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A possible association between missense polymorphism of the breakpoint cluster region gene and lithium prophylaxis in bipolar disorder

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Abstract

Lithium is one of the most commonly used drugs for the treatment of bipolar disorder. To prescribe lithium appropriately to patients, predictors of response to this drug were explored, and several genetic markers are considered to be good candidates. We previously reported a significant association between genetic variations in the breakpoint cluster region (BCR) gene and bipolar disorder. In this study, we examined a possible relationship between response to maintenance treatment of lithium and Asn796Ser single-nucleotide polymorphism in the BCR gene. Genotyping was performed in 161 bipolar patients who had been taking lithium for at least 1 year, and they were classified into responders for lithium monotherapy and non-responders. We found that the allele frequency of Ser796 was significantly higher in non-responders than in responders. Further investigation is warranted to confirm our findings.
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Keywords: BCR (breakpoint cluster region); Bipolar disorder; Lithium; SNP (single-nucleotide polymorphism)

1. Introduction

Bipolar disorder (BPD) is one of the most distinct syndromes in psychiatry, which is characterized by recurrent episodes of mania and depression. Three representative mood stabilizers, lithium, valproate and carbamazepine, are used worldwide for its treatment, and American Psychiatric Association guideline listed lithium as a first line agent (American Psychiatric Association, 2002). However, these treatments are associated with variable rates of efficacy and often with intolerable side effects. Therefore, many researchers explored psychopathological and biological markers for good response to lithium treatment (Gelenberg and Pies, 2003; Ikeda and Kato, 2003). To date, several studies investigated possible molecular predictors of lithium efficacy. The functional polymorphism in the upstream regulatory region of the serotonin transporter gene (5-HTTLPR) has been associated with lithium efficacy in two independent studies (Serretti et al., 2001;

Abbreviations: ANOVA, analysis of variance; BCR, breakpoint cluster region; BDNF, brain-derived neurotrophic factor; BPD, bipolar disorder; BP I, bipolar I disorder; BP II, bipolar II disorder; PH domain, pleckstrin homology domain; SNP, single-nucleotide polymorphism.

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Del Zompo et al., 1999), although the polymorphism associated with better lithium response was opposite. Other numerous genetic variants including catechol-*O*-methyltransferase were not associated with lithium response (Serretti et al., 2002). The association between prophylactic lithium response and the polymorphism of the brain-derived neurotrophic factor (BDNF) gene was reported (Rybakowski et al., 2005); however, this association was not replicated in subsequent studies (Masui et al., 2006; Michelon et al., 2006).

We previously reported a significant association between genetic variants in the breakpoint cluster region gene (*BCR*), which is located on chromosome 22q11, and BPD (Hashimoto et al., 2005). The *BCR* is highly expressed in hippocampal pyramidal cell layer and dentate gyrus (Fioretos et al., 1995), and encodes a Rho GTPase-activating protein (GAP), which inactivate the Rho GTPase playing an important role in neuronal development (Diekmann et al., 1991; Negishi and Katoh, 2002). The A2387G single-nucleotide polymorphism (SNP) in the *BCR* gene [National Center for Biotechnology Information (NCBI) SNP ID: rs140504] is the non-conservative SNP giving rise to an amino acid change of asparagine to serine at codon 796 (Asn796Ser; NCBI Protein ID: NP_004318). Ser796 allele showed a significant association with BPD and stronger evidence for an association with bipolar II disorder (BPII) than bipolar I disorder (BPI) (Hashimoto et al., 2005). It has been reported that patients with BPII have greater number of abnormal mood episodes and comorbidity of other psychiatric illnesses than patients with BPI (Ayuso-Gutierrez and Ramos-Brieva, 1982; Berk and Dodd, 2005). These clinical features of BPII have been also considered as markers for poor response to lithium treatment (Ikeda and Kato, 2003). Therefore, Ser796 allele of the *BCR* gene may contribute to poorer response to lithium therapy in BPD.

In this study, we examined the possible association between prophylactic effect of lithium and Asn796Ser SNP of the *BCR* gene in Japanese patients with BPD.

2. Methods

2.1. Subjects

Subjects were 161 patients with BPD (83 patients were BPI, and 78 patients were BPII). Consensus diagnosis was made for each patient by at least two psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders 4th edition (DSM-IV) criteria. The presence of concomitant diagnoses of mental retardation, drug dependence, or other Axis I disorder, together with somatic or neurological illnesses that impaired psychiatric evaluation, represented exclusion criteria. They were composed of 76 males and 85 females with mean age of 48.2±12.8 years (mean±S.D.). All the subjects were biologically unrelated Japanese. Patients had been treated with lithium carbonate and its serum concentration was maintained between 0.4 and 1.2 mEq/L at least for one year, in a completely naturalistic setting.

Response to lithium treatment was retrospectively determined for each patient from all available information including clinical interview and medical records, by at least two psychiatrists, and the patients were classified into lithium responders and non-responders. The phenotype definition of lithium prophylaxis is a very difficult issue. Lithium responders were defined as those patients without any affective episodes during the maintenance period of lithium mono-therapy. During the maintenance period, the addition of antidepressants, antipsychotics, or anticonvulsants was regarded as a relapse, and excluded from the responder group. However, coadministration of hypnotics for sleep disturbance was allowed, and was not regarded as a relapse when subsequent affective episode did not appear.

Our definition of response to lithium treatment is full response without any affective episode during lithium treatment. This definition is similar to "excellent lithium responders" used as clinical endophenotypic marker of BPD in some molecular-genetic research (Rybakowski et al., 2005; Mamdani et al., 2007). On the other hand, recurrence index [number of episodes/duration of illness (years)] before and during lithium treatment is a better method to measure the response to lithium including partial response (Gasperini et al., 1993; Serretti et al., 2002). However, more clinical information is necessary to calculate the recurrence index. We investigated the association between the change of recurrence index and clinical variables in parts of total subjects (24 patients) whose recurrence pattern were clearly established during more than 1 year [mean 5.8±5.0 (range 1.3–21.0) years] before lithium treatment. They were composed of 9 BPI and 15 BPII patients, whose age of onset was 35.4±9.5 years old, duration from onset of illness to lithium treatment was 9.5±7.0 (range 1.3–22.0) years, number of episodes which could be clearly identified before lithium treatment was 16.3±30.3 (range 3.0–150.0), duration of lithium treatment was 6.0±4.3 (range 1.0–14.3) years, number of episodes during lithium treatment was 6.8±6.0 (range 0.0–26.0) and recurrence index before and during lithium were 2.7±2.8 (range 0.6–14.2) and 1.8±1.5 (range 0.0–5.3), respectively.

After complete description of the study, written informed consent was obtained from every subject. The study protocol was approved by institutional ethics committees.

Table 1
Clinical characteristics of subjects, sorted by response to lithium treatment

	Response to lithium treatment		
	Responders (N=43)	Non-responders (N=118)	
Subtype			χ^2test
BPI	29 (34.9%)	54 (65.1%)	$p<0.05$
BPII	14 (18.0%)	64 (82.0%)	
Gender			
Male	25 (32.9%)	51 (67.1%)	NS
Female	18 (21.2%)	67 (78.8%)	
			t-test
Age at last observation	54.4±11.8	46.1±12.4	$p<0.01$
Age of onset	41.5±13.6	32.9±10.7	$p<0.01$
Duration of illness	12.9±9.0	13.2±9.9	NS

Continuous values were represented as the mean±SD.
BPI=bipolar I disorder, BPII=bipolar II disorder,
NS=not significant.

Table 2
Allele frequencies and genotype of the Asn796Ser polymorphism of the BCR gene and response to lithium treatment

Response to lithium treatment	Allele frequency		χ^2test	Genotype		χ^2test
	Asn	Ser	p value (OR)	Asn/Asn Asn/Ser	Ser/Ser	p value
Responders (n=43)	49 (57.0%)	37 (43.0%)		35 (81.4%)	8 (18.6%)	
Non-responders (n=118)	101 (42.8%)	135 (57.2%)	0.024 (1.77)	77 (65.3%)	41 (34.7%)	0.049
Total patients (n=161)	150 (46.6%)	172 (53.4%)		112 (69.6%)	49 (30.4%)	

OR: Odds ratio.

2.2. Genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to the standard procedures. The genotype of the Asn796Ser SNP (rs140504) of the *BCR* gene was determined by TaqMan 5′-exonuclease allelic discrimination assay, described previously (Hashimoto et al., 2005). Briefly, probes and primers for detection of the polymorphism were: forward primer 5′-AGCTGGACGCTTTGAAGATCA-3′, reverse primer 5′-TGGTGTGCACCTTCTCTCTCT-3′, probe 1 5′-VIC-CCAGATCAAGAATGACAT-MGB-3′, and probe 2 5′-FAM-CCAGATCAAGAGTGACAT-MGB-3′. PCR cycling conditions were: at 95 °C for 10 min, 50 cycles of 92 °C for 15 s and 60 °C for 1 min.

2.3. Statistical analysis

Difference in clinical characteristics between responders and non-responders to lithium treatment was analyzed using the χ^2 tests for categorical variables and t tests for continuous variables. The presence of Hardy–Weinberg equilibrium was examined by using the χ^2 test for goodness of fit. Subsequently, multiple logistic regression analysis was performed to correct background difference between responders and non-responders for lithium treatment. Possible predictors (genotype of the *BCR* gene, subtype of bipolar disorder, age of onset, age at last observation, and gender) were included in the original model. Backward stepwise regression was performed, and p-value greater than 0.10 was used for variable removal. Pearson coefficient of correlation test was used for comparison between recurrence index and clinical variables. The effect of the Asn796Ser SNP on recurrence index was assessed by analysis of variance (ANOVA). All p-values reported are two-tailed. Statistical significance was defined at $p<0.05$.

3. Results

Among 161 patients with BPD, 43 patients were determined as responders and 118 patients as non-responders for the maintenance treatment of lithium. The clinical characteristics sorted by response to lithium treatment and genotype distribution were presented in Table 1. There were significant differences between responders and non-responders in subtype of bipolar disorder (BPI and BPII), age at last observation, and age of onset.

The genotype distributions for the total patients, responders, and non-responders were in Hardy–Weinberg equilibrium (total patients: χ^2=0.94, df=1, p=0.33; responders: χ^2<0.001, df=1, p=0.98; non-responders: χ^2=0.81, df=1, p=0.37). Allele frequencies and genotype distributions of the Asn769Ser polymorphism of the *BCR* gene among responders and non-responders for lithium treatment are presented in Table 2. The Ser796 allele was in excess in the non-responders rather than responders (χ^2=5.09, df=1, p=0.024; OR 1.77, 95% CI 1.08–2.92). Then, we examined patients homozygous for the Ser796 allele and the Asn796 allele carriers, separately. Patients homozygous for the Ser796 allele were significantly more common in the non-responders than the Asn796 carriers (χ^2=3.88, df=1, p=0.049; OR 2.33, 95% CI 0.99–5.49). After backward stepwise regression, the final logistic regression model included subtype of bipolar (p<0.01), age of onset (p<0.01), and genotype which is separated to the Asn796 carrier and homozygous for the Ser796 (P=0.04).

We next investigated the association between lithium response using recurrence index and clinical variables in 24 subjects with BPD. The change of recurrence index before to during lithium treatment was not associated with subtype (t=0.79, df=22, p=0.44), age of onset (correlation coefficient=−0.29, p=0.17), duration from onset of illness to lithium treatment (correlation coefficient=0.12, p=0.57), duration during treatment (correlation coefficient=0.11, p=0.60), or the Asn796Ser SNP (df=2, F=0.03, p=0.97).

We also examined the association between age of onset and recurrence index before lithium treatment, which reflects severity of illness. There was a negative trend between age of onset and recurrence index (correlation coefficient=−0.37, p=0.074). Although difference among genotype of Asn796Ser SNP was not statistically significant, the number of Ser796 allele was associated with higher recurrence index before lithium treatment (Asn/Asn=1.63±1.19, Asn/Ser=2.89±0.84, and Ser/Ser=3.23±1.19. df=2, F=0.53, p=0.60). Therefore, the Ser796 allele might also be associated with both early onset and severity of illness, which could result in poorer lithium response.

4. Discussion

We investigated a possible association between the *BCR* gene and the prophylactic effect of lithium treatment in patients with BPD for the first time. As expected, our results suggested that lithium treatment might be less effective in patients homozygous for the Ser796 allele of the *BCR* gene than in patients with the Asn796 allele. In addition, allele frequencies of the Ser796 associated with poorer lithium response were 43.0%

in responders and 57.2% in non-responders. As allele frequency of the Ser796 in healthy subjects in our previous study was 48.1% (Hashimoto et al., 2005), allele frequency of the Ser796 of responders is similar to the general population.

Comparing clinical characteristics of responders and non-responders, there were more BPII patients in non-responder group. Clinical characteristics predicting poorer response to lithium therapy and that of BPII seem to overlap each other, but better lithium response in BPI is not universally accepted. We excluded any Axis I comorbidity in this study. This would leave in more typical bipolar II patients who would be more likely to respond to lithium, however, other clinical factors such as Axis II comorbidity might influence our results. The presence of positive family history of lithium responsive BPD has been reported as indicative of favorable response (Grof et al., 2002). However, it was not assumed that our sample size was enough to investigate this issue because only 8.7% of BPD had positive family history of the same disease in 1st degree relatives (Smoller and Finn, 2003). Therefore, information about family history of lithium response was not collected in this study.

Age at onset was also different between responders and non-responders, and early age of onset was associated with poorer response to lithium treatment in our subjects. This observation is consistent with recent meta-analysis (Kleindienst et al., 2005). As the objective of this study is to examine the association between response to lithium treatment and a SNP in the *BCR* gene, the differences in demographic parameters of responders and non-responders might not be preferable. Therefore, we conducted a multiple logistic regression analysis, and homozygous for the Ser796 allele of the *BCR* gene was still significantly associated with poorer response to lithium treatment.

The evaluation of lithium prophylaxis is considerably difficult because of complex clinical course of BPD, and each researcher has used different methodologies. Although our finding was based on the simple definition, in which lithium responders didn't have any affective recurrences during lithium, one of the limitation of this study is lack of detailed clinical information, e.g. duration from onset of illness to lithium treatment and number of episodes which could be clearly identified before lithium treatment in total subjects. To evaluate lithium efficacy including partial response, calculating recurrence index before and during lithium treatment is used in several researches. This would be a correct measure of lithium prophylaxis, but evaluating mood recurrence accurately before the first contact to mental professionals is difficult. We tried to evaluate lithium response with recurrence index; however, we could examine it in only 24 subjects out of 161 subjects due to the difficulty of collecting this clinical information. We did not found any association between the recurrence index and clinical variables and the SNP in the *BCR* gene, except for the trend between the recurrence index and age of onset. As these results were from subgroup analysis with smaller number, further investigation is needed in a larger sample size.

In this study, the same variant associated with the illness was also associated with poorer outcome. This situation is similar to that of the Val allele of the *BDNF* Val66Met polymorphism (Rybakowski et al., 2005), and it is possible that the *BCR* Ser796 and the *BDNF* Val66 alleles are associated with severer illness presentation. The trend between the recurrence index and age of onset in our subgroup analysis might imply this possibility. In case of the *BDNF* Val66Met SNP, the functional differences arisen from each allele were reported (Eagan et al., 2003). While biological functional of the *BCR* Asn796Ser SNP is still unknown, this SNP may produce functional difference in the brain, like the *BDNF* Val66Met SNP. To speculate this issue, it is noteworthy that this SNP is in the pleckstrin homology (PH) domain of the *BCR*. As PH domain is known for its ability to bind phosphatidylinositol and this binding regulates the activity of PH domain containing protein (Lemmon et al., 2002), signal transduction from inositol cycle to the *BCR* products might be affected by this SNP. As the *BCR* is RhoGAP, this change may influence on the activity of its downstream target, RhoGTPase, which activates many kind of effectors associated with constructing neuronal network, and subsequently influence on neuronal development. Additionally, as inositol cycle is considered as one of therapeutic targets of lithium (Harwood, 2005), this SNP could alter the clinical efficacy of lithium. To understand the mechanism of our findings, it is worth investigating whether the Asn796Ser SNP alters the binding ability of PH domain to inositol.

5. Conclusion

This is the first report demonstrating that long-term lithium treatment may be less effective in BPD patients homozygous for Ser796 allele of the *BCR* gene than in patients with the Asn796 allele. The limitations of this study are retrospective design without placebo control group, small sample size, and lack of clinical information such as presence of rapid cycling and/or psychotic symptoms, and detailed lithium levels. Further investigations are needed to confirm our findings.

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ORIGINAL ARTICLE

Pituitary adenylate cyclase-activating polypeptide is associated with schizophrenia

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Pituitary adenylate cyclase-activating polypeptide (PACAP, ADCYAP1: adenylate cyclase-activating polypeptide 1), a neuropeptide with neurotransmission modulating activity, is a promising schizophrenia candidate gene. Here, we provide evidence that genetic variants of the genes encoding PACAP and its receptor, PAC1, are associated with schizophrenia. We studied the effects of the associated polymorphism in the PACAP gene on neurobiological traits related to risk for schizophrenia. This allele of the PACAP gene, which is overrepresented in schizophrenia patients, was associated with reduced hippocampal volume and poorer memory performance. Abnormal behaviors in PACAP knockout mice, including elevated locomotor activity and deficits in prepulse inhibition of the startle response, were reversed by treatment with an atypical antipsychotic, risperidone. These convergent data suggest that alterations in PACAP signaling might contribute to the pathogenesis of schizophrenia.
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Keywords: schizophrenia; PACAP; SNP; hippocampus; memory; PPI

Introduction

Schizophrenia is a common neuropsychiatric disorder affecting 0.5–1% of the general population worldwide. This disease is characterized by psychosis and profound disturbances of cognition, emotion and social functioning. The pathophysiology of schizophrenia is still unclear; however, this disease is highly heritable[1] and several intermediate phenotypes such as neurocognitive dysfunction, abnormal brain morphology and deficits in prepulse inhibition (PPI) of the startle response are known to be useful to identify susceptibility genes for schizophrenia.[2,3]

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The adenylate cyclase-activating polypeptide 1 (ADCYAP1) gene encodes pituitary adenylate cyclase-activating polypeptide (PACAP), a neuropeptide, which is a member of the vasoactive intestinal peptide (VIP)/secretin/glucagon family. It exerts multiple activities as a neurotransmitter or neuromodulator via three heptahelical G-protein-linked receptors, one PACAP-specific (PAC1) receptor and two receptors that are shared with VIP (VPAC1 and VPAC2).[4–6] PACAP induces cyclic AMP accumulation through activation of these receptors.[4–6] We generated mice lacking the PACAP gene ($PACAP^{-/-}$); these mice had profound behavioral abnormalities including hyperactivity and explosive jumping in an open field, increased novelty-seeking behavior and deficits in PPI.[7,8] In addition, the PACAP gene is located on 18p11, which linkage studies have suggested as a locus for schizophrenia and bipolar disorder.[9] Although previous studies indicated that the PACAP gene could be a good candidate gene for schizophrenia, only one preliminary study has examined a

possible association with schizophrenia and reported negative results.[10] Here, we present data demonstrating a possible association between PACAP-PAC1 signaling and schizophrenia, using a multidisciplinary approach in both humans and rodents.

Materials and methods

Subjects

Subjects for the clinical association study were 804 patients with schizophrenia (51.1% males with a mean age of 44.2 years (s.d. 14.5) and a mean age of onset of 24.8 years (s.d. 8.8)) and 967 healthy controls (47.7% males with a mean age of 40.4 years (s.d. 16.1)). All the subjects were biologically unrelated Japanese. Three hundred and fifty-one patients with schizophrenia and 518 controls were from Tokyo Metropolitan (the east part of Japan), and 453 patients with schizophrenia and 449 controls were from Aichi prefecture (the central part of Japan). Patients were recruited at the National Center Hospital of Mental, Nervous, and Muscular Disorders; Nagoya University Hospital; Showa University Hospital and hospitals related to Department of Psychiatry, Nagoya University Graduate School of Medicine or Department of Psychiatry, Showa University School of Medicine. Healthy controls, including hospital and institutional staff, were recruited from local advertisements in Tokyo and Aichi. Magnetic resonance (MR) measurements and neurocognitive tests were performed only on some subjects (MR measurements: 81 patients with schizophrenia and 201 healthy controls; neurocognitive tests: 62 patients with schizophrenia and 139 healthy controls), all of whom were recruited at National Center of Neurology and Psychiatry. Demographic information for the subjects receiving MR measurements and neurocognitive tests is shown in detail in Supplementary Table 1 and Figure 1b. Consensus diagnosis was made for each patient by at least two trained psychiatrists, according to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria, based on clinical interview and other available information including medical records and other research assessments. No patient was diagnosed by medical records alone. Controls were healthy volunteers who had no current or past contact to psychiatric services. After a description of the study, written informed consent was obtained from every subject. The study protocol was approved by institutional ethics committees.

Genetic analysis

Venous blood was drawn from subjects and genomic DNA was extracted from whole blood according to standard procedures. Seven single nucleotide polymorphisms (SNPs) in the PACAP gene and three SNPs in the PAC1, VPAC1 and VPAC2 genes were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay, as described previously.[11,12] Primers and probes for the detection of the SNPs are available on request. Statistical analysis of genetic association studies was performed using SNPAlyse (DYNACOM, Yokohama, Japan). The presence of Hardy–Weinberg equilibrium was examined by using the χ^2 test for goodness of fit. Allele distributions between patients and controls were analyzed by the χ^2 test for independence. All P-values reported are two-tailed. Statistical significance was defined as $P < 0.05$.

Neuroimaging analysis

All MR studies were performed on a 1.5 T Siemens Magnetom Vision plus system (Siemens, Erlangen, Germany). A three-dimensional volumetric acquisition of a T1-weighted gradient echo sequence produced a gapless series of 144 sagittal sections using an MPRage sequence (TE/TR, 4.4/11.4 ms; flip angle, 15°; acquisition matrix, 256×256; 1NEX, field of view, 31.5 cm; slice thickness, 1.23 mm).

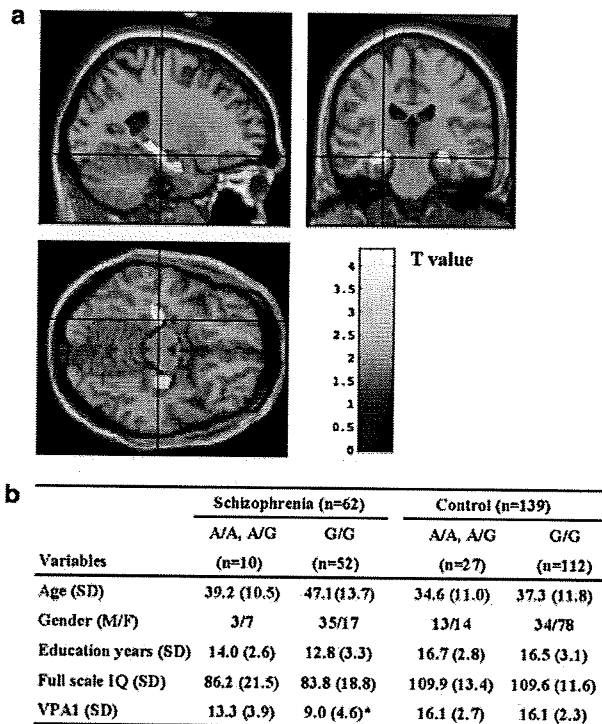
Data were analyzed with Statistical Parametric Mapping 2 (SPM2) running on MATLAB 6.5. MR images were processed using optimized voxel-based morphometry (VBM) in SPM2 as described in detail previously.[13,14] Normalized segmented images were modulated by multiplication with Jacobian determinants of the spatial normalization to encode the deformation field for each subject as tissue density changes in normal space. Following modulation, images were smoothed using a 12 mm full-width half-maximum of isotropic Gaussian kernel, because previous studies had proved that this should be a reasonable filter.[13,15,16] In addition, we confirmed that the results of statistical analyses with three different smoothing filters (6, 8 and 12 mm Gaussian kernels) were essentially the same.

Statistical analyses were performed with SPM2, which implemented a general linear model. A hypothesis-driven regions of interest (ROIs) approach was used to investigate the hippocampus, using an ROI from the Wake Forest University PickAtlas.[17] Our hypothesis is that the PACAP genotype related to the risk of developing schizophrenia is associated with hippocampal volume, because PACAP is associated with hippocampal function in rodents, and hippocampal volume is reported to be reduced in schizophrenia. The genotype and diagnostic effects on hippocampal gray matter volume change were assessed statistically using a single-subject condition and covariate model with a significance level set to 0.05 (corrected for multiple comparisons within the ROI). Age and gender were included in the model to control for confounds. Anatomic localization was according to both MNI coordinates and Talairach coordinates, obtained from M. Brett's transformations (http://www.mrccbu.cam.ac.uk/Imaging/Common/mnispace.shtml) and presented as Talairach coordinates.

Neurocognitive tests

Several memory tests, subscales of the Wechsler Memory Scale revised version (logical memory I, logical memory II, visual reproduction I, visual reproduction II, verbal paired associates I (VPAI),

verbal paired associates II, visual paired associates I and visual paired associates II) and the general intelligence IQ (from full scale of the Wechsler Adult Intelligence Scale, revised edition, WAIS-R), were performed by some of the subjects recruited at National Center of Neurology and Psychiatry. In association analysis between SNP3 of the PACAP gene and VPAI, group comparisons of demographic data were performed by using unpaired *t*-tests or χ^2, as appropriate. There were no differences between genotype groups and demographic variables, for example, age, gender, education years and full-scale IQ, except for gender distribution in patients with schizophrenia ($P = 0.026$) (Figure 1b). The effects of the SNP3 genotype of the PACAP gene and diagnosis on scores of memory tests were analyzed by a two-way analysis of covariance (ANCOVA), with age, gender and education years as covariates using SPSS 11.0J for Windows (SPSS Japan Inc., Tokyo, Japan).



Variables	Schizophrenia (n=62) A/A, A/G (n=10)	Schizophrenia (n=62) G/G (n=52)	Control (n=139) A/A, A/G (n=27)	Control (n=139) G/G (n=112)
Age (SD)	39.2 (10.5)	47.1 (13.7)	34.6 (11.0)	37.3 (11.8)
Gender (M/F)	3/7	35/17	13/14	34/78
Education years (SD)	14.0 (2.6)	12.8 (3.3)	16.7 (2.8)	16.5 (3.1)
Full scale IQ (SD)	86.2 (21.5)	83.8 (18.8)	109.9 (13.4)	109.6 (11.6)
VPA1 (SD)	13.3 (3.9)	9.0 (4.6)*	16.1 (2.7)	16.1 (2.3)

Figure 1 Genetic variation of PACAP is associated with hippocampal morphology and memory in humans. **(a)** Statistical maps of *t*-transformed hippocampal volume differences derived by optimized VBM of individuals homozygous for the G allele in SNP3 of the PACAP gene, relative to A-carriers, in all subjects, thresholded at $P < 0.05$ (corrected) in coronal, sagittal and axial views. These data show bilateral significant hippocampal volume reduction in individuals homozygous for the G allele. **(b)** Lower visual associate memory I score in individuals homozygous for the G allele in SNP3 of the PACAP gene, compared to A-carriers, in the schizophrenia group. Means $\pm$ s.d. are shown. VPAI, visual paired associates I. $^*P < 0.05$, compared with A-carriers.

When genotype effects on VPAI in controls or patients with schizophrenia were examined separately, a Mann–Whitney *U*-test and ANCOVA with gender as a covariate were used.

Animal study
All animal experiments were carried out in accordance with protocols approved by the Animal Research Committee of Osaka University and by the Ethics Review Committee for Animal Experimentation of the National Institute of Neuroscience. Generation of PACAP$^{-/-}$ mice by a gene targeting technique has been reported previously.[7] The null mutation was backcrossed onto the genetic background of Crlj:CD1 (Institute of Cancer Research) mice purchased from Charles River (Tokyo, Japan). All wild-type control mice and PACAP$^{-/-}$ mice (homozygous for the mutant PACAP gene) used in locomotor activity and PPI experiments were obtained from the intercross of heterozygous animals. C57BL/6J mice were purchased from Charles River and were allowed to acclimate in our animal facility for at least 5 days before initiation of experiments. Mice were housed in a temperature- (23 $\pm$ 1°C) and light-controlled room with a 12 h light–dark cycle (lights on from 0800 to 2000) and allowed free access to water and food, except during behavioral testing.

Locomotor activity was quantified using an infrared photocell beam detection system, Acti-Track (Panlab, Barcelona, Spain). Following intraperitoneal injection of risperidone (0.1 mg/kg) or an equivalent amount of saline, mice were placed in plastic activity monitoring boxes (30 $\times$ 30 $\times$ 30 cm) and tracked for 60 min, with data being stored permanently; parameters indicative of locomotor activity, such as distance traveled, were assessed. Each mouse was tested individually and had no contact with the other mice. The PACAP mutant cohort used in locomotor activity testing consisted of 12 wild-type mice and 12 PACAP$^{-/-}$ mice ($n = 6$ each for saline control and risperidone groups).

Acoustic startle responses for PPI were measured in a startle chamber (SR-LAB; San Diego Instruments, CA, USA) as described.[18] Mice were placed in the startle chamber for 30 min after intraperitoneal injection of risperidone (0.1 mg/kg) or an equal amount of saline. The testing session started with 5 min of acclimatization to the startle chamber in the presence of 65 dB background broadband (white) noise. Testing consisted of forty 120 dB pulses alone and 10 pulses preceded (100 ms) by a prepulse of 66, 68, 71 or 77 dB. Pulses were randomly presented with an average of 15 s between pulses. Twelve no-stimulus trials were included to assess spontaneous activity during testing. PPI was calculated as a percentage score: PPI (%) = (1−((startle response for pulse with prepulse)/(startle response for pulse alone))) $\times$ 100. The PACAP mutant cohort used in PPI testing consisted of 35 wild-type mice (saline control group = 22; risperidone group = 13) and 33 PACAP$^{-/-}$ mice (saline control group = 17; risperidone group = 16).

Male C57BL/6J mice weighing 20–25 g received once-daily injections intraperitoneally for 14 days with phencyclidine (PCP) (5 mg/kg; $n=13$) or saline for control ($n=12$). PACAP and PAC1 mRNA levels were measured by a real-time quantitative RT–PCR method (TaqMan assay, Applied Biosystems, Tokyo, Japan), using total RNA extracted from the frontal cortex or hippocampus of mice treated with PCP or saline, as described previously.[19] Statistically significant differences were assessed by the Mann–Whitney *U*-test.

Results

Genetic analysis

We examined the possible association between schizophrenia and genetic variations in the PACAP gene. Seven SNPs in the PACAP gene, selected from public databases, were genotyped, and the genotype distributions of all seven SNPs in the PACAP gene were in Hardy–Weinberg equilibrium in both controls and patients with schizophrenia (data not shown). The allele frequencies of the seven SNPs in patients and controls are shown in Table 1. The major allele of SNP3 and the minor allele of SNP5 were in excess in patients with schizophrenia when compared to controls (SNP3: $\chi^2=7.6$, $P=0.0059$, odds ratio = 0.74, 95% confidence interval (CI) 0.59–0.92; SNP5: $\chi^2=4.2$, $P=0.041$, odds ratio = 1.38, 95% CI 1.01–1.84), whereas no significant association of the other five SNPs with schizophrenia was observed (Table 1). SNP3 was significantly associated with schizophrenia after Bonferroni correction (corrected $P=0.041$). We next examined the possible association between schizophrenia and genes encoding the receptors for PACAP, such as the PAC1, VPAC1 and VPAC2 receptor genes. The genotype distributions of all three SNPs in the PAC1, VPAC1 and VPAC2 genes were in Hardy–Weinberg equilibrium in both controls and patients with schizophrenia, except for that of SNP3 of the VPAC1 gene in controls (data not shown). The allele frequencies of the three SNPs in each receptor gene in the patients and controls are shown in Table 2. There was significant evidence for an association between a genetic variant of the PAC1 gene and schizophrenia (SNP2: $\chi^2=6.0$, $P=0.014$, odds ratio = 1.18, 95% CI 1.03–1.35, corrected $P=0.042$), whereas none of the SNPs in the genes encoding VPAC1 or VPAC2 was associated with schizophrenia (Table 2). The evidence that the genes encoding PACAP and its receptor PAC1 are associated with schizophrenia suggests that signaling through PACAP and PAC1 might be associated with the pathophysiology of schizophrenia.

Intermediate phenotype

As the PACAP gene has been reported to play a role in learning and memory and hippocampal long-term potentiation in rodents,[20,21] we next examined the possible impact of SNP3 of the PACAP gene, which was associated with schizophrenia, on hippocampal volume in patients with schizophrenia and controls. A genotype effect was found as bilateral reductions of hippocampal volumes (right: $P=0.04$, $t=3.2$; left: $P=0.002$, $t=4.1$) in homozygous G subjects compared with A-carriers (Figure 1a). There was also a diagnostic effect, a significant reduction in left hippocampal volume in patients with schizophrenia compared with controls ($P=0.033$, $t=3.3$). Genotype–diagnosis interaction effects on brain morphology were not found, even at a lenient threshold (uncorrected $P=0.05$). We next estimated the effects of genotypes on hippocampal volume in the control groups and schizophrenic groups, separately. Schizophrenic patients homozygous for the G allele showed a significant reduction in bilateral hippocampal volumes (right: $P=0.013$, $t=3.5$; left: $P=0.005$, $t=3.9$). On the other hand, we found significantly decreased volumes of the bilateral hippocampi in homozygous G subjects compared with the A-carriers, at a lenient threshold (uncorrected $P=0.05$) in controls; however, no voxels could survive after the correction for multiple comparisons. These data

Table 1 Allele frequencies of seven SNPs in the PACAP gene between the patients with schizophrenia and controls

SNP-ID	*dbSNP*	*Distance from SNP1*	*Major/minor polymorphism*	*Location*	*Number of subjects*		*Minor allele frequency*		P-*value*	*Odds ratio (95% CI)*
					Controls	*Patients*	*Controls*	*Patients*		
SNP1	rs2846584	—	C/T	5′-region	967	804	0.362	0.373	0.54	
SNP2	rs2231181	712	G/C	5′-UTR	960	795	0.336	0.330	0.69	
SNP3	rs1893154	1071	G/A	Intron1	951	797	0.126	0.097	0.0059	0.74 (0.59–0.92)
SNP4	rs1893153	1149	T/A	Intron1	953	793	0.174	0.163	0.37	
SNP5	rs2856966	3656	A/G	Exon3 (D54G)	953	786	0.047	0.063	0.041	1.38 (1.01–1.84)
SNP6	rs928978	4481	C/A	Intron4	958	798	0.475	0.485	0.58	
SNP7	rs1610037	6581	A/G	3′-region	962	794	0.216	0.211	0.73	

Abbreviations: CI, confidence interval; PACAP, pituitary adenylate cyclase-activating polypeptide; SNPs, single nucleotide polymorphisms.
Minor allele frequencies in controls are shown. Significant results ($P<0.05$) are indicated with underline.

Table 2 Allele frequencies of SNPs in the PAC1, VPAC1 and VPAC2 gene between the patients with schizophrenia and controls

Gene name	SNP-ID	dbSNP	Distance from SNP1	Major/minor polymorphism	Location	Number of subjects		Minor allele frequency		P-value	Odds ratio (95% CI)
						Controls	Patients	Controls	Patients		
PAC1	SNP1	rs1468687	—	T/C	Intron2	950	796	0.287	0.264	0.12	
	SNP2	rs2302475	15553	C/T	Intron5	958	797	0.479	0.520	0.014	1.18 (1.03–1.35)
	SNP3	rs2267742	34598	A/G	Intron12	936	786	0.127	0.133	0.58	
VPAC1	SNP1	rs735773	—	C/G	Intron1	937	784	0.357	0.38	0.16	
	SNP2	rs406360	12972	A/G	Intron4	948	789	0.431	0.433	0.91	
	SNP3	rs3733055	22942	G/T	Exon13 (R445L)	958	801	0.041	0.035	0.33	
VPAC2	SNP1	rs885861	—	C/T	3′-UTR	963	802	0.208	0.232	0.090	1.15 (0.98–1.36)
	SNP2	rs3793224	55026	C/T	Intron4	944	791	0.247	0.232	0.29	
	SNP3	rs3812312	109228	C/T	Intron2	923	781	0.221	0.218	0.85	

Abbreviations: CI, confidence interval; SNPs, single nucleotide polymorphisms.
Minor allele frequencies in controls are shown. Significant results ($P<0.05$) are indicated with underline.

suggest that SNP3 in the PACAP gene could have an impact on hippocampal morphology.

As the human hippocampus is related to memory function, we also examined the association between SNP3 of the PACAP gene and several subscales of the Wechsler memory scale revised version in patients with schizophrenia and controls (Figure 1b). Two-way ANCOVA on VPAI revealed significant effects of diagnosis ($F=33.8$, $P<0.0001$) and genotype of SNP3 ($F=5.2$, $P=0.024$), and an interaction between diagnosis and genotype ($F=6.6$, $P=0.011$), whereas an effect of genotype was not found in other memory subscales (data not shown). Individuals homozygous for the G allele of SNP3, which was enriched in schizophrenia, had lower scores of VPAI than schizophrenic patients carrying the A allele (Mann–Whitney U-test: $P=0.015$); however, there was no difference between the two genotypes in the control group ($P>0.8$). ANCOVA with gender as a covariate did not alter the statistical significance of these results in patients with schizophrenia ($P=0.029$). These data suggest that the risk SNP of the PACAP gene could be associated with reduced hippocampal volume and poorer memory performance, which are neurobiological traits related to risk for schizophrenia.

Animal study

As our data indicate that PACAP might be associated with schizophrenia, PACAP knockout mice (PACAP$^{-/-}$ mice) could be a possible animal model for schizophrenia. Several schizophrenia-related behaviors in rodents, such as hyperactivity, deficits in PPI, locomotor response to antipsychotics, disturbance in social interaction and cognitive deficits, have been commonly observed in previous pharmacological and genetic animal models for schizophrenia.[22] Therefore, we examined the impact of an atypical antipsychotic, risperidone, on hyperactivity and deficits in PPI in PACAP$^{-/-}$ mice. PACAP$^{-/-}$ mice maintained high initial levels of locomotor activity during the open field test (Figure 2a and b), as reported previously.[7] When treated with risperidone, hyperlocomotion in PACAP$^{-/-}$ mice was attenuated almost to the normal levels seen in wild-type mice; however, treatment with risperidone had no significant effect on locomotor activity in wild-type mice (Figure 2a and b). Risperidone also reversed the diminished PPI in PACAP$^{-/-}$ mice[8] to the control level seen in wild-type mice (Figure 2c). Risperidone had no significant effect on PPI levels in wild-type mice (Figure 2c) and startle amplitudes in both PACAP$^{-/-}$ and wild-type mice (data not shown). These results suggest that the abnormal behaviors in PACAP$^{-/-}$ mice, which are believed to be schizophrenia-like phenotypes in rodents, can be rescued by an atypical antipsychotic, risperidone.

The abuse of PCP, an N-methyl-D-aspartic acid receptor antagonist, results in positive symptoms, negative symptoms and cognitive impairments, similar to those seen in patients with schizophrenia. Thus, mice chronically treated with PCP have been used as a potential animal model for schizophrenia.[23] To assess a possible change in the expression of PACAP and PAC1 receptor in the pathological state, we performed mRNA expression analysis for PACAP and PAC1 in the frontal cortex and hippocampus of mice chronically treated with PCP. The expression level of PACAP mRNA was significantly reduced in the frontal cortex, but not in the hippocampus (Supplementary Figure 1). On the other hand, increased expression of PAC1 mRNA was observed in both frontal cortex and hippocampus (Supplementary Figure 1). Although the altered expression of PACAP and PAC1 in mouse brains treated with PCP was subtle, these data are considered to be in line with the behavioral abnormalities in PACAP$^{-/-}$ mice, a possible animal model for schizophrenia.

These results using animal models support the notion that PACAP is associated with the pathophysiology of schizophrenia.

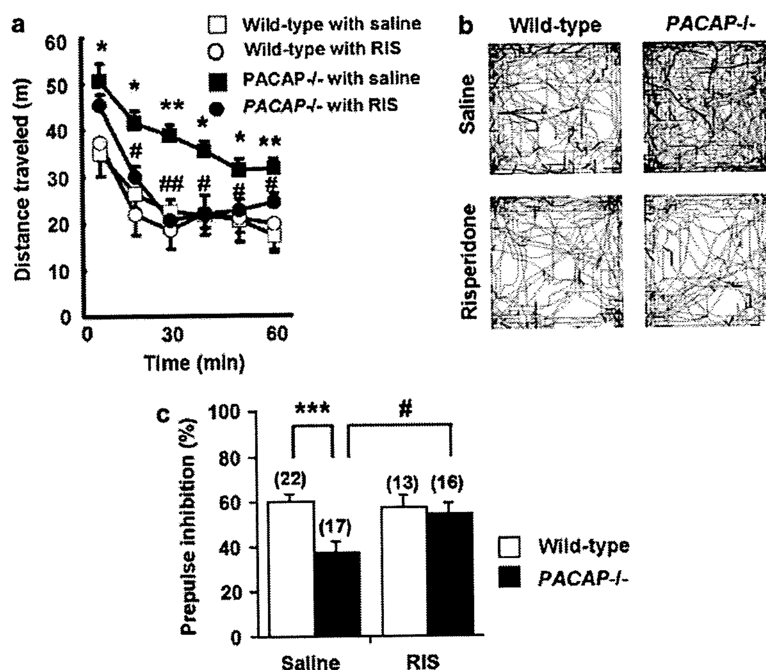


Figure 2 Hyperlocomotion and deficits in the PPI of PACAP$^{-/-}$ mice were normalized by risperidone treatment. (**a**) Locomotor activity in wild-type and PACAP$^{-/-}$ mice that received 0.1 mg/kg risperidone (RIS) or saline. $n = 6$ per group. (**b**) Representative locomotor patterns of saline- or 0.1 mg/kg risperidone-treated wild-type and PACAP$^{-/-}$ mice during 25–30 min of a 60 min recording in an open field test. (**c**) PPI levels induced by a 77 dB prepulse in wild-type and PACAP$^{-/-}$ mice after pretreatment with risperidone (0.1 mg/kg) or saline. Numbers of animals for experiments are shown in parentheses. Data are given as means ± s.e.m. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared to wild-type. #$P < 0.05$, ##$P < 0.01$, compared with saline in PACAP$^{-/-}$ mice.

Discussion

Our findings support the possibility that PACAP is a potential schizophrenia susceptibility gene. Clinical association between schizophrenia and the genes encoding PACAP and PAC1 and an association between intermediate phenotypes, hippocampal volume and visual associate memory performance and a risk SNP in the PACAP gene have been demonstrated in our study. There are several limitations in our results. We screened control subjects with no past or current visits to psychiatric services; however, we could not exclude the possibility that they have an undiagnosed or untreated psychiatric disorder. The obtained evidence for association was not very strong, especially in the association between the genotype and visual associate memory performance ($P < 0.05$ level). When we applied corrections for multiple testing for several memory tests, this positive association became negative. This association is not conclusive, although the association between the risk allele for schizophrenia and poorer memory performance might be attractive. Thus, replication studies should be conducted to confirm our findings. We do not know whether SNP3 alters the expression/function of the PACAP gene. Accordingly, there remains the possibility that other polymorphisms, which are in linkage disequilibrium to this polymorphism, are truly responsible for giving susceptibility.

Studies aiming to identify susceptibility genes for schizophrenia are faced with the confounds of subjective clinical criteria and the likelihood of allelic and locus heterogeneity. Although schizophrenia is substantially heritable, the mode of inheritance is complex, involving numerous genes of small effect and a nontrivial environmental component. The concept of intermediate phenotype (endophenotype) assumes that neurobiological deficits occur across the schizophrenia spectrum in schizophrenia patients, schizotypal patients and clinically unaffected relatives of schizophrenia patients. The intermediate phenotype approach is an alternative method for measuring phenotypic variation that may facilitate the identification of susceptibility genes in the context of complexly inherited traits. Using this approach, we showed an association between the PACAP gene and two intermediate phenotypes, hippocampal volume and visual associate memory, in addition to the genetic association with schizophrenia. Our study could be a successful example of using this strategy to find susceptibility genes for complex diseases.

The hyperactivity and deficits in PPI observed in PACAP$^{-/-}$ mice[7,8] are believed to be schizophrenia-like behaviors in rodents. PAC1 knockout mice also show abnormal behaviors, including elevated locomotor activity and abnormal social behavior.[24,25] Our genetic findings, which demonstrate an association

between schizophrenia and two genes, PACAP and PAC1, are supported by the abnormal behaviors in knockout mice of PACAP and PAC1. Risperidone, an atypical antipsychotic, has the advantage of better extrapyramidal tolerability than conventional antipsychotics, but also has advantages in cognitive disturbances and the treatment of negative and depressive symptoms.[26] Our previous study showed that haloperidol, a representative conventional antipsychotic, rescued hyperactivity,[7] but did not rescue deficits in PPI.[8] As risperidone treatment rescued both of these abnormalities in PACAP$^{-/-}$ mice, and as risperidone is a combined D2 and 5-HT$_{2A}$ receptor antagonist, either dopamine or serotonin signaling, or both, could be relevant to the abnormal behaviors in PACAP$^{-/-}$ mice.

Our convergent evidence suggests that investigation of PACAP-PAC1 signaling in the brain could provide a clue to elucidating the possible mechanisms of pathophysiology in schizophrenia.

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RESEARCH ARTICLE

Variants of dopamine and serotonin candidate genes as predictors of response to risperidone treatment in first-episode schizophrenia

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Aims: Abnormalities in dopaminergic and serotonergic transmission systems are thought to be involved in the pathophysiology of schizophrenia and the mechanisms underlying the therapeutic effects of antipsychotics. We conducted a pharmacogenetic study to evaluate whether variants in dopamine-related genes (*DRD1–DRD5, AKT1* and *GSK3β*) and serotonin receptor genes (*HTR1A, HTR1B, HTR1D, HTR2A, HTR2C, HTR6* and *HTR7*) can be used to predict the efficacy of risperidone treatment for schizophrenia. **Materials & methods:** A total of 120 first-episode neuroleptic-naive schizophrenia patients were treated with risperidone monotherapy for 8 weeks and clinical symptoms were evaluated by the Positive and Negative Syndrome Scale. **Results:** Among the 30 variants that we examined, two SNPs in *DRD2* (-241A>G [rs1799978] and TaqIA [rs1800497]) and two SNPs in *AKT1* (*AKT1*-SNP1 [rs3803300] and *AKT1*-SNP5 [rs2494732]) were significant predictors of treatment response to risperidone. **Conclusion:** These data suggest that the SNPs in *DRD2* and *AKT1* may influence the treatment response to risperidone in schizophrenia patients.

Keywords: dopamine, pharmacogenetics, risperidone, schizophrenia, serotonin

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Schizophrenia is a severe psychiatric disorder with a lifetime risk of 1%. Its pathophysiology is unknown, as are the mechanisms underling therapeutic response to treatment. Similarly, the reasons for variable individual responses to treatment are not known and, at present, the choice of particular antipsychotic treatments for individual patients is effectively a trial and error process. However, since genetic factors contribute to treatment response [1], pharmacogenetic approaches offer at least the potential for predicting treatment response at an individual level

With respect to the use of antipsychotic medication in schizophrenia, pharmacogenetic research has focused on dopamine- and serotonin-related genes. In particular, several groups [1–13] have targeted genes encoding the dopamine D2 receptor (*DRD2*: -141Ins/del [rs1799732] and TaqIA polymorphisms [rs1800497; although TaqIA is now known to be located in another proximal gene: X-kinase or ANKK1]), the D3 receptor (*DRD3*: Ser9Gly [rs6280]), the D4 receptor (*DRD4*: 48-bp repeat in exon III), and serotonin 5-HT2A (*HTR2A*: T102C [rs6313]) and 5-HT1A receptors (*HTR1A*: -1019C/G [rs6295]). However, the results of these studies have been discrepant [1,2]. This may be because variation in each individual gene may have weak effects [14]. It has been postulated that interaction between variants in dopaminergic and serotonergic systems may be of greater magnitude in predicting responses to treatment of schizophrenia [15]. The candidate polymorphisms in such genes should be integrated for precise analysis; therefore, we can evaluate the individual gene effects and gene–gene interaction to antipsychotic treatment.

Many candidate genes have been proposed to be of pharmacogenetic relevance to antipsychotic treatment; compared with typical antipsychotics, second-generation antipsychotics, including risperidone, have lower (to similar) affinity for the D2 receptor and a higher degree of occupancy at four other dopamine receptors (D1, D3, D4 and D5) and at some serotonin receptors (5-HT1A, 1B, 1D, 2A, 6 and 7, and other receptors) [16]. In addition, variation in signaling cascades downstream of D2 receptor blockage may be associated with treatment of schizophrenia [17]. Among these, the AKT/glycogen synthase kinase 3 (GSK3) signaling cascade is a particularly attractive candidate. First, antipsychotics, including risperidone, alter the expression level of GSK3 protein in the rat medial prefrontal cortex and striatum [18]. Second, increased phosphorylation of AKT1 and GSK3β have been reported in mice exposed to haloperidol [19]. Third, in a study of dopamine transporter knockout and wild-type mice, AKT1–GSK3β signaling cascades partially mediated DA-dependent behaviors in response to manipulation by exposure to lithium and amphetamine [20]. Lastly, genetic aspects of susceptibility to schizophrenia and antipsychotic response may be closely associated [21]. This is of

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relevance to AKT1–GSK3β signaling, since several case–control and family-based association studies have provided some evidence for association between *AKT1* and schizophrenia [19,22–26], although the findings are not universal [27–30].

The aim of this study was to conduct a pharmacogenetic study of risperidone response and variants in genes encoding dopamine and serotonin receptors (*D1–D5, HTR1A, HTR1B, HTR1D, HTR2A, HTR2C, HTR6* and *HTR7*), *AKT1* and *GSK3β*. In a small sample, we previously reported that diplotypes at *DRD2* were associated with clinical performance after risperidone treatment, although no association was found between risperidone response and gene variants in *5-HT2A* and *COMT* [12]. In this study, we expanded the sample size of first-episode neuroleptic-naive samples (from 31 to 120 patients) and controlled for nongenetic factors such as clinical charactistics and environmental variables (gender, age, duration of untreated psychosis [DUP] and baseline Positive and Negative Syndrome Scale [PANSS] total score) by multiple linear regression analysis.

Materials & methods

Subjects & collection of clinical data

In total, 131 first-episode, neuroleptic-naive schizophrenic patients were included in this open-label pharmacogenetic study. For *DRD2* and *HTR2A*, 31 patients were the same as those included in our previous report [12]. We excluded ten patients whose DUP was longer than 5 years in accordance with another study [31]. Genotypes could not be determined in one subject, and this patient was also excluded. Consequently, 120 patients were analyzed.

Patients were entered into the study if they met diagnostic and statistical manual of mental disorders (DSM)-IV-TR criteria for schizophrenia, were physically healthy and had all laboratory parameters within normal limits, or if they had neither current nor past DSM-IV-TR diagnosis of mood disorders or substance abuse. Consensus diagnoses were made by at least two experienced psychiatrists on the basis of unstructured interviews with patients and families and review of medical records. DUP was defined as the period from the onset of psychotic symptoms to that of first antipsychotic exposure.

Subjects received risperidone monotherapy (starting dosage: 0.5–4 mg/day; mean starting dosage: 2.5 mg) and dosage was adjusted in accordance with patients' symptoms by trained psychiatrists (1–8 mg/day; mean dosage: 3.4 mg at 8 weeks) for 8 weeks. Patients with insomnia were prescribed brotizolam 0.25 or 0.5 mg at bedtime. No other psychotropic drugs were permitted.

Clinical symptoms were evaluated at the first visit and after 8 weeks of treatment by the use of the PANSS. Evaluations were carried out by trained psychiatrists and a psychologist (inter-rater reliability: intraclass correlation coefficient [ICC's] = 0.90 [Yamanouchi & Iwata, Unpublished Data]).

The clinical characteristics of subjects that were used as potential covariates in the stepwise linear regression analysis were selected from a previous paper [32]: gender (58 male, 62 female), age (31.2 ± 8.7 years), DUP (13.7 ± 11.4 months) and baseline PANSS total score (79.1 ± 20.5).

All patients were unrelated and were ethnically Japanese. After explanation of the study, written informed consent was obtained from each subject. This study was approved by the Ethics Committee at Fujita Health University, University of Occupational and Environmental Health and Nagoya University Graduate School of Medicine.

Variant selection & genotyping

In total, 30 variants were selected from previous studies (Table 1). We specifically targeted potential functional polymorphisms and those which were previously associated with treatment response or with schizophrenia itself. These include: four SNPs for *DRD1* [33]; three variants for *DRD2* [12,34]; two SNPs for *DRD3* [35]; four variants for *DRD4* [36,37]; one SNP for *DRD5* [38]; five SNPs for *AKT1* [22]; two SNPs for *GSK3B* [39]; one SNP for *HTR1A* [40]; one SNP for *HTR1B* [41]; one SNP for *HTR1D* [42]; one SNP for *HTR2A* [12]; two SNPs for *HTR2C* [43,44]; one SNP for *HTR6* [10]; and two SNPs for *HTR7* [45]. Genotyping methods can be seen in Table 1 and primer sequences are available on request.

In *DRD1*, -1251*HaeIII* was not polymorphic while the three other SNPs (-800HaeIII, -48DdeI and +1403Bsp1286I) were in absolute linkage disequilibrium (LD; $r^2 = 1$) in our sample. Thus, we included only -800HaeIII in *DRD1* to the following regression analysis. At *DRD3*, -205G>A and Ser9Gly were similarly in LD ($r^2 = 1$), so we analyzed only Ser9Gly (Table 1). For the 48-bp repeat in exon III of *DRD4*, the allele frequencies of the variant differ considerably between populations, and the seven-repeat allele is quite rare in the Japanese population. Therefore, alleles with five or more repeats were grouped with the long L allele in accordance with another study [36]. For

Table 1. Distribution of genotypes and direct association between PANSS improvement and genotypes.

Gene symbol	SNP ID	Methods	n	Genotype			p-value*
				M/M	*M/m*	*m/m*	
DRD1‡	-1251HaeIII (G>C)	PCR-RFLP	120	120	0	0	NA
	-800HaeIII (C>T)	PCR-RFLP	120	99	21	0	0.686
DRD2	-241A>G	PCR-RFLP	120	96	24	0	0.447
	-141 Ins/Del (Ins>Del)	PCR-RFLP	120	90	30	0	0.435
	TaqIA (A2>A1)	PCR-RFLP	120	54	54	12	0.0239§
DRD3¶	Ser9Gly (Ser>Gly)	PCR-RFLP	120	60	55	5	0.989
DRD4	120 bp duplication (L>S)	PCR-RFLP	120	73	44	3	0.403
	-616G>C	Direct sequencing	120	56	57	7	0.193
	-521T>C	Direct sequencing	120	38	66	16	0.925
	48bp repeat in exon III (S>L)#	PCR	120	114	6	0	0.969
DRD5	1481C>T	PCR-RFLP	120	62	46	12	0.456
AKT1	SNP1 rs3803300 (G>A)	PCR-RFLP	120	34	56	30	0.102
	SNP2 rs1130214 (G>T)	PCR-RFLP	120	71	45	4	0.949
	SNP3 rs3730358 (C>T)	PCR-RFLP	120	89	31	0	0.676
	SNP4 rs2498799 (A>G)	PCR-RFLP	120	32	60	28	0.210
	SNP5 rs2494732 (C>T)	PCR-RFLP	120	65	44	11	0.0286§
GSK3B	SNP6 rs1574154 (C>T)	PCR-RFLP	120	37	56	27	0.525
	SNP8 rs2037547 (C>T)	PCR-RFLP	120	106	14	0	0.844
HTR1A	-1019C>G	TaqMan®	120	73	45	2	0.799
HTR1B	861G>C	PCR-RFLP	120	25	73	22	0.151
HTR1D	rs674386	TaqMan®	120	64	50	6	0.597
HTR2A	102T>C	PCR-RFLP	120	31	58	31	0.948
HTR2C	-759C>T	TaqMan®	120	105	7	8**	0.315
	-697C>G	TaqMan®	120	104	8	8**	0.222
HTR6	267C>T	PCR-RFLP	120	60	56	4	0.580
HTR7	SNP2 rs3808932	Primer extension	120	74	33	13	0.535
	SNP5 rs12412496	PCR-RFLP	120	60	40	20	0.0437§

*p-value for direct association (analysis of variance).
‡-48DdeI and +1403BspI286I were in absolute LD.
§Significant p-values.
¶-205G>C was in absolute LD.
#Five patients had 2/2 repeat, 25 patients had 4/2 repeat, 84 patients had 4/4 repeat, five patients had 4/5 repeat, one patient had 4/7 repeat.
**Hemizygotes.
LD: Linkage disequilibrium; M: Major allele; m: Minor allele; NA: Not analyzed; PANSS: Positive and Negative Syndrome Scale.

the two SNPs (-759C>T and -697C>G) in *HTR2C* that are located on the X chromosome, we applied a dominant genetic model to the following regression analysis: wild-type homozygote and the combined group of heterozygotes and mutant homozygotes (however, there is no sample with mutant homozygotes in our sample: eight samples in men have hemizygotes both of -759C>T and -697C>G. Seven and eight samples in women have heterozygotes of -759C>T and -697C>G, respectively).

Statistical analysis

All SNPs were tested for deviation from the Hardy–Weinberg equilibrium using an exact test (SAS/Genetics, release 8.2, SAS Institute Inc., Tokyo, Japan).

To check first if there was evidence for association between PANSS improvement and genotype, one-way analysis of variance (ANOVA) was applied (JMP, 6.J, SAS Institute Inc.). We next performed a stepwise backward selection procedure with a p-value threshold of 0.10 for excluding

covariates. In those analyses, the dependent variable was improvement rate in total scores of PANSS (calculated as shown below) and the independent variables included genotype, gender, age, DUP and baseline PANSS total score (JMP, 6.J).

$$\text{Improvement rate} = \frac{\left(\text{PANSS at week 0}\right) - \left(\text{PANSS at week 8}\right)}{\text{PANSS at week 0}}$$

For the baseline PANSS score, we expected that total symptoms, positive symptoms, negative symptoms and general psychopathology would be correlated. Therefore, we initially calculated the correlation by Spearman's rank correlation test. We also selected total PANSS score as the covariate due to its generality (all correlations between the total PANSS score and the other subscores showed significance, but other combinations were not always significant [data not shown]).

When the significant variables were obtained, the adjusted means of improvement rate in PANSS score for each genotype were estimated by the method of least squares, and the protected least square difference test was used to compare individual groups. The significance level for all statistical tests was set at a p-value of less than 0.05.

Results

The distributions of all SNPs were within the values expected from Hardy–Weinberg equilibrium.

The improvement in total PANSS scores followed a normal distribution (Shapillo–Wilk test: $W = 0.991$; $p = 0.629$). Using one-way ANOVA, we found that three polymorphisms (TaqIA, *AKT1*-SNP5 and *HTR7*-SNP5) were nominally associated with PANSS improvement (Table 1). By contrast, the stepwise regression analysis suggested that two SNPs in *DRD2* (-241A>G polymorphism: $p = 0.031$; TaqIA polymorphism: $p = 0.0075$) and two SNPs in *AKT1* (*AKT1*-SNP1: $p = 0.018$; *AKT1*-SNP5: $p = 0.02$) were significant contributors (Table 2). Considering clinical background, only the baseline PANSS total score was a significant contributor ($p = 0.0058$) to predicting response (worse scores at 0 week showed better response). We found no difference in diagnosis subtypes between genotype groups for the four SNPs that were found to be predictors for treatment response (data not shown).

To evaluate the quantitative risk of predictor genotypes, the adjusted improvement rate among each genotype was compared by *t*-statistic. The patients with the A/A genotype in -241A>G, the A1/A1 genotype in TaqIA or the T/T genotype in AKT1-SNP5 showed significantly better improvement in total PANSS score than those without the above genotypes in each SNP. For AKT1-SNP1, individuals with the G/A genotype showed a significantly better improvement than patients with the A/A genotype, and a nearly significant improvement compared with those with the G/G genotype ($p = 0.0697$) (Figure 1).

Discussion

In this study, we found that SNPs in *DRD2* and *AKT1* (-241A>G [rs1799978] and TaqIA [rs1800497] in *DRD2*, and *AKT1*-SNP1 [rs3803300] and *AKT1*-SNP5 [rs2494732] in *AKT1*) were significant predictors of the improvement in total PANSS score after risperidone treatment.

However, we found a number of disagreements between the ANOVA and the regression analyses (-241A>G, *AKT1*-SNP1 and *HTR7*-SNP5 in *HTR7* [rs12412496]), although two findings, TaqIA and *AKT1*-SNP5 were consistent between the two analyses. It is not surprising, and we hypothesize that this is derived from the effects of gene–gene or gene–environmental interaction, since multiple linear regression analysis can reflect or adjust the above effects.

Our data support a recent report regarding an association between -241A>G in *DRD2* and treatment response for schizophrenia [11], but do not support previous significant associations between other polymorphisms in dopamine- and serotonin-related genes [1]; in the Chinese population, the 'A' allele of the -241A>G polymorphism is the predictor allele of better treatment response to risperidone [11]. Our data replicated this finding, suggesting that it might be a true predictor for risperidone treatment in Asian populations in general. However, we need further replications for clinical use. In another study, the authors found that the 'G' allele (and -141Ins/Ins homozygote) is associated with a faster response time to antipsychotic treatment [9], but we cannot directly compare our present results with this finding, as our study did not contain multiple longitudinal assessment points.

Our previous pharmacogenetic study suggested that diplotypes in *DRD2* were associated with clinical response: compared with patients with the Ins-A2/Ins-A2 diplotype, PANSS total scores of patients with Ins-A2/Del-A1 showed 40% greater improvement [12]. In the present study, we decided not to use this type of analysis