brain: (1) the choice of an appropriate brain region for investigation; (2) the heterogeneity of cell types within brain tissue; (3) the reliance on relatively small samples; and (4) the impact of cause of death and/or postdeath handling of the tissues on gene expression [Marcotte et al., 2003]. Thus, the use of postmortem brain tissue is compounded by a range of confounding factors (age, race, gender, different microarray platforms, and analysis methods) and may be the cause of the relative lack of gene/transcript-level consistency among expression studies. To overcome some of these problems, several groups have considered the use of lymphoblasts rather than the postmortem brain [Matigian et al., 2008; Slonimsky et al., 2010; Yamamori et al., 2011; Yasuda et al., 2011]. Lymphoblasts are useful for schizophrenia researchers because blood-based tissue (lymphoblasts) can be obtained with ease from living subjects, which allows larger case–control studies with optimal matching of key variables (age, sex, and race). In addition, immortalized lymphoblasts in culture are considered an effective tool for studying cells in the absence of the effect of antipsychotic treatments and duration of illness, both of which could mask the genetic differences in RNA expression. Thus, lymphoblasts could be good tool to investigate the impact of a gene in the absence of the impact of any confounding factors. On the other hand, there were some demerits of using lymphoblasts. In immortalized lymphocytes, it might be difficult to observe the effects of genes on their neuron-specific functions, for example, the effects of genes on glutamate and dopamine release and on the formation of synaptic vesicles. When isolation and immortalization procedures of lymphocytes from blood were performed or immortalized lymphocytes were grown in culture media, a genetic mutation might be inserted into genomic DNA in the cultured lymphoblasts and alter DNA sequences. It remains still controversial whether immortalized lymphocytes are an appropriate alternative to neuronal tissue, because there was a little evidence of analysis using immortalized lymphocytes from patients with schizophrenia. In this study, the difference in the association of gene expression with genetic variants between previous study and present study could be explained by the difference in the gene expression profile between immortalized lymphoblast and postmortem brain tissue. Other possible factors contributing to differences in association between studies could be a difference in the SNPs and haplotypes investigated or ethnic differences between Japanese and Caucasian populations.

Smith et al. [2011] performed mutation searches of all four exons of *NRGN* gene in 14 Caucasian subjects with schizophrenia and of the cording exons of *NRGN* gene in 1,113 Bulgarians individuals, 699 of whom had schizophrenia. However, they did not find any novel common polymorphism in the region. Thus, we did not perform a systematic mutation search in this study because there has been no novel common genetic variant in the region. If we perform sequencing and find a novel rare polymorphism, we cannot analyze association between the rare polymorphism and gene expression for only a small number of individuals with rare variant. A genetic variant, particularly a SNP not listed in the HapMap database, that is likely to be more strongly associated with schizophrenia may exist in the rs12807809–rs12278912 haplotype region. Sequencing the entire gene in individuals with risk haplotype in comparison with the protective haplotype carriers with larger sample sizes could provide further information underlying the genomic mechanism for this risk haplotype.

There are several limitations to interpreting our results. Because a number of statistical analyses supported the association of the *NRGN* gene and schizophrenia, such as genotypic and allelic associations for five SNPs (total 5×2), haplotype analysis using a window fashion analysis (total 10) and expression analysis for three individual haplotypes (total 3×4), a correction for multiple testing should be considered. In this study, the overall number of genetic association tests was 32; however, not all tests were independent, and several hypotheses were included. Thus, Bonferroni correction, a method to correct for multiple independent tests for one hypothesis, might not be appropriate. The consensus how to correct such multiple testing has not been reached in this research field. Thus, we applied SNPSpD correction for genotypic and allelic association analysis, permutation method for haplotype analysis and Bonferroni correction for expression analysis (three tests). However, even though we applied these methods of correcting such multiple testing, they might cause false positive results. We did not control for geographical variation of control origin because there is little possibility for ethnic/genetic difference among four geographical regions for feature of homogeneous race in Japan [Yamaguchi-Kabata et al., 2008]. Our significant results may be derived from sample bias owing to population stratification and non-sex-matched samples. In the present study, our results support an association between the *NRGN* gene and schizophrenia. We suggest that the functional haplotype of the *NRGN* gene, which is associated with *NRGN* expression, could be related to the pathogenesis of schizophrenia.

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A promoter variant in the *chitinase 3-like 1* gene is associated with serum YKL-40 level and personality trait

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ABSTRACT

The *chitinase 3-like 1* (*CHI3L1*) gene, a cellular survival factor against several environmental and psychosocial stresses, has been sown to be more highly expressed in the hippocampus and prefrontal cortex of patients with schizophrenia than unaffected individuals. We recently reported a significant association between schizophrenia and SNP rs4950928, which is located in the promoter region of the *CHI3L1* gene, in a Japanese population. The G-allele at this SNP in the gene has been associated with higher transcriptional activity in a luciferase reporter assay and with higher mRNA levels in the peripheral blood cells of patients with schizophrenia. We investigated the impact of the *CHI3L1* polymorphism rs4950928 on serum YKL-40 levels, the protein product of *CHI3L1*. We found that individuals with the G-allele, who were more prevalent among patients with schizophrenia, had significantly higher serum YKL-40 levels ($p = 0.043$). Personality traits are considered to be an important aspect of schizophrenia primarily because they may influence symptoms and social functioning. Personality trait analyses using the temperament and character inventory (TCI) indicated that schizophrenic patients have a unique personality profile that appears to be present across cultures. We hypothesized that higher serum YKL-40 levels are associated with personality trait in patients with schizophrenia. Thus, we next examined the impact of the risk *CHI3L1* polymorphism on personality traits using the TCI. We found that individuals with the G-allele had significantly higher self-transcendence scores ($p = 0.0054$). These findings suggest possible associations between the SNP in the *CHI3L1* gene, the risk for schizophrenia, and higher serum YKL-40 levels and personality traits in a Japanese population.

Schizophrenia is a common and complex psychiatric disease. Many genes have been implicated in the pathogenesis of schizophrenia [8,9,19,30,33], and the *chitinase 3-like 1* gene (*CHI3L1*) gene has been reported to be associated with the disease [34,35]. We have recently reported a significant association between schizophrenia and a SNP rs4950928 ($p = 0.009$) located in the promoter region of the *CHI3L1* gene (the most significant $p < 0.001$) in a Japanese population using the largest sample size to date (1463 cases and 1795 controls) [25]. Elevated expression of the *CHI3L1* gene has been indicated in the schizophrenic hippocampus and prefrontal cortex in independent postmortem studies [1,5]. The G-allele of the gene at rs4950928, which was found to be more prevalent in patients with schizophrenia, has been associated with higher transcriptional activity in a luciferase reporter assay and higher mRNA levels in peripheral blood cells in patients with schizophrenia [35]. *CHI3L1* gene acts as a cellular survival factor in responses to a variety of adverse environments, including various types of physiologic stress such as inflammation, hypoxia and nutrient deprivation. These stressors may induce high expression of *CHI3L1* [15,26]. The protein product of the *CHI3L1* gene was named YKL-40 [14]. YKL-40 is a secreted protein, produced by activated macrophages and neutrophils in different tissues characterized by inflammation and increased remodeling of the extracellular matrix [16,28,32]. YKL-40 initiates phosphoinositide-3 kinase (PI-3K) signaling cascades

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in fibroblasts [27]. The PI-3K pathway, in particular the phosphorylation of protein kinase B (AKT), is strongly associated with cell survival [2], which suggests a role for YKL-40 as an anti-apoptotic protein. The genetic variants of the *CHI3L1* gene and the higher serum YKL-40 levels are associated with several inflammatory diseases, such as sarcoidosis, asthma and inflammatory bowel diseases [13,17,18,24]. It has been hypothesized that YKL-40 plays a protective role in inflammatory processes in patients with schizophrenia and is highly expressed in patients with schizophrenia. In this study, we investigated whether the G-allele has an effect on YKL-40 levels in schizophrenic patients. To achieve this goal, we measured the serum YKL-40 levels of patients with schizophrenia and control subjects.

Personality traits are considered to be an important aspect of schizophrenia primarily because they may influence symptoms and social functioning [20,21]. The temperament and character inventory (TCI) is a well-established self-report questionnaire. It measures four temperament dimensions [novelty seeking (NS), harm avoidance (HA), reward dependence (RD) and persistence (PS)] and three character dimensions [self-directedness (SD), cooperativeness (CO) and self-transcendence (ST)] [6]. Personality trait analyses using the TCI have indicated that schizophrenic patients have a unique personality profile that appears to be present across cultures [higher scores of ST and HA and lower scores of NS, RD, SD and CO in schizophrenia] [3,4,7,11,29]. We hypothesized that higher serum YKL-40 levels would be associated with personality traits in patients with schizophrenia. Thus, we secondly examined the impact of the risk *CHI3L1* polymorphism on personality traits using the TCI.

For serum YKL-40 measurements, 20 patients with schizophrenia and 19 controls were enrolled. The subjects for personality trait analysis consisted of 99 patients with schizophrenia and 179 controls. All controls and 18 of 20 patients with schizophrenia enrolled for serum YKL-40 measurements were also enrolled for personality trait analysis. Cases were recruited at Osaka University hospitals. Each schizophrenic research subject had been diagnosed and assessed by at least two trained psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria based on unstructured clinical interview. Symptoms of schizophrenia were assessed using the positive and negative syndrome scale (PANSS). Three of 20 patients with schizophrenia enrolled for serum YKL-40 measurements were not treated with anti psychotic drugs and 7 of 99 patients with schizophrenia enrolled for personality trait analysis were not treated with anti psychotic drugs. Cases of schizophrenia with the comorbidities of substance-related disorders or mental retardation were excluded. Controls were recruited through local advertisements. Psychiatrically, medically and neurologically healthy controls were evaluated using the DSM-IV structured clinical interview, non-patient version. Subjects were excluded if they had neurological or medical conditions that could potentially affect the central nervous system, such as atypical headache, head trauma with loss of consciousness, chronic lung disease, kidney disease, chronic hepatic disease, thyroid disease, active stage cancer, cerebrovascular disease, epilepsy or seizures. Written informed consent was obtained for all subjects after the procedures had been fully explained. This study was carried out in accordance with the World Medical Association's Declaration of Helsinki and approved by the Research Ethical Committee of Osaka University.

Serum YKL-40 was measured using an enzyme-linked immunosorbent assay kit (Metra YKL-40, Quidel Corporation, San Diego, CA, USA), in accordance with the manufacturer's instructions. All samples were run in duplicate and mean values were used for analysis. The average intra-assay coefficient of variation determined by triplicate of 10 CSF samples was 3.2%. To examine reproducibility, four CSF samples were analyzed in each of two experiments. After normalization, the average inter-assay coefficient of variation was 5.6%.

The TCI is administered through a self-report questionnaire based on 240 items requiring a true or false item response [6]. We only examined the main scores of the four temperaments (HA, NS, RD and PS) and three characters (SD, CO and ST) dimensions of the scale. The concepts of each dimension are as follows: NS is the activation of behavior in response to novelty and signals of reward or relief of punishment; HA is the inhibition of behavior in response to signals of punishment or non-reward; RD is the maintenance of behavior that was previously rewarded; PS is the perseveration with behavior despite frustration and fatigue; SD is the concept of the self as an autonomous individual; CO is the concept of the self as an integral part of humanity or society; and ST is the concept of the self as an integral part of the universe and its source [6].

Venous blood was collected from the subjects and genomic DNA was extracted from whole blood according to standard procedures. The timing of blood collection was not consistent among the samples. Genotyping of the SNP was carried out via TaqMan assays (Applied Biosystems, Foster City, CA, USA) as previously described [10,23]. The TaqMan probe and Universal PCR Master Mix were obtained from Applied Biosystems. The TaqMan probe ID for the SNP rs4950928 was C__27832042_10. Allelic-specific fluorescence was measured using an ABI PRISM 7900 Sequence Detector System (Applied Biosystems).

Statistical analyses were performed using SNPAlyze V5.1.1 Pro software (DYNACOM, Yokohama, Japan) and SPSS 16.0J software (SPSS Japan Inc., Tokyo, Japan). Differences in clinical characteristics between patients and controls or between genotype groups were analyzed using χ^2 tests for categorical variables and the Mann–Whitney *U*-test for continuous variables. Deviation from Hardy–Weinberg equilibrium (HWE) was tested separately in cases and controls. The analysis revealed age and gender differences in some dimensions. Therefore, the effect of the *CHI3L1* genotype and the effect of diagnosis on the serum YKL-40 levels were analyzed by a two-way analysis of covariance (ANCOVA), with age and gender as covariates. In previous personality traits analyses using the TCI, it has been suggested that possible confounding factors affect personality traits [22,31]. The number of years of education was lower in patients with schizophrenia than in healthy controls in a Japanese population [11]. Therefore, with age, gender and education years as covariates, the effect of *CHI3L1* genotype and the effect of diagnosis on personality traits were analyzed by a two-way ANCOVA. The significant level for statistical tests of genetic and personality association was set at $p < 0.05$.

We examined possible associations between the *CHI3L1* genotype at rs4950928 and serum YKL-40 levels in patients with schizophrenia and controls, because this variant was indicated to have a significant association with schizophrenia in a previous study. Supplementary Table 1 shows the characteristics of the subjects and the distribution of genotypes. There was no difference in demographic variables, age, gender, years of education, chlorpromazine equivalents of total antipsychotics (CPZeq) and positive and negative symptom scale (PANSS) scores between *CHI3L1* genotype groups. Given that there was only one CC homozygous individual in subjects for serum YKL-40 analysis, we removed the data of the subject with CC genotype and compared GG genotype with GC genotype. The effects of *CHI3L1* genotype and diagnosis on serum YKL-40 levels were shown in Table 1. Two-way ANCOVA revealed significant effects of genotype ($F = 4.46$, $p = 0.043$, $\eta^2 = 0.122$). No effect of diagnosis ($p > 0.70$) or genotype–diagnosis interaction was found ($p > 0.80$). Individuals homozygous for the G-allele, which was more common in the patient group, showed higher serum YKL-40 levels than the C-carriers (Fig. 1). There was no genotype effect when we separately analyzed the effect of genotype on YKL-40 in patients ($p > 0.30$) and controls ($p > 0.10$).

Table 1
Effects of *CHI3L1* genotype and diagnosis on serumYKL-40.

Variables	Schizophrenia ($n=19$)		Control ($n=19$)		ANCOVA p-values (F-values)		
	G/G	G/C	G/G	G/C	Diagnosis effect	Genotype effect	Interaction
Serum YKL-40 (ng/ml)	109.8 ± 63.5	75.3 ± 15.6	101.7 ± 37.3	75.0 ± 24.0	0.696 (0.156)	*0.043 (4.46)*	0.785 (0.076)

Means ± SD are shown. ANCOVA, two-way analysis of covariance. Significant p-values are italicized.

Table 2
Effects of *CHI3L1* genotype, diagnosis and their interaction on personality traits using TCI.

	Schizophrenia		Control		ANCOVA p-values (F-values)		
	G/G($N=70$)	G/C, C/C($N=29$)	G/G($N=118$)	G/C, C/C($N=61$)	Diagnosis effect	Genotype effect	Interaction
NS	17.6 ± 4.8	18.4 ± 4.7	21.4 ± 4.4	20.5 ± 4.7	*2.21×10^{-6} (23.40)*	0.82 (0.05)	0.46 (0.54)
HA	22.6 ± 7.4	23.7 ± 6.3	16.3 ± 5.5	17.2 ± 5.8	*2.93×10^{-11} (48.13)*	0.24 (1.42)	0.89 (0.02)
RD	14.3 ± 3.5	14.2 ± 3.7	16.4 ± 2.9	15.9 ± 3.5	*8.08×10^{-4} (11.48)*	0.65 (0.21)	0.49 (0.48)
PS	4.4 ± 1.6	4.2 ± 1.7	4.6 ± 1.9	4.4 ± 1.7	0.96 (<0.01)	0.36 (0.83)	0.93 (0.01)
SD	24.3 ± 7.1	23.3 ± 8.8	29.4 ± 5.7	29.6 ± 5.7	*1.40×10^{-7} (29.24)*	0.72 (0.13)	0.96 (<0.01)
CO	27.2 ± 5.7	26.8 ± 6.3	29.3 ± 4.1	29.2 ± 5.2	*0.0090 (6.92)*	0.88 (0.02)	0.70 (0.15)
ST	13.9 ± 7.0	11.2 ± 6.1	9.7 ± 5.2	8.2 ± 4.2	*5.95×10^{-5} (16.64)*	*0.0054 (7.86)*	0.35 (0.89)

TCI, temperament and character inventory; NS, novelty seeking; HA, harm avoidance; RD, reward dependence; PS, persistence; SD, self directedness; CO, cooperativeness; ST, self transcendence. Means ± SD are shown. The effects of CHI3L1 genotype, diagnosis and their interaction on the personality trait were analyzed by a two-way analysis of covariance (ANCOVA) with age, gender and education years as covariates. Significant p-values are italicized.

We next examined possible associations between the *CHI3L1* genotype at rs4950928 and personality traits in patients with schizophrenia and in controls. There was no difference in demographic variables, age, gender, years of education, CPZeq and PANSS scores between the *CHI3L1* genotype groups (Supplementary Table 2). Given that there were few homozygous CC individuals, we divided the participants into two groups (individuals with GG genotype and C-carriers). The effects of *CHI3L1* genotype and diagnosis on personality traits as measured by TCI are shown in Table 2. Two-way ANCOVA revealed significant effects of diagnosis (NS: $F=23.40$, $p<0.001$; HA: $F=48.13$, $p<0.001$; RD: $F=11.48$, $p<0.001$; SD: $F=29.24$, $p<0.001$; CO: $F=6.92$, $p<0.001$ and ST: $F=16.64$, $p<0.001$) and genotype (ST: $F=7.86$, $p=0.0054$, $\eta^2=0.03$). No genotype–diagnosis interaction was found ($p>0.20$). Individuals homozygous for the G-allele had higher ST scores than the C-carriers (Fig. 2).

To our knowledge, this is the first report showing an association between the G-allele at rs4950928 and higher serum YKL-40 levels in both patients with schizophrenia and controls. The G-allele may be related to the part of pathophysiology of schizophrenia through its effect on serum YKL-40 levels. Higher serum YKL-40 levels may be the response to the environmental and psychological stresses that have been shown to be sensitive in schizophrenia [12].

We first performed the association study to assess *CHI3L1* genotype and personality traits using 99 patients and 179 controls, which is the largest sample size to date for an association study examining a risk genotype and the TCI. Similar to the previous studies, we found significantly lower scores for NS, RD and SD and higher scores on HA and ST in patients than in controls (diagnosis effect). Moreover, higher ST scores were also revealed in individuals with the risk GG genotype at rs4950928 compared to the C-carriers (genotype effect). For personality traits, however, no genotype–diagnosis interaction was detected. ST is an important dimension for schizophrenia, as higher ST scores have been shown in unaffected relatives of schizophrenic patients [3]. Therefore, this dimension might be an intermediated-phenotype for schizophrenia.

This study has several limitations. The sample size was relatively small for both the serum YKL-40 and TCI analyses. In the previous multi-center case–control study which showed the significant association between schizophrenia and SNP rs4950928 in

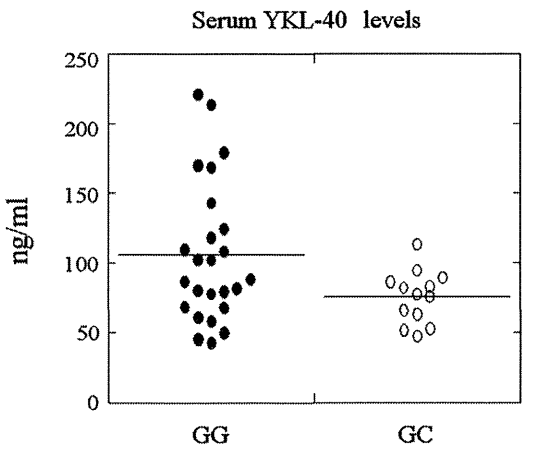

Fig. 1. Effect of the risk *CHI3L1* genotype at SNP rs4950928 on serum YKL-40 levels. *X* axis represents *CHI3L1* genotypes at SNP rs4950928. *Y* axis represents serum YKL-40 levels.

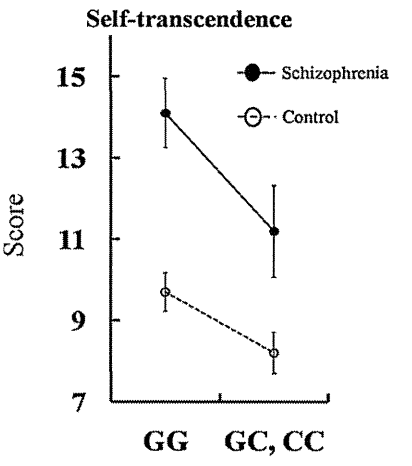

Fig. 2. Effect of the risk *CHI3L1* genotype at SNP rs4950928 and effect of diagnosis on personality trait (self-transcendence) using TCI. *X* axis represents *CHI3L1* genotypes at SNP rs4950928. *Y* axis represents self-transcendence scores. Closed circles represent subjects with schizophrenia. Open circles represent healthy controls. Bars represent the standard error.

a Japanese population [25], 1463 cases and 1795 controls were enrolled, however, serum samples and personality traits data were available only in limited cases and controls from our laboratory. For TCI analysis, we had enough power of 0.802 (sample size: 278, effect size: $\eta^2 = 0.03$, statistical significance = 0.05). For serum YKL-40 analysis, we did not have enough power: 0.599 < 0.8 (sample size: 38, effect size: $\eta^2 = 0.122$, statistical significance = 0.05), however, we fortunately found the significant association. Because of the small sample size, only one CC homozygous individual was included in serum YKL-40 analysis and we removed the data of the subject with CC genotype and compared GG genotype with GC genotype. A much larger sample size would be needed to definitively test the associations between the *CHI3L1* genotype and YKL-40 and TCI in schizophrenia. Circadian rhythms and medications may affect the serum YKL-40 levels, however, the timing of blood collection was not consistent among the samples. In addition, personality traits were assessed in patients after the onset of symptoms. A careful interpretation of our results is called for because we did not consider whether the findings reflect pre-clinical personality traits versus pre- or post-therapeutic personality traits. Further replication studies in other ethnic populations and an association study between the risk *CHI3L1* genotype and serum YKL-40 levels and personality trait in patients with schizophrenia are required to establish a definitive relationship between *CHI3L1* and schizophrenia.

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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.neulet.2012.02.039.

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Impact of the Genome Wide Supported *NRGN* Gene on Anterior Cingulate Morphology in Schizophrenia

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Abstract

Background: The rs12807809 single-nucleotide polymorphism in *NRGN* is a genetic risk variant with genome-wide significance for schizophrenia. The frequency of the T allele of rs12807809 is higher in individuals with schizophrenia than in those without the disorder. Reduced immunoreactivity of *NRGN*, which is expressed exclusively in the brain, has been observed in Brodmann areas (BA) 9 and 32 of the prefrontal cortex in postmortem brains from patients with schizophrenia compared with those in controls.

Methods: Genotype effects of rs12807809 were investigated on gray matter (GM) and white matter (WM) volumes using magnetic resonance imaging (MRI) with a voxel-based morphometry (VBM) technique in a sample of 99 Japanese patients with schizophrenia and 263 healthy controls.

Results: Although significant genotype-diagnosis interaction either on GM or WM volume was not observed, there was a trend of genotype-diagnosis interaction on GM volume in the left anterior cingulate cortex (ACC). Thus, the effects of *NRGN* genotype on GM volume of patients with schizophrenia and healthy controls were separately investigated. In patients with schizophrenia, carriers of the risk T allele had a smaller GM volume in the left ACC (BA32) than did carriers of the non-risk C allele. Significant genotype effect on other regions of the GM or WM was not observed for either the patients or controls.

Conclusions: Our findings suggest that the genome-wide associated genetic risk variant in the *NRGN* gene may be related to a small GM volume in the ACC in the left hemisphere in patients with schizophrenia.

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Introduction

Schizophrenia is a common and complex psychiatric disorder that has a strong genetic component; the estimated heritability is 81% [1]. Many genes have been implicated in the pathogenesis of schizophrenia [2].

A genome-wide association study (GWAS) of single-nucleotide polymorphisms (SNPs) conducted by accessing thousands of DNA samples from patients and controls can be a powerful tool for identifying common risk factors for such a complex disease. Stefansson et al. examined a combined sample of 12,945 patients with schizophrenia and 34,591 controls from three large GWASs (the SGENE-plus, the International Schizophrenia Consortium and the Molecular Genetics of Schizophrenia) and a follow-up with 4,999 patients and 15,555 controls from four additional sample sets from various areas of Europe (including the Netherlands, Denmark, Germany, Hungary, Norway, Russia, Sweden, Finland and Spain) [3]. The researchers identified several

significant association signals. Seven markers gave p values smaller than the genome-wide significance threshold of approximately 1.6×10^{-7} in the combined samples. Five of these markers—rs6913660, rs13219354, rs6932590, rs13211507 and rs3131296—span the major histocompatibility complex (MHC) region on chromosome 6p21.3–22.1; one marker, rs12807809, is located 3,457 bases upstream from the neurogranin (*NRGN*) gene on 11q24.2; one additional marker, rs9960767, is located in intron four of the transcription factor 4 (*TCF4*) gene on 18q21.2. Of these seven SNPs, four SNPs, rs6913660, rs13219354, rs13211507 and rs9960767, were not polymorphic in samples from the HapMap Japanese in Tokyo (JPT) project. Minor allele frequencies (MAF) of two SNPs, rs6932590 and rs3131296, were under 5%. Because only one marker, rs12807809 in *NRGN*, was a common SNP in HapMap JPT samples (MAF>5%), we focused on this SNP in the present study.

NRGN is the human homolog of the neuron-specific rat gene RC3/neurogranin. *NRGN* encodes a postsynaptic protein kinase substrate that binds to calmodulin (CaM) in the absence of calcium [4]. The *NRGN* gene spans 7.3 kb of genomic DNA and contains four exons that transcribe a protein of 78 amino acids [5]. Exons 1 and 2 encode the protein, and exons 3 and 4 contain untranslated sequences. NRGN plays an important role in the Ca^{2+}–CaM signaling pathway [6]. A Ca^{2+} influx-induced oxidation of NRGN leads to postsynaptic activation of CaM-dependent protein kinase II (CaMKII) by CaM, which is associated with strengthened *N*-methyl-D-aspartate (NMDA) receptor signaling [7]. Altered NRGN activity may therefore mediate the effects of the NMDA hypofunction implicated in the pathophysiology of schizophrenia.

Many attempts have been made to minimize clinical and genetic heterogeneity in studies of schizophrenia. One strategy for gene discovery uses neurobiological quantitative traits (QT) as intermediate phenotypes rather than the diagnosis of schizophrenia [8,9]. This strategy has the potential to reduce clinical and genetic heterogeneity by examining intermediate phenotypes that reflect underlying genetic vulnerability better than diagnostic categorization [10]. Structural brain phenotypes are QT that show considerable variation in human populations [11]. A voxel-wise meta-analysis of gray matter (GM) alterations in patients with schizophrenia indicated that they had a reduced GM density in the bilateral insular cortex, anterior cingulate, left parahippocampal gyrus, left middle frontal gyrus, postcentral gyrus, and thalamus and had an increased GM density in the striatal regions relative to the control subjects [12]. A voxel-wise meta-analysis of white matter (WM) alterations in patients with schizophrenia indicated that these patients had a decreased WM volume in the frontal regions and internal capsule relative to control subjects [13]. Heritability estimates indicate a moderate (40–70%) to high (70–95%) genetic influence on brain structure volumes in the frontal and temporal brain regions, such as the middle frontal and the anterior cingulate cortices [11,14]. Some studies have shown that abnormalities in brain structure are intermediate phenotypes that bridge the gap between the genotype and diagnostic categorization [10,15,16]. Our research group has a long-standing interest in the effects of genetic variants on brain structure (i.e., *COMT*, *DISC1*, *PACAP*, *BDNF*, *APOE* and *AKT1*) [17,18, 19,20,21,22] and on prefrontal activity as measured by near-infrared spectroscopy (NIRS) (*TBP* and *SIGMAR1*) in psychiatric disorders [23,24]. *NRGN* is expressed exclusively in the brain, especially in the dendritic spines. Reduced NRGN immunoreactivity has been observed in prefrontal areas 9 and 32 of post-mortem schizophrenic brains [25]. To date, no study has investigated the effects of the *NRGN* polymorphism and the genotype-diagnosis interaction on brain morphology at the whole brain level. In this study, we examined the impacts of the *NRGN* polymorphism and the genotype-diagnosis interaction on GM volumes and WM volumes in patients with schizophrenia and in healthy volunteers.

Materials and Methods

Ethics statement

Written informed consent was obtained from all subjects after the procedures had been fully explained. This study was carried out in accordance with the World Medical Association's Declaration of Helsinki and approved by the Research Ethical Committee of Osaka University.

Subjects

Voxel-based morphometry (VBM) analyses were conducted on 99 patients with schizophrenia [52.5% males (52 males and 47 females); mean age ± SD, 38.4±12.9 years] and 263 healthy controls [44.5% males (117 males and 146 females); mean age ± SD, 36.7±11.6 years]. All subjects were biologically unrelated within the second-degree of relationship and of Japanese descent [23,26]. The subjects were excluded if they had neurological or medical conditions that could potentially affect the central nervous system, such as atypical headache, head trauma with loss of consciousness, chronic lung disease, kidney disease, chronic hepatic disease, thyroid disease, active cancer, cerebrovascular disease, epilepsy, seizures, substance-related disorders or mental retardation. Cases were recruited from the university hospital. Each patient with schizophrenia had been diagnosed by at least two trained psychiatrists according to the criteria of the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* (DSM-IV) based on the Structured Clinical Interview for DSM-IV (SCID). Controls were recruited through local advertisements at Osaka University. Psychiatrically, medically and neurologically healthy controls were evaluated using the non-patient version of the SCID to exclude individuals who had current or past contact with psychiatric services or who had received psychiatric medication. Current symptoms of schizophrenia were evaluated using the positive and negative syndrome scale (PANSS) [27]. Mean age, sex ratio and handedness did not differ significantly between cases and controls ($p>0.17$), while the years of education, estimated premorbid intelligence quotient (IQ) and GM volumes were significantly lower in the patients with schizophrenia than in the controls ($p<0.001$) (Table S1). When the genotype groups were compared, we found no differences in the demographic variables, except for years of education and duration of illness in patients with schizophrenia (Table S1).

SNP selection and SNP genotyping

We selected rs12807809 in the *NRGN* gene as described in the introduction. This polymorphism is reported as T/C and was previously described in the GWAS [3]. Venous blood was collected from the subjects, and genomic DNA was extracted from whole blood according to standard procedures. The SNP was genotyped using the TaqMan 5′-exonuclease allelic discrimination assay (Assay ID: C__32029000_20, Applied Biosystems, Foster City, California, USA) as previously described [18,19]. Detailed information on the PCR conditions is available upon request. No deviation from Hardy-Weinberg equilibrium (HWE) in the examined SNP was detected in the patients or in the controls ($p>0.05$).

Magnetic resonance imaging procedure

All magnetic resonance (MR) studies were performed on a 1.5T GE Sigma EXCITE system. A three-dimensional volumetric acquisition of a T1-weighted gradient echo sequence produced a

gapless series of 124 sagittal sections using a spoiled gradient recalled acquisition in the steady state (SPGR) sequence (TE/TR, 4.2/12.6 ms; flip angle, 15°; acquisition matrix, 256×256; 1NEX, FOV, 24×24 cm; slice thickness, 1.4 mm). MR images were processed using optimized VBM in Statistical Parametric Mapping 5 (SPM5) running on MATLAB R2010b (MathWorks, Natick, MA) according to the VBM5.1-Manual (http://dbm.neuro.uni-jena.de/vbm/vbm5-for-spm5/manual/) and as previously described [28,29]. We screened all scans and found no gross abnormalities, such as infarcts, hemorrhages or brain tumors, in any of the subjects. Each image was visually examined to eliminate images with motion or metal artifacts, and then the anterior commissure-posterior commissure line was adjusted. The normalized segmented images were modulated by multiplication with Jacobian determinants of the spatial normalization function to encode the deformation field for each subject as tissue volume changes in the normal space. Finally, images were smoothed with a 12-mm full-width, half-maximum isotropic Gaussian kernel.

Statistical analyses were performed with SPM8 software (http://www.fil.ion.ucl.ac.uk/spm/software/spm8/). First, we performed whole brain searches to explore the effects of the *NRGN* genotype and the genotype-diagnosis interaction on GM or WM volume in total subjects. Second, we performed separate whole brain searches to explore the effect of the *NRGN* genotype on GM or WM volume in patients with schizophrenia and in controls. The genotype effect on GM or WM volume was assessed statistically using a multiple regression model in SPM8. We contrasted GM or WM volume between the genotype groups (coded as the number of rs12807809 risk T alleles: 0, 1, or 2); GM or WM volumes were correlated with the number of risk T alleles, either positively (CC<CT<TT) or negatively (TT<CT<CC). The genotype-diagnosis interaction on GM or WM volumes was assessed full factorial model with diagnosis as a factor and genotype status as a covariate interacted with the diagnosis in SPM8. Age, sex and years of education were included as covariates of no interest into all analyses to control for confounding variables. Non-sphericity was estimated. These analyses yielded statistical parametric maps {SPM (t)} based on a voxel-level height threshold of $p<0.001$ (uncorrected for multiple comparisons). Clusters of more than 100 contiguous voxels were considered in the analyses. Family-wise error (*FWE*) correction was applied for multiple testing to avoid type I errors. The significance level was set at $p<0.05$ (*FWE* corrected). Anatomic localization was performed according to both MNI coordinates and Talairach coordinates, which were obtained from M. Brett's transformations (http://www.mrccbu.cam.ac.uk/Imaging/Common/mnispace.shtml) and presented as Talairach coordinates.

Statistical analyses

The presence of Hardy-Weinberg equilibrium was examined by the χ^2 test for goodness-of-fit using SNPAlyze V5.1.1 Pro software (DYNACOM, Yokohama, Japan). Statistical analyses of demographic variables were performed using PASW Statistics 18.0 software (SPSS Japan Inc., Tokyo, Japan). Differences in clinical characteristics between patients and controls or between genotypes were analyzed using χ^2 tests for categorical variables and the Mann-Whitney *U*-test or Kruskal-Wallis test for continuous variables. The significance level for all statistical tests was set at two-tailed $p<0.05$.

Results

Effects of the genotype and diagnosis-genotype interaction on GM or WM regions in total subjects

First, we investigated the effects of genotype and diagnosis-genotype interaction on GM or WM volumes in the whole brain analyses of total subjects. We found significant effects of the risk T allele on decreased GM volume in the right fusiform gyrus (uncorrected $p<0.001$, Table 1 and blue regions in Figure 1), and on increased WM volume in the inferior parietal lobule among total subjects (uncorrected $p<0.001$, Table 1). We also found significant genotype-diagnosis interaction on GM volume in the left anterior cingulate gyrus and the bilateral precuneus (uncorrected $p<0.001$, Table 1 and red regions in Figure 1). However, the effects of genotype and genotype-diagnosis interaction on these GM or WM regions did not survive after the *FWE*-correction for multiple tests (*FWE*-corrected $p>0.05$). There was no significant effect of the risk T allele on increased GM volumes, the risk T allele on decreased WM volumes, or genotype-diagnosis interaction on WM volume among total subjects (uncorrected $p>0.001$).

Effect of the risk T allele on decreased GM regions (TT<CT<CC)

Second, we separately investigated the effects of genotype on GM or WM volumes in the whole brain analyses of patients with schizophrenia and healthy controls. We found significant effects of the *NRGN* genotype on GM volume in the left anterior cingulate gyrus, the bilateral middle temporal gyrus and the left inferior frontal gyrus among the patients with schizophrenia (uncorrected $p<0.001$, Table 2 and red regions in Figure S1). We found significant effect of the *NRGN* genotype on GM volume in the right fusiform gyrus among the healthy controls (uncorrected $p<0.001$, Table 2 and blue regions in Figure S1). The genotype effect on the left anterior cingulate gyrus (BA32) in the patients with schizophrenia remained significant even after the *FWE*-correction for multiple tests at the whole brain level ($T_{94}=5.63$, *FWE*-corrected $p=0.0042$, Table 2); genotype effects on other regions did not survive the *FWE*-correction (*FWE*-corrected $p>0.05$). In patients with schizophrenia, the risk T carriers had a smaller GM volume in the left anterior cingulate gyrus than did the non-risk C carriers (Figure 2).

Researchers have suggested that the volume reduction of the anterior cingulate cortex (ACC) is associated with the duration of the illness (the length of time the patient has had schizophrenia) [30]. In our samples, the duration of illness differed significantly among the genotype groups in patients with schizophrenia (Table S1). Thus, we corrected for the duration of illness. The genotype effect on the left anterior cingulate gyrus remained significant even after controlling for the duration of illness ($T_{93}=5.86$, *FWE*-corrected $p=0.0017$).

Effect of the risk T allele on increased GM regions (CC<CT<TT)

We found significant effects of the *NRGN* genotype on GM volume in the bilateral precuneus among the patients with schizophrenia (uncorrected $p<0.001$, Table 2 and red region in Figure S2); however, the genotype effects on these regions did not survive after the *FWE*-correction for multiple tests (*FWE*-corrected $p>0.05$). There was no significant effect of the *NRGN* genotype on GM volume among the healthy controls (uncorrected $p>0.001$).

Effects of the risk T allele on WM regions

We found no significant effect of the risk T allele on any decreased WM regions (TT<CT<CC) for either the patients or controls (uncorrected $p<0.001$). On the other hand, we found significant effects of the risk T allele on increased WM region (CC<CT<TT) in the bilateral insula and middle frontal gyrus

Table 1. Effects of *NRGN* genotype and genotype-diagnosis interaction on GM and WM volumes in total subjects.

						p values		Talairach coordinates		
	Brain regions	**R/L**	**BA**	**CS**	***T***	**Uncorrected**	***FWE***	***x***	***y***	***z***
GM	***NRGN* genotipe-diagnosis interaction**									
	Limbic Lobe									
	Anterior Cingulate	L	32	219	4.17	<0.001	0.33	−12	40	−10
	Occipital Lobe									
	Precuneus	R	31	118	3.63	<0.001	0.90	15	−64	20
	Precuneus	L	31	165	3.54	<0.001	0.95	−7	−72	25
GM	**Total subjects; TT<CT<CC (higher risk<lower risk)**									
	Temporal Lobe									
	Fusiform Gyrus	R	20	290	4.28	<0.001	0.25	45	−30	−23
GM	**Total subjects; TT>CT>CC (higher risk>lower risk)**									
	no suprathreshold clusters									
WM	***NRGN* genotipe-diagnosis interaction**									
	no suprathreshold clusters									
WM	**Total subjects; TT<CT<CC (higher risk<lower risk)**									
	no suprathreshold clusters									
WM	**Total subjects; TT>CT>CC (higher risk>lower risk)**									
	Parietal Lobe									
	Inferior Parietal Lobule	R		616	3.72	<0.001	0.34	44	−41	25

GM: gray matter, WM: white matter, R: right, L: left, BA: Brodmann area, CS: Cluster size, FWE: family-wise error.
doi:10.1371/journal.pone.0029780.t001

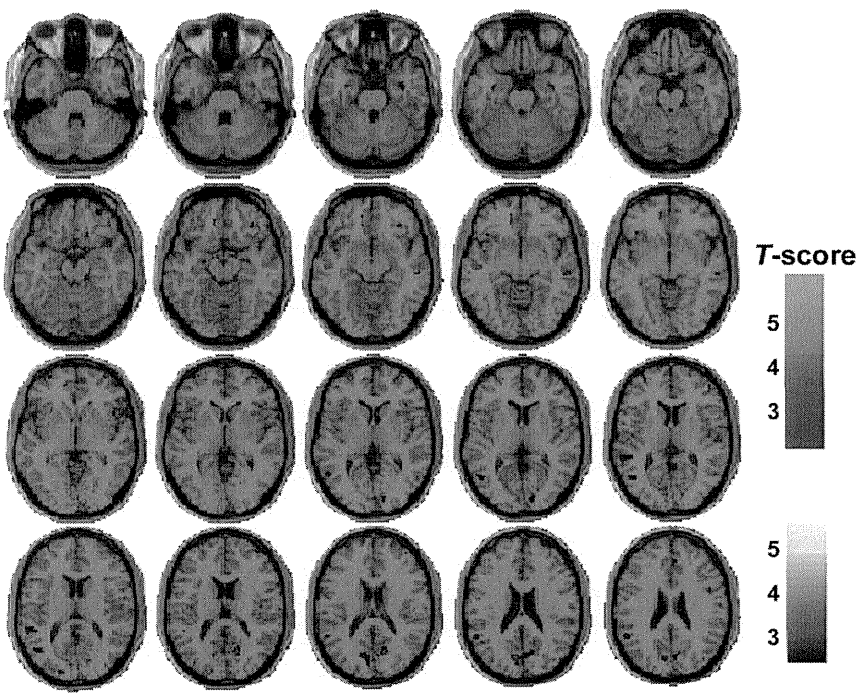

Figure 1. Effects of the risk-T-allele on decreased GM regions and diagnosis-*NRGN* genotype interaction on GM regions. Effects of the risk T allele on decreased GM regions (TT<CT<CC) in total subjects were shown by whinter colormap (blue areas). Diagnosis-*NRGN* genotype interaction on GM regions was shown by hot colormap (red areas). There was no significant effect of the risk T allele on increased GM regions (CC<CT<TT) among the total subjects. Each colormap shows *t* values corresponding to the color in the figure.
doi:10.1371/journal.pone.0029780.g001

Table 2. Effects of *NRGN* genotype on GM volumes in patients with schizophrenia and in healthy controls.

					p values		Talairach coordinates		
Brain regions	**R/L**	**BA**	**CS**	***T***	**Uncorrected**	***FWE***	***x***	***y***	***z***
SZ; TT<CT<CC (higher risk<lower risk)									
Limbic Lobe									
Anterior Cingulate	L	32	525	5.63	<0.001	**0.0042**	−12	42	−9
Temporal Lobe									
Middle Temporal Gyrus	L	21	143	3.87	<0.001	0.80	−66	−19	−5
Middle Temporal Gyrus	R	21	106	3.69	<0.001	0.93	59	−24	−6
Frontal Lobe									
Inferior Frontal Gyrus	L	10	102	3.88	<0.001	0.80	−36	45	4
HC; TT<CT<CC (higher risk<lower risk)									
Temporal Lobe									
Fusiform Gyrus	R	20	334	4.4	<0.001	0.19	45	−31	−23
SZ; TT>CT>CC (higher risk>lower risk)									
Parietal Lobe									
Precuneus	L	7	182	4	<0.001	0.68	−15	−64	38
Occipital Lobe									
Precuneus	R	31	143	3.81	<0.001	0.86	15	−64	19
HC; TT>CT>CC (higher risk>lower risk)									
no suprathreshold clusters									

GM: gray matter, R: right, L: left, BA: Brodmann area, CS: Cluster size, FWE: family-wise error, SZ: patients with schizophrenia, HC: healthy controls. Significant results [$p<0.05$ (*FWE* corrected)] are shown as bold face and underline.
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among the patients with schizophrenia (uncorrected $p<0.001$, Table S2 and red regions in Figure S3). However, the genotype effects on these regions did not survive after the *FWE*-correction (*FWE*-corrected $p>0.05$). There was no significant genotype effect on any increased WM region for the controls (uncorrected $p<0.001$). These findings suggest that *NRGN* may not play a major role in the morphology of WM.

Discussion

This is the first study to identify brain morphology associated with genome-wide significant risk variants in *NRGN* for schizophrenia at the whole brain level. Genotype-diagnosis interaction on GM volume in the left ACC was found, even though the effect did not survive after the *FWE*-correction. When we separately

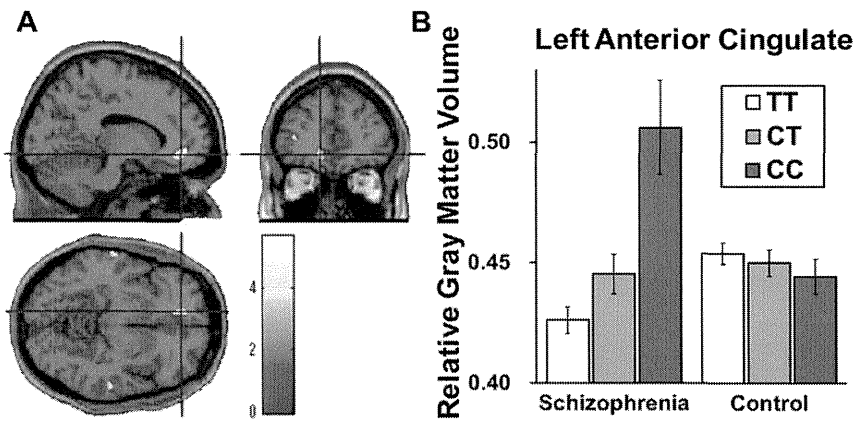

Figure 2. Impact of the *NRGN* genotype on GM volume of left anterior cingulate gyrus in schizophrenia. (**A**) Anatomical localizations are displayed on coronal, sagittal, and axial sections of a normal MRI spatially normalized into the Montreal Neurological Institute template (uncorrected $p<0.001$, cluster size>100). A significant cluster of the genotype effect was in the left anterior cingulate gyrus in the patients with schizophrenia, after controlling for differences in the duration of illness among genotypes. The region is shown as cross-hairline. The color bars show *t* values corresponding to the color in the figure. (**B**) Each column shows relative gray matter volumes extracted from the left anterior cingulate gyrus (Talairach coordinates; −12, 42, −9). We extracted a sphere with a 10 mm volume-of-interest (VOI) radius from the significant region to compare the effects of the genotype in both the patients with schizophrenia and healthy subjects. Error bars represent the standard error.
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investigated the effects of the interaction on GM volume of patients with schizophrenia and healthy controls, carrying the risk T allele of rs12807809 was associated with reduced GM volume in the left ACC in patients with schizophrenia. The genotype effect survived a correction for multiple comparisons at the whole brain level. This finding applies to the patients with schizophrenia but not to the healthy controls, and it is present even after controlling for differences in the duration of illness among genotypes. Significant difference on WM volume between genotypes was not observed for any region in patients or controls.

The ACC is a functionally heterogeneous region involved in diverse cognitive processes [30]. The functional diversity of the ACC encompasses executive, attention, social cognitive, affective and skeleton- and viscera-motor functions. Most MRI studies suggest that patients with schizophrenia show reduced GM in the ACC [30]. These reductions extend across the dorsal and rostral divisions of the limbic and paralimbic regions of the ACC. Some studies suggest that relatives of schizophrenia patients also show bilateral reductions in GM volume or thickness in the ACC [31,32]. Post-mortem findings indicate that these imaging-related changes are accompanied by reductions in neuronal, synaptic, and dendritic density as well as increased afferent input [30]. These findings suggest that the GM differences observed with MRI arise from alterations in both neuronal and non-neuronal tissue compartments.

The GM reductions in the ACC precede the onset of psychosis in some categories of high-risk individuals. Cross-sectional and longitudinal studies suggest that the earliest ACC changes in schizophrenia appear in the rostral paralimbic regions of the ACC prior to the onset of psychosis, extend across the paralimbic regions of the ACC during the transition to a first episode psychosis, and spread to engulf the limbic regions of the ACC with continued illness [30]. The regions of the genotype effect in the present study were the paralimbic regions of the ACC. A mean duration of illness in patients included in this study was 13.0 ± 10.4 years; these patients are considered to have established schizophrenia. As the duration of illness has been related to the degree of reduction of the ACC and because it significantly differed among the genotype groups in our subjects, we ascertained whether the genotype effect in the ACC is affected by variation in the duration of illness. However, the genotype effect in the left ACC was robust even after controlling for the duration of illness. These findings suggest that part of the paralimbic regions of the ACC may be attributed to the effects of the genome-wide supported variant of *NRGN* in patients with schizophrenia, regardless of the duration of illness.

NRGN is especially enriched in CA1 pyramidal neurons in the hippocampus [33]. *NRGN* produced severe deficits in hippocampus-dependent tasks in knock-out mice [34,35]. This evidence suggests that *NRGN* may be important in neurocognitive tasks such as learning and memory and in the morphology and function of the hippocampus. Based on this hypothesis, Donohoe et al. tested the relationship between schizophrenia associated with the *NRGN* variant rs12807809 and cognition in Irish and German case-control samples [36]. They did not find a significant association between the *NRGN* variant and cognition in the samples. Pohlack et al. found that homozygous T carriers had decreased activation of the left hippocampus during contextual fear conditioning but did not find the same result in the hippocampal structure of Caucasian healthy volunteers [37]. We did not find a significant association between the *NRGN* variant and hippocampal volume, consistent with recent study using the ROI approach [37]. These findings suggest that *NRGN* may play an important role in hippocampal activity but not play a major role in the neurocognition of learning and memory or in the morphology of the hippocampus.

There were several limitations to this study. A false-positive association could not be excluded from our study despite the precautions for ethnic matching and corrections for multiple testing. It is necessary to conduct further investigations to confirm our findings in other samples with much larger sample sizes and/or with different ethnicities and/or in relatives with schizophrenia. A false-negative association could not be excluded in our study because we applied a strict correction for multiple comparisons at the whole brain level (*FWE*-corrected $p<0.05$). The regions shown in the Supporting Information (uncorrected $p<0.001$) might be helpful in further studies. It is still unclear whether this genetic variant of the *NRGN* gene is associated with the expression, transcription, splicing or translation of the gene. The lack of a clear association makes it difficult to determine whether our results are directly linked to the *NRGN* polymorphism rs12807809, to other polymorphisms in linkage disequilibrium with this variant, or to interaction between this genetic variant of the *NRGN* and other polymorphism. As with other risk variants for schizophrenia, clarifying the biological role of this variant through *in vitro* and *in vivo* studies is important to improve the understanding of the pathophysiology of schizophrenia. In addition, an extensive search for other functional variants at this locus is needed to determine whether rs12807809 is the most strongly associated variant for schizophrenia in this gene.

In conclusion, we found that a genome-wide supported variant of *NRGN* may be associated with brain morphological vulnerability of the left ACC in patients with schizophrenia. Abnormalities in ACC may partly explain the disturbances in cognitive and emotional integration in patients with schizophrenia. Further research will be required to clarify the function of the risk *NRGN* variant on the pathophysiology of schizophrenia.

Supporting Information

Figure S1 Effect of risk-T-allele on decreased GM regions in patients with schizophrenia and in healthy controls. Effect of the risk T allele on decreased GM regions (TT<CT<CC) in the patients with schizophrenia was shown by hot colormap (red areas), while effect of the T allele on decreased GM regions in the healthy controls was shown by winter colormap (blue areas).
(TIF)

Figure S2 Effect of the risk-T-allele on increased GM regions in the patients with schizophrenia. Effect of the risk T allele on increased GM regions (CC<CT<TT) in the patients with schizophrenia was shown by hot colormap (red areas). There was no significant effect of the *NRGN* genotype on GM volume among the healthy controls.
(TIF)

Figure S3 Effect of the risk-T-allele on increased WM regions in the patients with schizophrenia. Effect of the risk T allele on increased WM regions (CC<CT<TT) in the patients with schizophrenia was shown by hot colormap (red areas). There was no significant effect of the *NRGN* genotype on WM volume among the healthy controls.
(TIF)

Table S1 Demographic information for patients with schizophrenia and healthy controls included in the VBM analysis.
(DOC)

Table S2 Effects of the *NRGN* genotype on WM volumes in patients with schizophrenia and healthy controls.
(DOC)

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Author Contributions

Conceived and designed the experiments: KO RH. Performed the experiments: HY SU TOakada KN TOhnishi. Analyzed the data: KO RH KN TOhnishi HY SU TOakada. Contributed reagents/materials/analysis tools: YY MF MI HK MT. Wrote the paper: KO RH YY MF MI HK MT.

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ORIGINAL INVESTIGATION

The *AKT1* gene is associated with attention and brain morphology in schizophrenia

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Abstract

Objectives. A meta-analysis of the associations between genetic variants in the *AKT1* gene and schizophrenia found that a single nucleotide polymorphism (SNP5; rs2494732) was associated with schizophrenia in Asian populations. *Methods.* In this study, we investigated the effects of this SNP on memory and attentional performance and brain structure using magnetic resonance imaging in a Japanese population (117 patients with schizophrenia and 189 healthy subjects). *Results.* The memory performance, particularly attention/concentration score, measured by the Wechsler Memory Scale-Revised in A carriers of SNP5, which was found to be enriched in patients with schizophrenia, was lower than that in individuals with the G/G genotype. We confirmed the association of the SNP with attentional performance using the Continuous Performance Test, which assessed sustained attention and vigilance of attentional function. Patients with A allele demonstrated lower attentional performance than patients with the G/G genotype. Patients with the A allele had smaller gray matter volumes in the right inferior parietal lobule related to attentional processes and in the frontostriatal region related to different SNPs in *AKT1* than patients with the G/G genotype. *Conclusions.* Our results suggest that a genetic variant of *AKT1* might be associated with attentional deficits and brain morphological vulnerability in patients with schizophrenia.

Key words: AKT1, *schizophrenia, single nucleotide polymorphism (SNP), attention, VBM*

Introduction

V-akt murine thymoma viral oncogene homolog 1 (AKT1, also known as PKB; protein kinase B) belongs to a serine/threonine kinase family and is highly expressed in the brain. AKT1 serves as a central node in cell signaling downstream of growth factors, cytokines, and other external stimuli. AKT1 contributes to several cellular functions such as cell growth, survival, and metabolism (Grimes and Jope 2001). Several studies in rodents demonstrated involvement of AKT1 in memory formation and synaptic plasticity (Lin et al. 2001; Mizuno et al. 2003; Horwood et al. 2006; Sui et al. 2008). In addition, *Akt1*-knockout mice showed poorer working memory performance

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under dopaminergic agonist challenge (Lai et al. 2006).

Schizophrenia is a common and complex psychiatric disease with strong genetic components. Schizophrenia has an estimated heritability of approximately 80% (Cardno and Gottesman 2000; Tsuang 2000) and many genes have been implicated in the pathogenesis of schizophrenia (Sun et al. 2008). Attention and memory are specifically impaired in schizophrenia (Green 2006). The attention and memory deficits are prominent trait markers for schizophrenia, with impairments also observed in first-degree relatives (Snitz et al. 2006). Susceptibility genes have been hypothesized to contribute to the disorder at least partly by influencing cognitive performance (Weinberger et al. 2001a). The *AKT1* gene located on chromosome 14q32.32 has been reported as a susceptibility gene for schizophrenia in various populations from the United States, the UK, Ireland, Switzerland, France, Bulgaria, Australia, Iran, China and Japan (Emamian et al. 2004; Ikeda et al. 2004; Schwab et al. 2005; Bajestan et al. 2006; Norton et al. 2007; Xu et al. 2007; Shi et al. 2008; Thiselton et al. 2008; Betcheva et al. 2009; Karege et al. 2010; Mathur et al. 2010), but not in all studies (Ohtsuki et al. 2004; Ide et al. 2006; Liu et al. 2006; Turunen et al. 2007; Sanders et al. 2008; Lee et al. 2010). A genetic variant, rs2494732 (SNP5), in the *AKT1* gene was associated with schizophrenia in Asian populations in a recent meta-analysis (Shi et al. 2008). This polymorphism was also found to predict treatment response to risperidone in Japanese patients with schizophrenia (Ikeda et al. 2008). The other genetic variant, rs1130233 (SNP4), in the *AKT1* gene was associated with AKT1 protein levels in lymphoblast (Harris et al. 2005; Tan et al. 2008b). The haplotypes comprising rs1130214 (SNP2), rs3730358 (SNP3) and SNP4 was associated with AKT1 protein levels in postmortem brain tissues (Karege et al. 2010). The interactions between SNP4 and rs1076560 in the dopamine D2 or a functional polymorphism (Val158Met) in catechol-*O*-methyltransferase (*COMT*) have been also investigated (Tan et al. 2008b). The interactions between SNP4 and rs1076560 were associated with AKT1 protein levels, phosphorylation of GSK3β, cingulate response, behavioural accuracy during attentional processing and response to olanzapine treatment (Blasi et al. 2011). The interactions between SNP4 and Val158Met were related to AKT1 phosphorylation (Sei et al. 2010), prefrontal physiology during executive function, and frontostriatal gray matter volume (Tan et al. 2008b). In this study, we focused on the single marker SNP5 associated with schizophrenia in Asian populations, but not these interactions to elucidate the genotype effect as simple as possible.

Many attempts have been made to minimize clinical and genetic heterogeneity for schizophrenia. A strategy for gene discovery proposes using quantitative neurobiological traits as intermediate phenotypes instead of the diagnosis of schizophrenia (Meyer-Lindenberg and Weinberger 2006; Tan et al. 2008a). This strategy has the potential to reduce clinical and genetic heterogeneity by applying intermediate phenotypes that reflect underlying genetic vulnerability better than diagnostic categorization. Memory, attention and brain structure are potential intermediate phenotypes that bridges the gap between genotype and diagnostic categorization (Chen and Faraone 2000; Weinberger et al. 2001b; Skelley et al. 2008). In recent years, a number of attempts to explain the link between intermediate phenotypes and a specific gene have been made. Indeed, *AKT1* was shown to be associated with verbal learning and memory in twin pairs of healthy controls, in patients with schizophrenia and in patients with bipolar disorder (Pietilainen et al. 2009). *AKT1* has also been associated with behavioral accuracy during attentional processing in healthy subjects (Blasi et al. 2011) and associated with IQ/processing speed in subjects with European ancestry (Tan et al. 2008b), although one study did not find an association between *AKT1* and verbal memory, working memory, visual memory or vigilance in patients with schizophrenia (Pinheiro et al. 2007). It was also reported that *AKT1* was associated with prefrontal structures in healthy controls (Tan et al. 2008a,b). In this study, we examined possible impacts of a genetic variant (SNP5) of *AKT1* on performances of memory and attention and brain structure in a Japanese population.

Methods and materials

Subjects

The subjects for this study consisted of 117 unrelated patients with schizophrenia (50.4% males (59/58), mean age ± SD; 35.9 ± 11.5 years) and 189 unrelated healthy controls (49.2% males (93/96), mean age ± SD; 38.3 ± 12.1 years). The Wechsler Memory Scale-Revised (WMR-R) was administered to 94 patients and 121 controls (Table I). The Continuous Performance Test – Identical pairs version (CPT-IP) was administered to 60 patients and 121 controls (Table II). Neuroimaging analysis was performed for 55 patients and 159 controls (Table III). Although we attempted to examine WMS-R, CPT-IP and magnetic resonance (MR) imaging from all subjects as much as we could, all tests were available only for 26 patients with schizophrenia and 91

Table I. Demographic information for subjects included in WMS-R analysis.

Variables	Schizophrenia ($N=94$) G/G ($N=46$)	Schizophrenia ($N=94$) A carriers ($N=48$)	Schizophrenia ($N=94$) P values (z)	Control ($N=121$) G/G ($N=57$)	Control ($N=121$) A carriers ($N=64$)	Control ($N=121$) P values (z)	Group difference P values (z)
Age (years)	39.9 ± 12.0	39.2 ± 12.0	0.90 (–0.12)	35.1 ± 12.3	34.9 ± 11.5	0.98 (–0.03)	**0.0022 (–3.06)**
Sex (male/female)	26/20	23/25	0.40 (0.70)[a]	32/25	31/33	0.31 (1.04)[a]	0.99 (<0.01)[a]
Education (years)	13.8 ± 2.4	14.2 ± 2.2	0.28 (–1.07)	15.4 ± 2.3	15.6 ± 2.1	0.70 (–0.38)	**3.37×10^{-6} (–4.65)**
Estimated premorbid IQ	100.9 ± 9.9	99.9 ± 9.9	0.69 (–0.40)	106.0 ± 8.0	104.2 ± 8.5	0.30 (–1.05)	**4.77×10^{-4} (–3.49)**
CPZeq (mg/day)	601.4 ± 479.7	583.8 ± 450.7	0.97 (–0.04)	–	–	–	–
Age at onset (years)	23.5 ± 9.0	24.0 ± 7.3	0.59 (–0.55)	–	–	–	–
Duration of illness (years)	16.4 ± 10.8	15.3 ± 11.6	0.59 (–0.53)	–	–	–	–
PANSS positive symptoms	16.7 ± 5.5	17.7 ± 6.8	0.66 (–0.44)	–	–	–	–
PANSS negative symptoms	17.9 ± 7.2	18.1 ± 7.2	0.93 (–0.08)	–	–	–	–
PANSS general psychopathology	34.1 ± 9.5	36.1 ± 10.7	0.41 (–0.82)	–	–	–	–

Means ± SD and P values are shown. [a]χ^2-test. PANSS, Positive and Negative Syndrome Scale; CPZ-eq, chlorpromazine equivalent of total antipsychotics. There was no significant difference between genotypes for any variable in each genotype group. Complete demographic information was not obtained for all patients with schizophrenia (estimated premorbid IQ: A carriers, $N=43$; PANSS: A carriers, $N=46$).

Table II. Demographic information for subjects included in CPT analysis.

Variables	Schizophrenia ($N=60$) G/G ($N=35$)	Schizophrenia ($N=60$) A carriers ($N=25$)	Schizophrenia ($N=60$) P values (z)	Control ($N=121$) G/G ($N=57$)	Control ($N=121$) A carriers ($N=64$)	Control ($N=121$) P values (z)	Group difference P values (z)
Age (years)	40.0 ± 12.4	40.4 ± 11.6	0.65 (–0.46)	35.1 ± 12.3	34.9 ± 11.5	0.51 (–0.65)	**0.0035 (–2.92)**
Sex (male/female)	22/13	12/13	0.25 (1.31)[a]	32/25	31/33	0.40 (0.72)[a]	0.56 (0.34)[a]
Education (years)	14.0 ± 2.5	14.2 ± 2.0	0.47 (–0.72)	15.4 ± 2.3	15.6 ± 2.1	0.87 (–0.17)	**9.91×10^{-5} (–3.89)**
Estimated premorbid IQ	100.0 ± 10.4	99.8 ± 11.1	0.99 (–0.01)	106.0 ± 8.0	104.2 ± 8.5	0.34 (–0.95)	**0.0011 (–3.25)**
CPZeq (mg/day)	542.9 ± 477.6	544.0 ± 394.2	0.76 (–0.31)	–	–	–	–
Age at onset (years)	23.7 ± 9.2	23.9 ± 6.6	0.50 (–0.67)	–	–	–	–
Duration of illness (years)	16.3 ± 11.0	16.5 ± 12.1	0.98 (–0.02)	–	–	–	–
PANSS positive symptoms	16.8 ± 5.9	18.8 ± 7.6	0.42 (–0.80)	–	–	–	–
PANSS negative symptoms	18.5 ± 7.3	18.5 ± 8.1	0.91 (–0.11)	–	–	–	–
PANSS general psychopathology	35.5 ± 9.8	36.2 ± 11.5	0.90 (–0.13)	–	–	–	–

Means ± SD and P values are shown. [a]χ^2-test. PANSS, Positive and Negative Syndrome Scale; CPZ-eq, chlorpromazine equivalent of total antipsychotics. There was no significant difference between genotypes for any variable in each genotype group.

Table III. Demographic information for patients with schizophrenia and healthy controls included in the ROI analysis.

Variables	Schizophrenia ($N=55$)			Control ($N=159$)			Group difference
	G/G ($N=25$)	A carriers ($N=30$)	P values (z)	G/G ($N=71$)	A carriers ($N=88$)	P values (z)	P values (z)
Age (years)	35.3 ± 12.2	36.5 ± 11.0	0.59 (−0.53)	36.8 ± 10.9	36.7 ± 11.5	0.89 (−0.14)	0.64 (−0.47)
Sex (male/female)	15/10	14/16	0.32 (0.97)[a]	33/38	42/46	0.88 (0.02)[a]	0.48 (0.51)[a]
Education (years)	12.9 ± 2.5	14.6 ± 1.8	**0.0054 (−2.78)**	15.4 ± 2.5	15.4 ± 2.4	0.88 (−0.15)	**3.50×10^{-4} (−3.58)**
Estimated premorbid IQ	97.3 ± 9.0	101.0 ± 9.4	0.15 (−1.43)	106.8 ± 7.6	105.9 ± 9.0	0.57 (−0.57)	**1.91×10^{-6} (−4.76)**
Handedness (rt./lt.)	23/2	29/1	0.45 (0.48)[a]	67/4	85/3	0.50 (0.46)[a]	0.75 (0.10)[a]
Gray matter volume (mm3)	693.0 ± 78.6	683.3 ± 81.3	0.51 (−0.66)	709.7 ± 68.4	709.8 ± 84.5	>0.99 (<0.01)	0.067 (−1.83)
CPZeq (mg/day)	648.8 ± 540.9	518.8 ± 502.8	0.30 (−1.04)	–	–	–	–
Age at onset (years)	24.2 ± 11.2	23.9 ± 8.7	0.67 (−0.42)	–	–	–	–
Duration of illness (years)	11.1 ± 6.0	12.6 ± 9.9	0.87 (−0.16)	–	–	–	–
PANSS positive symptoms	16.6 ± 6.9	18.5 ± 6.4	0.24 (−1.17)	–	–	–	–
PANSS negative symptoms	19.4 ± 8.5	18.2 ± 6.3	0.79 (−0.26)	–	–	–	–
PANSS general psychopathology	35.8 ± 12.2	38.5 ± 10.4	0.32 (−1.00)	–	–	–	–

Means ± SD and P values are shown. [a]χ^2-test. PANSS, Positive and Negative Syndrome Scale; CPZ-eq, chlorpromazine equivalent of total antipsychotics. Complete demographic information was not obtained for all subjects (estimated premorbid IQ and PANSS: A carriers in patients, $N=29$; estimated premorbid IQ: A carriers, $N=87$ in controls).

healthy subjects. The subjects met criteria as follows: all subjects were biologically unrelated Japanese individuals and were recruited at Osaka University. The subjects were excluded from the analyses if they had neurological or medical conditions that could potentially affect the central nervous system, such as atypical headache, head trauma with loss of consciousness, chronic lung disease, kidney disease, chronic hepatic disease, thyroid disease, active cancer, cerebrovascular disease, epilepsy or seizures. Cases were recruited from both outpatients and inpatients at the university hospital. Each subject with schizophrenia had been diagnosed by at least two trained psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV), based on an unstructured clinical interview. Schizophrenics with comorbid substance-related disorders or mental retardation were excluded. Controls were recruited through local advertisements. Psychiatrically, medically and neurologically healthy controls were evaluated using the structured clinical interview for DSM-IV-Non-Patient to exclude individuals who had current or past contact with psychiatric services. Written informed consent was obtained for all subjects after the procedures had been fully explained. This study was carried out in accordance with World Medical Association's Declaration of Helsinki and approved by the Research Ethical Committee of Osaka University.

SNP selection and genotyping

A genetic variant, rs2494732 (SNP5), in the *AKT1* gene was selected for this study, as described in the introduction. The designation of the SNP in parenthesis was according to original study (Emamian et al. 2004). Venous blood was collected from the subjects and genomic DNA was extracted from whole blood according to standard procedures. Genotyping of SNP5 was carried out using TaqMan assays as previously described (Hashimoto et al. 2006, 2007). Detailed information on the PCR conditions and the primer pairs is available upon request. No deviation from Hardy-Weinberg equilibrium in the examined SNP was detected in the patients or controls ($P>0.05$) (patients with schizophrenia; G/G: 56; A/G: 52; A/A: 9, healthy controls; G/G: 86; A/G: 78; A/A: 25). Because of the small number of subjects homozygous for the A allele, the A/A and A/G genotype groups were combined and treated as the A carriers for this study.

Cognitive measures

A full version of the WMS-R (Sugishita 2001; Wechsler 1987), a measure that is generally used to

measure memory and attentional functions, was administered to the subjects. The five indices, attention/concentration, verbal memory, visual memory, delayed recall and general memory, of the WMS-R were used to the analysis. The scores of indices were corrected by age. To assess sustained attention and vigilance, we used the CPT-IP. Compared to the simple version CPT-X, the CPT-IP is considered to be highly attention demanding (Cornblatt et al. 1988). This version is a prominent measure of attention in schizophrenia research (Cornblatt et al. 1989; Cornblatt and Keilp 1994). The CPT-IP was presented in a computerized version. The stimuli were two-, three-, or four-digit numbers in separate conditions. We performed each condition within a 1-min interval in order of two-, three-, or four-digit numbers. Each condition contained 150 stimuli. Each stimulus appeared on the monitor for 50 ms, followed by a dark time of 950 ms, for a total trial time of 1 s. A 150-trial condition, therefore, takes approximately 2.5 min to administer. Subjects were asked to respond (via a finger lift from a reaction-time key) whenever the same digit numeral appeared twice in succession during the sequence. In each condition, 20% of the stimuli are target pairs that are exactly alike and require a response; 20% are "catch" trials (pairs that are almost alike but are not quite identical); and 60% are randomly organized fillers. The parameter *D'* is used as outcome variable which is a measure of sensitivity composed of hits and false alarms. *D'2*, *D'3*, and *D'4* correspond to the number of digits in each number. *D'3* is more difficult than *D'2*, and *D'4* is the most difficult condition among the three different conditions.

Magnetic resonance imaging procedure

All MR studies were performed on a 1.5 T GE Sigma EXCITE system. A three-dimensional volumetric acquisition of a T1-weighted gradient echo sequence produced a gapless series of 124 sagittal sections using a spoiled gradient recalled acquisition in the steady state (SPGR) sequence (TE/TR, 4.2/12.6 ms; flip angle, 15°; acquisition matrix, 256 × 256; 1NEX, FOV, 24 × 24 cm; slice thickness, 1.4 mm). Statistical analyses were performed with Statistical Parametric Mapping 5 (SPM5) software (http//www.fil.ion.ucl.ac.uk/spm) running on MATLAB R2007a (MathWorks, Natick, MA). MR images were processed using optimized voxel-based morphometry (VBM) in SPM5 according to VBM5.1-Manual (http://dbm.neuro.uni-jena.de/vbm/vbm5-for-spm5/manual/) as previously described in detail (Ashburner and Friston 2000; Good et al. 2001). We screened all scans and found no gross abnormalities such as infarct, haemorrhage and brain tumours in any of the subjects. Each image was visually confirmed to eliminate images with motion or metal artifacts, and then the anterior commissure-posterior commissure line was adjusted. The normalized segmented images were modulated by multiplication with Jacobian determinants of the spatial normalization function to encode the deformation field for each subject as tissue volume changes in the normal space. Finally, images were smoothed with a 12-mm full-width half-maximum of isotropic Gaussian kernel.

We first performed an exploratory whole brain search to investigate the effects of diagnosis, genotype and their interaction on gray matter volume in patients with schizophrenia and controls. These effects on gray matter volume were assessed statistically using the full factorial model for a 2 × 2 ANOVA in SPM5. We contrasted gray matter volumes between the genotype groups (smaller volume region in A carriers relative to individuals with G/G genotype, and larger volume region in A carriers relative to those with G/G genotype), the diagnosis groups (smaller volume region in patients with schizophrenia relative to controls) and their interaction. Age, sex and education years were included to control for confounding variables in the analysis. Since it is desirable to adjust for each subject's global gray matter volume (Good et al. 2001), adjustment was performed by entering the global gray matter values as a covariate. Non-sphericity estimation was used. The exploratory whole brain analysis yielded statistical parametric maps {SPM (t)} based on a voxel-level height threshold of $P < 0.001$ (uncorrected for multiple comparisons). To reduce the type I error due to small clusters, only clusters of more than 180 contiguous voxels were considered in the analysis. Given the a priori hypothesis, small volume correction (SVC) was applied to protect against type I error using family wise error (FWE). The significance level was set as $P < 0.05$ (*FWE* corrected) after SVC for spheres with a radius of 10 mm around the peak. Based on the priori hypothesis, we next performed volume-of-interest (VOI) approach to further compare significant regions of diagnosis–genotype interaction in the exploratory whole brain analysis. We extracted a sphere of 10 mm VOI radius from regions of interest because SVC applied for spheres with a radius of 10 mm around the peak. Anatomic localization was performed according to both MNI coordinates and Talairach coordinates, which were obtained from M. Brett's transformations (http://www.mrccbu.cam.ac.uk/Imaging/Common/mnispace.shtml) and presented as Talairach coordinates.

Statistical analysis

Statistical analyses were performed using PASW Statistics 18.0 software (SPSS Japan Inc., Tokyo, Japan). Differences in clinical characteristics between patients and controls or between genotypes were analyzed using χ^2-tests for categorical variables and the Mann–Whitney *U*-test for continuous variables. The presence of Hardy–Weinberg equilibrium was examined using the χ^2-test for goodness of fit. Multivariate analysis of covariance (MANCOVA) is an extension of ANCOVA methods to cover cases where there is more than one dependent variable and where the dependent variables cannot simply be combined and are correlated with each other. The effects of the diagnosis, genotype and their interaction on memory performance in the WMS-R and attentional performance in the CPT were analyzed by two-way MANCOVA. Diagnosis and genotype statuses were included in the model as independent variables. Each WMS-R subscale score (attention/concentration, verbal memory, visual memory, delayed recall and general memory) on memory performance or each *D'* (*D'2*, *D'3* and *D'4*) on attentional performance was included as dependent variables. Complete demographic information about estimated premorbid IQ was not obtained for all subjects. As years of education are correlated with the premorbid IQ, we used the education years in place of the premorbid IQ as covariates. For the WMS-R, sex and years of education were treated as covariates, as they were possible confounding factors. For the CPT, sex, age, and years of education were treated as covariates. Post hoc analyses were performed as analyses of covariance (ANCOVA). Pearson's correlation coefficients were used to assess relationships between neurocognitive domains. We extracted relative gray matter volume as the "*y*" values from maxima voxel in the region of interest, and used these values in the VOI analysis using PASW. The effects of the variation in *AKT1* on the extracted VOI were tested by analyses of variance (ANOVA) without covariates, as the extraction of VOI was performed after confounding factors including age, sex, education years and total gray matter volumes were included in the whole brain search analysis. Statistical significance was defined as $P<0.05$.

Results

Association between a genetic variant in AKT1 and memory performances measured by the WMS-R

We examined the possible impact of SNP5 on memory performance measured by the WMS-R in 94 patients with schizophrenia and 121 healthy subjects. There was no difference in demographic variables between *AKT1* genotype groups (Table I). A two-way MANCOVA revealed significant effects of diagnosis ($F_{1,205}=28.55$, $P=6.35\times10^{-22}$) and marginally significant effects of genotype ($F_{1,205}=2.22$, $P=0.054$) on memory performance (Table IV and Figure 1). No genotype–diagnosis interaction was found ($P=0.52$). As expected, memory performance in patients with schizophrenia was poorer than in controls. Memory performance in A-carriers was poorer than in subjects homozygous for the G-allele. Post hoc two-way ANCOVA revealed significant effects of diagnosis (attention/concentration: $F_{1,209}=44.97$, $P=1.84\times10^{-10}$, verbal memory: $F_{1,209}=120.28$, $P=2.12\times10^{-22}$, visual memory: $F_{1,209}=53.39$, $P=5.69\times10^{-12}$, delayed recall: $F_{1,209}=130.70$, $P=7.97\times10^{-24}$ and general memory: $F_{1,209}=127.57$ $P=2.11\times10^{-23}$) and genotype (attention/concentration: $F_{1,209}=5.01$, $P=0.026$ and delayed recall: $F_{1,209}=3.99$, $P=0.047$). No genotype–diagnosis interaction was found ($P>0.39$). Genotype effects were found in the scores of attention/concentration and delayed recall (Figure 1), while there was no association between the genetic variation of *AKT1* and the scores on the other three indices (Figure 1). The attention/concentration and delayed recall scores in A allele carriers were lower than in homozygous G subjects. These results suggest that the A allele carriers could have poorer memory performance, particularly

Table IV. Association of a genetic variant of *AKT1* with the five indices of WMS-R.

	Schizophrenia ($N=94$)		Control ($N=121$)		*P* values ($F_{1,209}$ values)		
	G/G ($N=46$)	A carriers ($N=48$)	G/G ($N=57$)	A carriers ($N=64$)	Diagnosis effect	Genotype effect	Interaction
MANCOVA					**6.35×10^{-22} (28.55)**	0.054 (2.22)	0.52 (0.84)
Attention/concentration	94.3 ± 14.0	89.6 ± 17.0	110.0 ± 15.7	106.0 ± 13.1	**1.84×10^{-10} (44.97)**	**0.026 (5.01)**	0.85 (0.04)
Verbal memory	85.0 ± 17.9	85.1 ± 18.2	111.5 ± 15.0	110.6 ± 10.9	**2.12×10^{-22} (120.28)**	0.59 (0.29)	0.85 (0.03)
Visual memory	91.5 ± 19.7	90.3 ± 20.8	108.8 ± 11.4	109.1 ± 9.0	**5.69×10^{-12} (53.39)**	0.54 (0.38)	0.68 (0.17)
Delayed recall	85.0 ± 19.2	79.7 ± 20.0	112.0 ± 14.2	110.3 ± 12.4	**7.97×10^{-24} (130.70)**	**0.047 (3.99)**	0.39 (0.76)
General memory	85.0 ± 19.2	84.3 ± 19.6	113.1 ± 14.5	111.4 ± 10.1	**2.11×10^{-23} (127.57)**	0.35 (0.86)	0.86 (0.03)

MANCOVA, multivariate analysis of covariance. Means ± SD and *P* values are shown. Significant *P* values are indicated in bold.