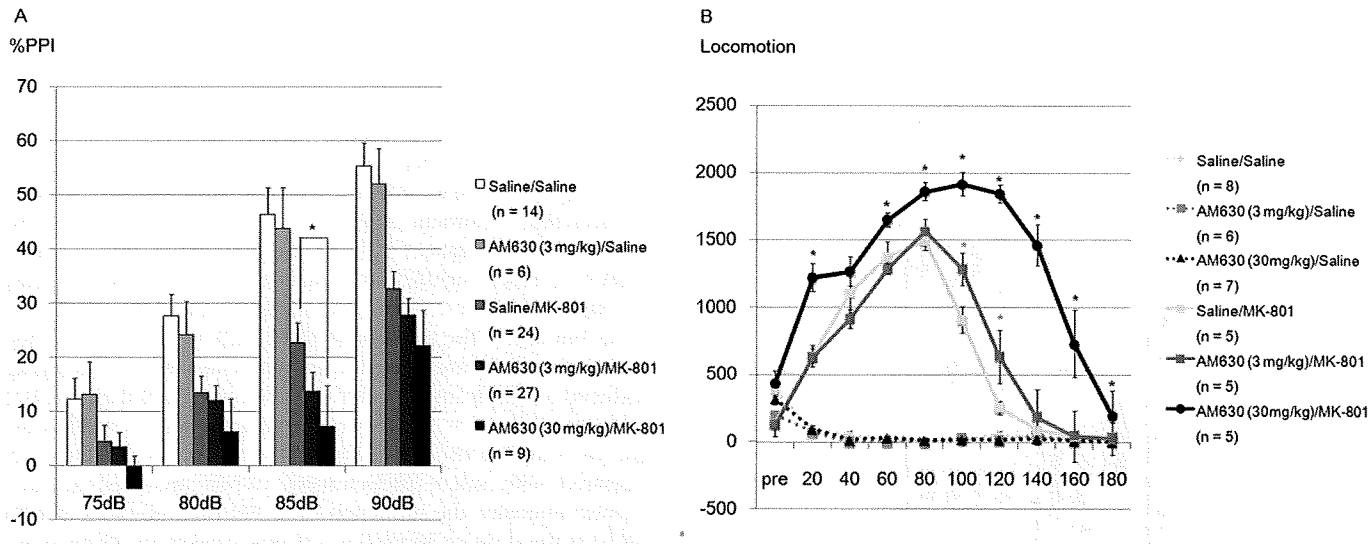

Figure 4. MK-801 induced alteration of prepulse inhibition (PPI) and locomotion influenced by AM630. **(A)** The vertical axis shows % PPI. Saline pretreated and treated before PPI test (Saline/Saline, $n = 14$), AM630 (3 mg/kg intraperitoneal [IP]) pretreated and saline treated before PPI test (AM630 [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.

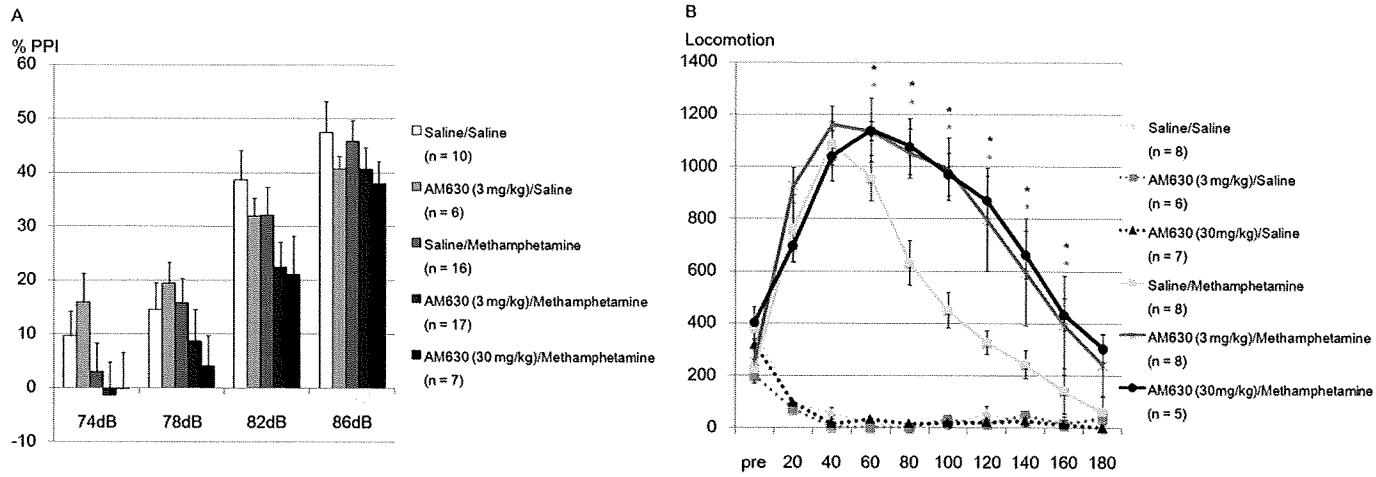

Figure 5. Methamphetamine-induced alteration of prepulse inhibition (PPI) and locomotion influenced by AM630. **(A)** The vertical axis shows % PPI. Saline pretreated and treated before PPI test (Saline/Saline, $n = 10$); AM630 (3 mg/kg intraperitoneal [IP]) pretreated and saline treated before PPI test (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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Supplementary material cited in this article is available online.

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RESEARCH ARTICLE

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Association Study of Bromodomain-Containing 1 Gene With Schizophrenia in Japanese Population

Itaru Kushima,[1,2]* Branko Aleksic,[1,2] Masashi Ikeda,[2,3,4] Yoshio Yamanouchi,[2,3] Yoko Kinoshita,[2,3] Yoshihito Ito,[1,2] Yukako Nakamura,[1,2] Toshiya Inada,[5] Nakao Iwata,[2,3] and Norio Ozaki[1,2]

[1]Department of Psychiatry, Graduate School of Medicine, Nagoya University, Aichi, Japan

[2]CREST, Japan Science and Technology Agency, Saitama, Japan

[3]Department of Psychiatry, Graduate School of Medicine, Fujita Health University, Aichi, Japan

[4]Department of Psychological Medicine, School of Medicine, Cardiff University, Health Park, Cardiff, UK

[5]Seiwa Hospital, Institute of Neuropsychiatry, Tokyo, Japan

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Chromosome 22q13 region has been implicated in schizophrenia in several linkage studies. Genes within this locus are therefore promising genetic and biologic candidate genes for schizophrenia if they are expressed in the brain or predicted to have some role in brain development. A recent study reported that bromodomain-containing 1 gene (*BRD1*), located in 22q13, showed an association with schizophrenia in a Scottish population. Except for being a putative regulator of transcription, the precise function of *BRD1* is not clear; however, expression analysis of *BRD1* mRNA revealed widespread expression in mammalian brains. In our study, we explored the association of *BRD1* with schizophrenia in a Japanese population (626 cases and 770 controls). In this association analysis, we first examined 10 directly genotyped single-nucleotide polymorphisms (SNPs) and 20 imputed SNPs. Second, we compared the *BRD1* mRNA levels between cases and controls using lymphoblastoid cell lines (LCLs) derived from 29 cases and 30 controls. Although the SNP (rs138880) that previously has been associated with schizophrenia showed the same trend in the Japanese population, no significant association was detected between *BRD1* and schizophrenia in our study. Similarly, no significant differences in *BRD1* mRNA levels in LCLs were detected. Taken together, we could not strongly show that common SNPs in the *BRD1* gene account for a substantial proportion of the genetic risk for schizophrenia in the Japanese population. © 2009 Wiley-Liss, Inc.

Key words: association analysis; imputation; gene expression analysis; meta-analysis

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INTRODUCTION

Schizophrenia is a severe, debilitating disorder characterized by delusional beliefs, hallucinations, disordered speech, and deficits in emotional and social behavior. It is strongly familial, and heritability is around 80% based on twin studies [Sullivan et al., 2003]. However, the pattern of inheritance is complex, with most studies suggesting an interaction of multiple genes. There are now several positional candidate regions all over the genome that have been shown to be related to schizophrenia in genetic studies [Badner and Gershon, 2002; Williams et al., 2003].

One promising region is chromosome 22q. Initial evidence for linkage to chromosome 22q came from three markers spanning ~23 cM in the 22q13.1 region in the Maryland family sample [Pulver et al., 1994]. Additional interest in 22q13 came from a genome scan of catatonic schizophrenia pedigrees, which showed suggestive evidence for linkage ($P = 1.8 \times 10^{-3}$; non-parametric logarithms of the odds [LOD] score 1.85) on 22q13 [Stober et al., 2000]. Furthermore, a multicenter linkage study that evaluated 10 microsatellite markers spanning 22q in 779 schizophrenia pedigrees

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Itaru Kushima and Branko Aleksic contributed equally to this work.

*Correspondence to:
Dr. Itaru Kushima, Department of Psychiatry, Nagoya University Graduate School of Medicine, 65 Tsurumai-Cho, Showa-ku, Nagoya, Aichi 466-8550, Japan. E-mail: kushima.itaru@a.mbox.nagoya-u.ac.jp

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showed linkage of borderline significance to D22S1169 at 22q13.32 in the total sample when intersample heterogeneity was taken into account [Mowry et al., 2004].

A recent study [Severinsen et al., 2006] looked into this 22q13 region and reported that two single-nucleotide polymorphisms (SNPs) (rs4468 and rs138880) located within bromodomain-containing 1 gene (*BRD1*) were associated with schizophrenia in a single-marker association analysis. This gene, expressed in mammalian brain tissue, encodes a protein of unknown function that contains a bromodomain, a motif often found in transcriptional coactivators. The motif represents an evolutionarily conserved nucleotide sequence found in many chromatin-associated proteins and in nearly all known nuclear histone acetyltransferases. It is therefore thought that *BRD1* is related to transcriptional regulation [Zeng and Zhou, 2002].

BRD1 is an attractive candidate gene for schizophrenia for two reasons. First, *BRD1* as a putative transcriptional cofactor might have functional implications for susceptibility to schizophrenia. Second, it also maps to the 22q13.33 locus, the region with evidence for linkage to schizophrenia.

As mentioned, a single study has implicated genetic variants within *BRD1* locus as contributing factor to schizophrenia in a Scottish population [Severinsen et al., 2006]. To further investigate this possible association, we selected SNPs within the *BRD1* locus and carried out a case–control study in a Japanese population. In terms of understanding the relationship between *BRD1* and schizophrenia, our study brings additional information from a genetic point of view: a larger sample size, a different population, and better coverage (in terms of SNPs selected for analysis).

MATERIALS AND METHODS

Subjects

All subjects were of Japanese descent and recruited from the main island of Japan. For the association analysis, 626 patients with schizophrenia and 770 healthy controls were used (Supplementary Table I). For the expression analysis, 29 patients with schizophrenia and 30 healthy controls were used (Supplementary Table II). All patients were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, criteria based on the consensus of at least two experienced psychiatrists using an unstructured interview and review of medical records. All healthy controls were psychiatrically screened using an unstructured interview to exclude subjects with any brain disorder or psychotic disorder or who had first-degree relatives with psychotic disorders. The present study was approved by the Ethics Committees of Nagoya University, Fujita Health University. All subjects provided written informed consent after the study was described to them.

Tagging SNP Selection, SNP Genotyping, and Quality Control

The International Haplotype Mapping (HapMap) (www.hapmap.org) SNP database and ABI (Applied Biosystems) SNP browser were used to select tagging SNPs in the BRD1 locus. The screened region was extended 5 kb upstream of the annotated transcription start site and downstream at the end of the last *BRD1* exon [Neale and Sham, 2004]. The tagging SNP selection criteria were that polymorphisms had a minor allele frequency >5% in the Japanese population (release #21; phase II; July 2006). Then, we took advantage of observed linkage disequilibrium [Barrett et al., 2005] in the *BRD1* locus to exclude redundant SNPs from genotyping. In other words, if the correlation coefficient between two loci (r^2) was 0.9 or higher, then only one of the two loci was selected for the association study [Barrett et al., 2005]. Based on our criteria, 10 SNPs were selected for the analysis. The promoter SNP rs138880, which was one of the two SNPs associated with schizophrenia in the previous study [Severinsen et al., 2006], was included in these 10 SNPs. The 3′ UTR SNP rs4468, the other SNP associated with schizophrenia in the previous study, was also added to the tagging SNPs despite a lack of information on frequency of this polymorphism in a Japanese population in the HapMap database. Therefore, 11 SNPs made up the tagging set. All SNPs were genotyped by TaqMan assay (Applied Biosystems Japan Ltd, Tokyo, Japan). For quality control, three strategies were employed. First, we checked deviation from the Hardy–Weinberg equilibrium (HWE). Second, we genotyped 20 randomly selected samples for each SNP in duplicate in order to evaluate the genotype error rate. Third, we confirmed whether the minor allele frequency for each SNP genotyped in control samples was consistent with that in the Japanese population in the HapMap database.

Imputation of Ungenotyped SNPs

Because tagging SNPs was selected based on r^2, we included imputation as an exploratory method to compute genotypes of SNPs that were not selected for genotyping (untyped SNPs). The advantage of imputing untyped SNPs is that the coverage of common variants within the locus of interest can be enhanced, boosting the statistical power [Marchini et al., 2007]. The MACH program (http://www.sph.umich.edu/csg/abecasis/MACH/) was used to calculate the genotypic prediction of 20 untyped SNPs using directly typed SNP information (10 SNPs used in the screening scan) and the HapMap database (recombination map and haplotype data for the Japanese/Chinese population, release #23a; phase II; March 2008). The MACH program was recently reported to have similar imputation accuracy rates to IMPUTE and to outperform fastPHASE, PLINK, and Beagle [Pei et al., 2008]. The targeted region for imputation was limited to the *BRD1* locus as defined above.

Power Calculation

Power was calculated according to the methods described by Skol et al. [2006]. In brief, for a predefined alpha level, in the disease prevalence and inheritance model, statistical power of any given sample is a function of sample size and effect size. In other words, power is directly proportional to sample size on one side and minor allele frequency and genotype relative risk on the other side.

Statistical Methods for Association Study

Deviation from the HWE was tested by chi-square analysis. All single marker association analyses were done by calculating the