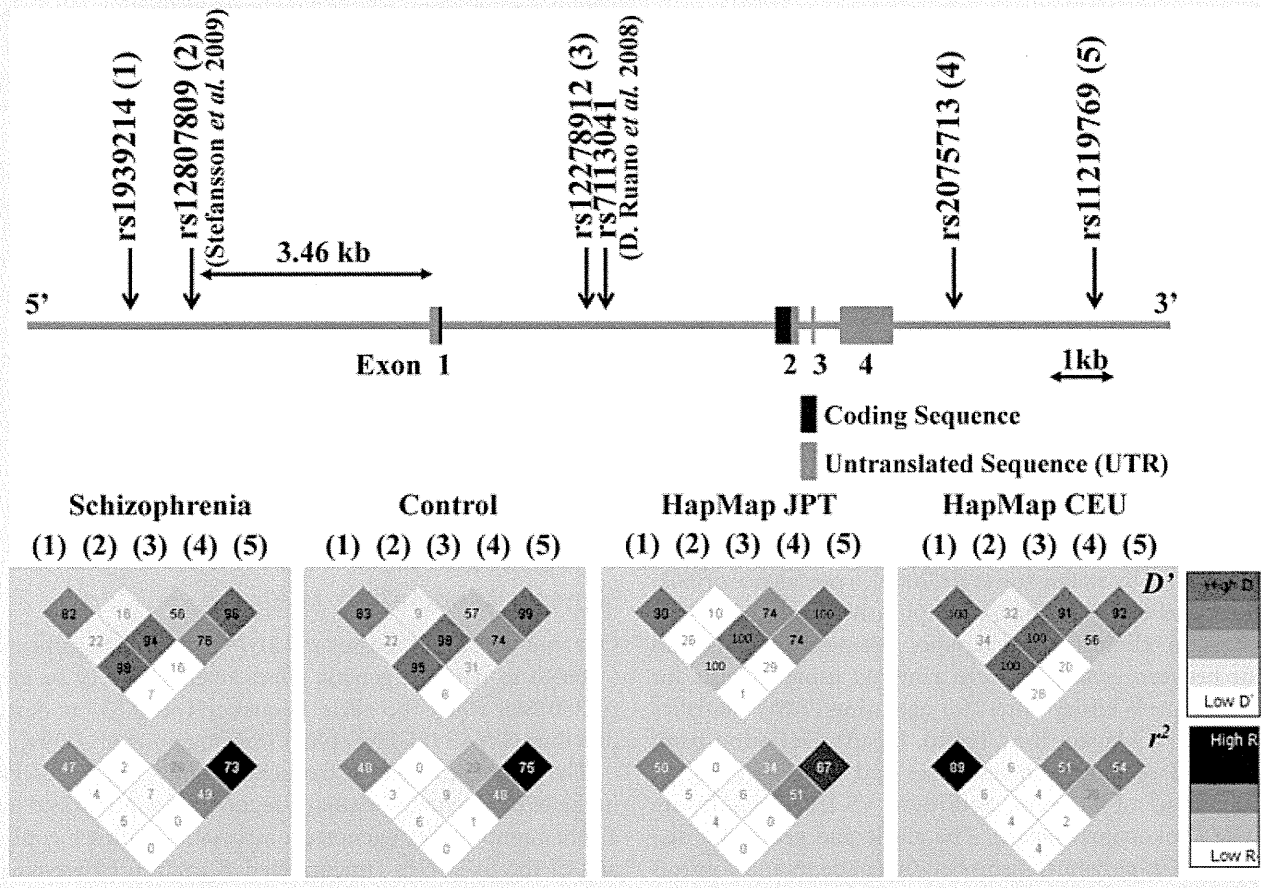

FIG. 1. The genomic structure of *NRGN*, including the locations of the five tagging SNPs studied and linkage disequilibrium of these SNPs in the patient, control, HapMap JPT, and CEU groups. Based on an entry in the Entrez Gene database (National Center for Biotechnology Information), the genomic structure of *NRGN* is shown above. The locations of the SNPs analyzed in this study are indicated by arrows, with numbers indicated in parentheses. The numbers indicated in parentheses refer to the numbering of the SNPs in the linkage disequilibrium (LD) diagram. The distances of exons–introns and intermarkers are drawn to scale. The LDs between pairwise SNPs are shown using the D′ (upper) and r^2 (lower) values at the bottom of the map of the gene structure separately for cases, controls, the HapMap JPT samples and the HapMap CEU samples. High levels of LD are represented by black (D′ and r^2) coloring, with increasing color intensity from 0 to 100, as shown by color bars.

First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Detailed information on the PCR conditions is available upon request.

To measure mRNA expression levels of housekeeping (β-*actin*) and *NRGN* genes, we used the Pre-Developed TaqMan Assay Reagent kit (Applied Biosystems). Primer information (gene name: assay ID, transcript ID, target region) is as follows; *NRGN*: Hs00382922_m1, NM_001126181.1 and NM_006176.2, Exon1-2; β-actin: 4326315E, NM_001101, no region indicated (Applied Biosystems). Expression levels of these genes were measured by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) using an ABI Prism 7900 Sequence Detection System (Applied Biosystems) with a 384-well format as previously described [Yamamori et al., 2011; Yasuda et al., 2011]. PCR data were obtained using Sequence Detector software (SDS version 2.1; Applied Biosystems) and quantified using a standard curve. This software plotted the real-time fluorescence intensity and selected the threshold within the linear phase of the amplicon profile. The software plotted a standard curve of the cycle at threshold C_t, which is where the fluorescence generated within a reaction crossed the threshold, versus the quantity of RNA. All samples were measured using a single plate per target gene, and their C_t values were in the linear range of the standard curve. The quantity of each sample was predicted by C_t values. The qRT-PCR reaction was performed in triplicate, and the expression level of the gene was taken as the average of three independent measurements. Standard curves were obtained using serial dilutions (1:4) of pooled complementary DNA prepared from 300 ng total RNA derived from immortalized lymphocytes. The standard curves of β-*actin* and *NRGN* showed that these genes were expressed in immortalized lymphocytes. In each experiment for β-*actin* and *NRGN*, the R^2 value of the standard curve was >0.99, and no-template control assays resulted in no detectable signal. The individual expression levels of the *NRGN* gene were normalized to the housekeeping gene (raw target gene expression level divided by raw housekeeping gene expression level) and were used for statistical analysis.

Haplotype Associated With *NRGN* Expression (eQTL)

To identify whether the haplotypes in *NRGN* associated with schizophrenia may be expression quantitative trait loci (eQTL), we analyzed *NRGN* expression in two datasets of lymphoblast-derived HapMap JPT samples and in the Japanese case–control samples. For the HapMap JPT samples, we extracted genotypes and *NRGN* lymphoblastoid expression data from the HapMap JPT samples (n = 45) deposited in GeneVar (http://www.sanger.ac.uk/humgen/genevar/ [Stranger et al., 2007]). For the Japanese case–control samples, we used our genotypes and *NRGN* lymphoblastoid expression data obtained using the method described above.

Statistical Analyses

We performed power calculations using the Power Calculator for Two-Stage Association Studies (http://www.sph.umich.edu/csg/abecasis/CaTS/ [Skol et al., 2006]). The power estimate was based on an allele frequency of 0.83 at rs12807809, an odds ratio of 1.19, which was indicated by Stefansson et al. [2009], a prevalence of 0.01, and an alpha level of 0.05 using a multiplicative model.

Differences in clinical characteristics between patients and controls or between genotypes were analyzed using χ^2 tests for sex and the Mann–Whitney *U*-test for age using PASW Statistics 18.0 software (SPSS Japan, Inc., Tokyo, Japan). Deviation from HWE was tested separately in test cases and controls using χ^2 tests for goodness of fit using SNPAlyze V5.1.1 Pro software (DYNACOM, Yokohama, Japan). The allelic and genotypic distributions of *NRGN* polymorphisms between patients and controls were analyzed using χ^2 tests with SNPAlyze V5.1.1 Pro software. The number of effective independent SNPs assayed was estimated to correct for multiple testing by the spectral decomposition method of Nyholt using the SNPSpD software [Nyholt, 2004]. The effective number of independent marker loci was 4.13 and corrected *P*-value for allelic and genotypic associations was set at $P < 0.012$. Pairwise linkage disequilibrium (LD) analyses expressed by D′ and r^2 were applied to detect the intermarker relationships in each group using Haploview 4.2 software (http://www.broad.mit.edu/mpg/haploview/contact.php). Haplotype frequencies were estimated using the method of maximum likelihood with genotyping data using the expectation–maximization (EM) algorithm from SNPAlyze V5.1.1 Pro software. Rare haplotypes detected in less than 3% of patients and controls were excluded from the haplotypic association analysis, as previously described [Ohi et al., 2009, 2010]. We performed 10,000 permutations for significance tests to determine empirical significance using a 2×2 contingency table approach. We used a 2- to 5-window fashion analysis. Since Bonferroni correction for multiple testing is considered to be too conservative to apply to genetic association analyses [Nyholt, 2001], method of Nyholt [Nyholt, 2004] for allelic and genotypic associations and permutation tests [Dudbridge, 2003] for haplotypic associations are considered to be appropriate for these analyses.

The difference in expression levels between Japanese patients with schizophrenia and controls was analyzed using linear regression in PASW Statistics 18.0 software. Age and sex, which may influence gene expression, were corrected for in the expression analysis. HPlus (http://qge.fhcrc.org/hplus) is a software application for estimating haplotype frequencies and inferring individual haplotypes based on EM and progressive ligation (PL) algorithms [Li et al., 2003], and most significantly assessing haplotypic associations with various types of phenotypes using linear regression. Differences of expression levels among haplotypes were analyzed using linear regression in HPlus software. Each genotype was treated as the number of major alleles (0, 1, and 2) in the expression analysis. For the joint haplotype analysis in HPlus software, each haplotype was tested against the reference haplotype (equal to most frequent haplotype) using linear regression. As age and sex were not available for the HapMap samples, these confounding factors were not corrected for in the expression analysis. Expression levels in Japanese cases, control samples and in the combined samples were corrected for age and sex in the analyses. We applied a Bonferroni correction in expression analysis (three tests). The significance level for statistical tests was set at two-tailed $P < 0.05$.

RESULTS

Genetic Association Analysis

Our study size of 2,019 cases and 2,579 controls had sufficient power (>80%) to detect a genetic effect at ORs of 1.19 or greater for rs12807809 when the allele frequency was 0.83, as described in previous GWAS (SGENE-plus) [Stefansson et al., 2009].

The genotype and allele frequencies of five tagging SNPs located in the *NRGN* gene and flanking regions are summarized in Table I. There was no allelic or genotypic association with schizophrenia for any of the five SNPs (uncorrected $P > 0.05$). However, nominal differences in allele frequencies between patients and controls were observed in rs12278912 ($\chi^2 = 3.6$, $P = 0.057$, corrected $P = 0.24$) and rs2075713 ($\chi^2 = 3.6$, $P = 0.057$, corrected $P = 0.24$). The major allele frequencies of both SNPs were higher in patients than in controls. Consistent with previous GWAS [Stefansson et al., 2009], the frequency of the major T allele of rs12807809 was higher in patients (75.4%) than in controls (74.4%) in our Japanese population, although the results did not reach statistical significance [$\chi^2 = 1.3$, $P = 0.25$, OR (95% confidence interval (CI)) = 1.06 (0.96–1.16)].

We focused on haplotypic association between patients with schizophrenia and healthy subjects using a 2- to 5-window fashion analysis. Haplotype analysis showed a significant association with schizophrenia (rs12807809–rs12278912, $\chi^2 = 13.1$, global $P = 0.0042$) (Supplementary Table I). The frequency of the major TG haplotype of rs12807809–rs12278912 was higher in patients (62%) than in controls (58%) [$\chi^2 = 9.4$, $P = 0.0019$, OR (95% CI) = 1.14 (1.05–1.24)] (Table II). On the other hand, the frequency of the TA haplotype of rs12807809–rs12278912 was lower in patients (14%) than in controls (16%) [$\chi^2 = 7.3$, $P = 0.0053$, OR (95% CI) = 0.85 (0.76–0.96)] (Table II). There was no haplotypic association with schizophrenia for any other haplotypes. These findings suggest that the major TG haplotype of rs12807809–rs12278912 may be related to an increased risk of schizophrenia, and the TA haplotype may have a protective role against the susceptibility to schizophrenia. These results of allelic, genotypic, or haplotypic associations were not affected by excluding 86 samples used for expression analyses (data not shown).

TABLE II. Differences in the rs12807809–rs12278912 Haplotype Between Patients With Schizophrenia and Healthy Subjects

Haplotype rs12807809[a]–rs12278912[b]	Frequency Patients	Frequency Controls	Individual P (χ^2)	OR (95%CI)	Global P (χ^2)
					0.0042 (13.1)
TG	0.62	0.58	**0.0019 (9.4)**	1.14 (1.05–1.24)	
CG	0.17	0.18	0.07 (3.4)	0.90 (0.81–1.01)	
TA	0.14	0.16	**0.0053 (7.3)**	0.85 (0.76–0.96)	
CA	0.08	0.08	0.57 (0.3)	1.05 (0.90–1.22)	

Significant P values are shown as bold-faced and underlined type.
[a]The genome-wide supported SNP for schizophrenia [Stefansson et al., 2009].
[b]Because a high linkage disequilibrium between rs12278912 and rs7113041 [Ruano et al., 2008] was found in the HapMap JPT samples ($r^2 = 0.93$), rs12278912 was selected as the tagging SNP by the TAGGER program.

The LD relationships between the markers are provided in Figure 1. The LD pattern observed in our controls was similar to our patients and the JPT HapMap samples; however, it was different from that of the CEU HapMap samples. The strengths of the LD patterns of rs1939214–rs12807809 and rs12278912–rs2075713–rs11219769 were different between Japanese populations and the CEU HapMap samples. The low LD pattern of rs12807809–rs12278912 was similar among the groups ($D' < 0.50$, $r^2 < 0.10$).

NRGN Gene Expression Analysis

The *NRGN* expression level was lower in patients with schizophrenia (n = 42, mean ± SD, 0.86 ± 0.58) than in controls (n = 44, 1.00 ± 0.75). However, the results did not reach statistical significance (r = −0.14, beta = −0.11, SE = 0.14, t = −0.97, $P = 0.34$).

Based on the results from the genetic association analysis, we investigated whether the rs12807809–rs12278912 haplotype of the *NRGN* gene was an eQTL in two datasets. The rs12807809–rs12278912 haplotype related to schizophrenia was significantly associated with *NRGN* expression in healthy HapMap JPT samples. The *NRGN* gene expression of the high-risk TG haplotype of rs12807809–rs12278912 was significantly lower than that of the protective TA haplotype (z = 2.69, $P = 0.007$). We confirmed that the rs12807809–rs12278912 haplotype was significantly associated with *NRGN* expression normalized to the β-*actin* expression in the controls and combined samples (Fig. 2 and Table III, control samples: z = 2.30, $P = 0.021$, combined samples: z = 3.09, $P = 0.002$). The association occurred in the same direction among the HapMap JPT, control, and combined samples. In case samples, the expression level of rs12807809–rs12278912 was lower in samples with the high-risk TG haplotype than in those with the protective TA haplotype, although the result did not reach statistical significance (z = 1.49, $P = 0.14$). The association in the HapMap JPT and combined samples remained significant after correction for multiple tests (HapMap JPT samples: corrected $P = 0.021$, combined samples: corrected $P = 0.006$). However, there was no significant association after applying the correction in control samples (corrected $P = 0.063$).

DISCUSSION

In this study, we provided evidence that haplotypes, including the genome-wide-screen-supported SNP of the *NRGN* gene, were associated with an increased risk of schizophrenia. Our in silico analysis showed that the high-risk rs12807809–rs12278912 haplotype of the *NRGN* gene may be associated with a low expression level of the *NRGN* gene in lymphoblasts derived from the HapMap JPT samples. We confirmed the association between the haplotype and *NRGN* expression in the combined case–control samples. Our results suggest that the schizophrenia-associated haplotype at the

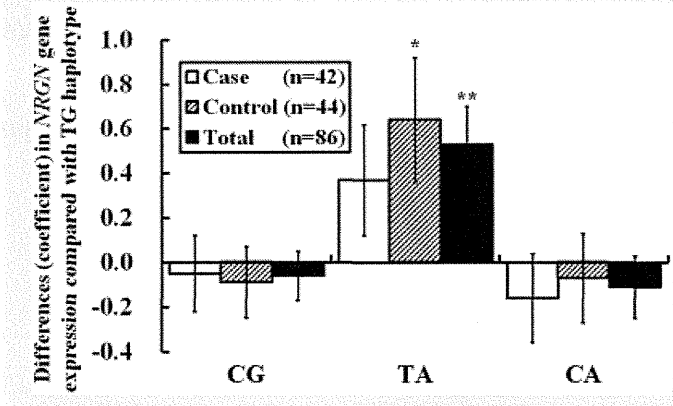

FIG. 2. The association between the rs12807809–rs12278912 haplotype of the *NRGN* gene and *NRGN* expression in lymphoblasts. Expression of the protective TA haplotype of rs12807809–rs12278912 was significantly higher than that of the high-risk TG haplotype in controls and combined case–control samples. The error bars represent standard errors of the coefficient. Estimated frequencies of each haplotype were as follows—TG haplotype: Case, 69%; Control, 61%; Total, 65%; CG haplotype: Case, 16%; Control, 20%; Total, 18%; TA haplotype: Case, 7%; Control, 11%; Total, 9%; CA haplotype: Case, 8%; Control, 9%; Total, 8%. *$P < 0.05$, **$P < 0.01$.

NRGN gene may be a functional variant, and the results support an association between the *NRGN* gene and schizophrenia.

This report is the first investigation of the association of tagging SNPs and haplotypes covering the *NRGN* gene with schizophrenia. To our knowledge, five genetic studies have investigated whether the *NRGN* gene is implicated in schizophrenia. A genome-wide linkage study has shown that the chromosomal region 11q23.3-24 including the *NRGN* gene is linked to schizophrenia in British and Icelandic populations [Gurling et al., 2001]. Subsequently, an association study determined that rs7113041, which displays high LD with rs12278912 and is located on intron 1 in the *NRGN* gene, is related to the risk of developing schizophrenia in male subjects of Portuguese origin [Ruano et al., 2008]. In addition, three GWAS and follow-up studies have shown that rs12807809 is associated with schizophrenia in large European samples [Stefansson et al., 2009]. However, two studies reported no association between *NRGN* and schizophrenia in Bulgarian or Chinese populations [Betcheva et al., 2009; Li et al., 2010]. In the present study, we determined that the rs12807809–rs12278912 haplotype is associated with an increased risk of schizophrenia in a Japanese population. However, there were no significant associations between any SNP, including rs12807809 and rs12278912, and schizophrenia in the population. The inconsistency of association among the previous studies and the present study might result from ethnic differences or type I or II errors for the different sample sizes: Portuguese, 315 cases, 295 controls and 73 trios [Ruano et al., 2008]; European Caucasian, 12,945 cases and 34,591 controls [Stefansson et al., 2009]; Japanese, 2,019 cases and 2,579 controls (present study); Bulgarian, 185 cases and 184 controls [Betcheva et al., 2009]; and Chinese, 2,496 cases and 5,184 controls [Li et al., 2010]. In addition, the SNPs investigated in each study were different. Ruano et al. [2008] and Betcheva et al. [2009] examined rs7113041, which has high LD with rs12278912 but not with rs12807809, whereas Stefansson et al. [2009] and Li et al. [2010] examined rs12807809 but not rs12278912. However, none of these studies examined haplotypes for the *NRGN* gene. Because the rs12807809–rs12278912 haplotype may be the most significant genetic variant in this region, further study is required to confirm the association between the rs12807809–rs12278912 haplotype and schizophrenia in other populations.

Differences in the relative *NRGN* expression levels between patients with schizophrenia and healthy subjects were not demonstrated. This result may be due to the small sample sizes in this study, which may have resulted in the failure to identify a modest difference in *NRGN* expression in this complex disease. We determined that the major TG haplotypic and the TA haplotypic frequencies of rs12807809–rs12278912 were higher and lower, respectively, in patients with schizophrenia than in healthy controls. In addition to these findings, we found that *NRGN* gene expression of the high-risk TG haplotype was significantly lower than that of the protective TA haplotype in lymphoblasts derived from our Japanese case–control subjects as well as the JPT HapMap sample. The low LD patterns of rs12807809–rs12278912 were similar across populations. This region may be vulnerable to recombination. Combinations of the TG and TA of rs12807809–rs12278912 could play an important role in the pathogenesis of schizophrenia. In this study, gene expression data derived from lymphoblasts raised the possibility that the rs12807809–rs12278912 haplotype may be a functional variant of *NRGN*. Further biological studies of the function of rs12807809–rs12278912 are required to verify the expression results.

Smith et al. [2011] analyzed *NRGN* expression in several brain tissues derived from a dataset of at least 130 individuals of European ancestry. However, they showed that neither the genome-wide supported SNP nor any individually correlated SNPs were associated with *NRGN* expression. They did not examine any association between haplotype and *NRGN* expression. There are several challenges in investigating expression findings in the postmortem

TABLE III. The Association Between the rs12807809–rs12278912 Haplotype and mRNA Expression

Haplotypes	Frequency	Coefficient	SE	CI	*P*-value (*Z*-score)
Schizophrenia (*n* = 42)					
TG	0.69	0 (ref)	—	—	—
CG	0.16	−0.05	0.17	(−0.39–0.29)	0.76 (−0.30)
TA	0.07	0.37	0.25	(−0.12–0.86)	0.14 (1.49)
CA	0.08	−0.16	0.20	(−0.55–0.24)	0.43 (−0.78)
Healthy control (n = 44)					
TG	0.61	0 (ref)	—	—	—
CG	0.20	−0.09	0.16	(−0.39–0.22)	0.58 (−0.55)
TA	0.11	0.64	0.28	(0.09–1.18)	**0.021 (2.30)**
CA	0.09	−0.07	0.20	(−0.46–0.32)	0.73 (−0.34)
Total subjects (n = 86)					
TG	0.65	0 (ref)	—	—	—
CG	0.18	−0.06	0.11	(−0.28–0.15)	0.57 (−0.57)
TA	0.09	0.53	0.17	(0.19–0.87)	**0.002 (3.09)**
CA	0.08	−0.11	0.14	(−0.39–0.17)	0.45 (−0.75)

Joint Association Analysis (the reference haplotype is the most frequent haplotype).
For the joint haplotype test, several haplotypes were tested against the reference haplotype.
Significant *P* values are shown as bold-faced and underlined type.

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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The *p250GAP* Gene Is Associated with Risk for Schizophrenia and Schizotypal Personality Traits

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Abstract

Background: Hypofunction of the glutamate *N*-Methyl-d-aspartate (NMDA) receptor has been implicated in the pathophysiology of schizophrenia. p250GAP is a brain-enriched NMDA receptor-interacting RhoGAP. p250GAP is involved in spine morphology, and spine morphology has been shown to be altered in the post-mortem brains of patients with schizophrenia. Schizotypal personality disorder has a strong familial relationship with schizophrenia. Several susceptibility genes for schizophrenia have been related to schizotypal traits.

Methods: We first investigated the association of eight linkage disequilibrium-tagging single-nucleotide polymorphisms (SNPs) that cover the *p250GAP* gene with schizophrenia in a Japanese sample of 431 schizophrenia patients and 572 controls. We then investigated the impact of the risk genetic variant in the *p250GAP* gene on schizotypal personality traits in 180 healthy subjects using the Schizotypal Personality Questionnaire.

Results: We found a significant difference in genotype frequency between the patients and the controls in rs2298599 ($\chi^2 = 17.6$, $p = 0.00015$). The minor A/A genotype frequency of rs2298599 was higher in the patients (18%) than in the controls (9%) ($\chi^2 = 15.5$, $p = 0.000083$). Moreover, we found that subjects with the rs2298599 risk A/A genotype, compared with G allele carriers, had higher scores of schizotypal traits ($F_{1,178} = 4.08$, $p = 0.045$), particularly the interpersonal factor ($F_{1,178} = 5.85$, $p = 0.017$).

Discussion: These results suggest that a genetic variation in the *p250GAP* gene might increase susceptibility not only for schizophrenia but also for schizotypal personality traits. We concluded that the *p250GAP* gene might be a new candidate gene for susceptibility to schizophrenia.

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Introduction

Schizophrenia is a common and complex psychiatric disease. The lifetime morbidity rate is 0.5–1.0% across distinct populations. Family, twin and adoption studies of schizophrenia have indicated that there are strong genetic factors and have estimated the rate of schizophrenia heritability at 80% [1,2]. Although genes implicated in the pathogenesis of schizophrenia have been found using several approaches, such as through association studies of candidate genes, genome-wide association studies (GWAS), copy number variation (CNV) studies and pedigree studies [3,4], the exact genetic factors of this complex disease remain to be explained.

Hypofunction of the glutamate *N*-Methyl-d-aspartate (NMDA) receptor is strongly implicated in the pathophysiology of schizophrenia. NMDA receptor antagonists, such as phencyclidine (PCP) and ketamine, mimic symptoms of the disorder in humans and exacerbate symptoms in patients with schizophrenia [5]. These NMDA receptor antagonists induce schizophrenia-like symptoms in humans. Preclinically, they have been shown to

induce similar symptoms and to induce neural circuitry changes reminiscent of schizophrenia [6]. The ability of these NMDA receptor antagonists to induce a schizophrenia-like phenotype supports the concept that schizophrenia may be the result of reduced or abnormal functioning of NMDA receptors. Altered NMDA receptor binding density in several brain regions, such as in the anterior cingulate cortex, has been reported in schizophrenia [7,8]. The NR2 subunits of the NMDA receptor are spatially and developmentally regulated, and they provide an important level of receptor regulation [9,10]. NR2A and NR2B are the predominant subunits in the cortex, striatum and hippocampus [11,12,13]. In particular, these three areas are closely associated with the pathology of schizophrenia and with the neural circuits within and between these regions [14]. In patients with schizophrenia, alterations have been observed in the NR2 subunit mRNA and protein in the prefrontal cortex, including a reduction in NR2A mRNA and NR2B protein levels [15,16]. Additionally, the NR2B subunit mRNA levels were increased in the hippocampus [17]. Therefore, different expression of NR2 subunits could play an important role in the pathophysiology of schizophrenia.

The NMDA receptor regulates activity-dependent spine morphological plasticity by modulating the actin cytoskeleton [18]. As the key regulators of actin cytoskeleton dynamics, the Rho family of GTPases, including RhoA, Cdc42, and Rac1 and their regulators, play an important role in NMDA receptor-mediated spine morphogenesis [18,19]. In our previous study, we identified the *p250GAP* gene (also known as *p200RHOGAP*, *GRIT*, *KIAA0712*, *RICS*, or *ARHGAP32*: OMIM 608541) as a novel NMDA receptor-interacting RhoGAP [20,21,22,23]. This gene spans approximately 56.17 kb of the genomic DNA and is located on chromosome 11q24.3. p250GAP is highly enriched in the central nervous system, is concentrated in the post-synaptic densities in neurons and is co-localized with the NR2B subunit of the NMDA receptor [20]. Knockdown of p250GAP increased spine width and elevated the endogenous RhoA activity in primary hippocampal neurons, suggesting that p250GAP regulates spine morphogenesis through its RhoGAP activity for RhoA [24]. Importantly, p250GAP activity and localization within neurons are regulated by NMDA receptor activity [20,24], suggesting that p250GAP, together with the NMDA receptor, regulates NMDA receptor-mediated spine morphogenesis. Given that neuropathological studies of schizophrenia have shown alterations in spine morphology [25,26], we hypothesized that the *p250GAP* gene may be related to the pathophysiology of schizophrenia. In this study, we investigated the association between the *p250GAP* gene and schizophrenia in a Japanese population using a gene-based approach.

Schizotypal personality disorder (SPD) is one of the schizophrenia spectrum disorders and is characterized by social avoidance, ideas of reference, vagueness, magical thinking, odd speech, illusions and paranoid ideation. The lifetime prevalence of SPD has been estimated at 3.9% [27], making it one of the more common psychiatric disorders. The prevalence rate of SPD in relatives of individuals with schizophrenia (6.9%) was higher than the prevalence rates found either in relatives of individuals with other psychiatric disorders or in mentally healthy subjects [28]. Twin studies have estimated that the heritability of the latent liability to SPD is 61–72% [29,30]. Premorbid SPD is related to the development of schizophrenia [31]. These findings suggest that SPD shares common genetic influences with schizophrenia. The traits of SPD were incorporated in the SPD criteria in the *Diagnostic and Statistical Manual of Mental Disorders*, third edition (DSM-III), and the traits are listed in the DSM-IV-TR on Axis II. These traits can be identified using a well-validated questionnaire, such as the Schizotypal Personality Questionnaire (SPQ) [32]. The heritability rates of three schizotypal trait factors, cognitive/perceptual, interpersonal and disorganization, have been estimated at 40 to 60% [33,34]. We recently demonstrated that a genome-wide genetic variant for schizophrenia in the *ZNF804A* gene was associated with schizotypal personality traits [35]. Additionally, we investigated whether a genetic variant in the *p250GAP* gene was associated with schizotypal personality traits in healthy subjects.

Materials and Methods

Ethics statement

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

Subjects

The subjects of our genetic association study were 431 patients with schizophrenia (48.7% male (210 males, 221 females), mean age ± SD was 49.7±15.4 years) and 572 healthy controls (46.7% male (267 males, 305 females), mean age ± SD was 61.9±20.4 years). The sex ratio did not differ significantly between the groups ($\chi^2 = 0.41$, $p = 0.52$), but the mean age was significantly different ($z = -11.49$, $p < 0.001$). The subjects were all biologically unrelated and were Japanese. The subjects were recruited from both outpatient and inpatient units at Osaka University Hospital and other psychiatric hospitals. Each patient with schizophrenia had been diagnosed by at least two trained psychiatrists by unstructured clinical interviews, according to the criteria of the DSM-IV. When the diagnosis of the two trained psychiatrists was discordant, they discussed the diagnosis. When the diagnostic disputes were resolved and the patient was diagnosed as schizophrenic, we included the patient. When the diagnostic disputes were not resolved by discussion or the patient was not diagnosed as schizophrenia, we excluded the patient. Controls were recruited through local advertisements. Psychiatrically healthy controls were evaluated using unstructured interviews to exclude individuals with current or past contact with psychiatric services, with experience with psychiatric medications or who were not Japanese. We did not assess the controls for their family history of mental disorders, such as schizophrenia, bipolar disorder, or major depressive disorder. The ethnicity was determined by self-report and was not confirmed by genetic analyses.

Data for the schizotypal personality trait analysis were available for 180 healthy subjects [48.3% male (87/93), mean age ± SD: 36.5±11.5 years]. The subjects were included in the genetic association analysis. The subjects included in the analysis met additional criteria. Psychiatrically, medically and neurologically healthy controls were evaluated using the Structured Clinical Interview for DSM-IV-Non-Patient Edition (SCID-I/NP) to exclude individuals who had received psychiatric medications or who had first- or second-degree relatives with psychiatric disorders. Additionally, subjects were excluded from this study if they had neurological or medical conditions that could have potentially affected their central nervous system, such as atypical headaches, 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.

SNP selection, genotyping and genomic sequencing

This study was designed to examine the association between the *p250GAP* gene and schizophrenia by tagging single-nucleotide

polymorphisms (SNPs) in the *p250GAP* gene and its flanking regions (±5 kb). Of the 31 SNPs in the *p250GAP* gene and flanking regions, we selected eight tagging SNPs using the TAGGER algorithm (Paul de Bakker, http://www.broad.mit.edu/mpg/tagger) with the criteria of r^2 greater than 0.5 in 'pair-wise tagging only' mode and a minor allele frequency (MAF) greater than 5%. The selection was implemented in Haploview 4.2 using HapMap data release 24/PhaseII Nov 08, on NCBI B36 assembly, dbSNP b126 (Japanese in Tokyo (JPT), Chr 11: 128,338,052..128,404,222) (Table S1). The eight tagging SNPs were rs493172, rs10893947, rs2276027, rs3796668, rs581258, rs3740829, rs546239 and rs2298599. The markers are shown in Table 1; the orientation and the alleles are reported on the genomic minus strand. The positions of the eight SNPs analyzed in the present study and the LD relationships between the SNPs in a HapMap JPT population are shown in Figure 1. Venous blood was collected from the subjects. Genomic DNA was extracted from the whole blood using standard procedures. The SNPs were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay (Applied Biosystems; Foster City, California, USA) as previously described [36,37]. Detailed information on the PCR conditions is available upon request. Genotyping call rates were 99.3% (rs493172), 98.9% (rs10893947), 99.1% (rs2276027), 99.7% (rs3796668), 98.4% (rs581258), 99.2% (rs3740829), 98.5% (rs546239) and 99.3% (rs2298599). No deviations from the Hardy-Weinberg equilibrium (HWE) in the examined SNPs were detected ($p>0.05$). Additionally, with 48 subjects with schizophrenia, we confirmed a SNP significantly associated with schizophrenia, genotyped by the TaqMan method, using direct DNA sequencing. These subjects were included in the genetic association analysis. The genomic regions were amplified by PCR using a pair of primers for rs2298599, 5′- AAGTCAGCCCA-GACTCTCCA -3′ and 5′- GAGGGAGGAAGGGATTTTTG -3′. PCR for each sample was carried out in a total volume of 40 µl using a Gene Amp® PCR System 9700 (Applied Biosystems, CA, U.S.A). The PCR cycling conditions were 94°C for 10 minutes, 30 cycles at 94°C for 1 minute, 60°C for one minute and 72°C for 1 minute, followed by an incubation at 72°C for 10 minutes. The PCR products were purified using a QIA quick® PCR Purification Kit (QIAGEN, CA, USA), and the purification products were sequenced using a Big Dye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA). Cycle sequencing conditions were 96°C for 2 minutes, 25 cycles of 96°C for 20 seconds, 50°C for 30 seconds and 60°C for 2 minutes, using a Gene Amp® PCR System 9700. The PCR products from the cycle sequencing were purified using a Big Dye® XTerminator™ Purification Kit (Applied Biosystems, CA, U.S.A.), and they were sequenced using an ABI PRISM® 3700 DNA Analyzer (Applied Biosystems, CA, U.S.A.). The sequencing was checked with SEQUENCHER ver. 4.7 (Gene Codes, U.S.A.).

Schizotypal personality trait analysis

To assess schizotypal personality traits, a full Japanese version of the SPQ was administered to healthy subjects [38,39]. The SPQ is a 74-item self-report questionnaire with a "yes/no" response format [40]. All items answered "yes" were scored 1. The SPQ measures nine subscales of specific schizotypal features, which are ideas of reference, odd beliefs/magical thinking, unusual perceptual experiences, suspiciousness/paranoid ideation, social anxiety, no close friends, constricted affect, eccentric/odd behavior and odd speech. The total SPQ score was obtained by summing the scores from all of the items. The three schizotypal trait factors, cognitive/perceptual, interpersonal and disorganization, were derived by summing the related subscale raw scores according to the three-factor model of Raine and colleagues [32]. Full-scale IQ was assessed using the Wechsler Adult Intelligence Scale, Revised or Third edition.

Statistical analysis

Differences in clinical characteristics between the patients and the controls or between the genotype groups were analyzed using the χ^2 test for categorical variables and the Mann-Whitney *U*-test for continuous variables, using the PASW Statistics 18.0 software (SPSS Japan Inc., Tokyo, Japan). We performed power calculations using the Power Calculator for Two Stage Association Studies (http://www.sph.umich.edu/csg/abecasis/CaTS [41]). The power estimates were based on the allele frequency of 0.35 (rs2298599) in the controls and an alpha level of 0.05. Power was calculated under a prevalence of 0.01 using a multiplicative model that assumed varying degrees of the odds ratio (OR). Statistical analyses for the genetic associations were performed using the SNPAlyze V5.1.1 Pro software (DYNACOM, Yokohama, Japan). Deviation from the HWE was tested using χ^2 tests for goodness of

Table 1. Genotypic and allelic distributions for SNPs in the *p250GAP* between patients with schizophrenia and controls.

Marker				SCZ ($n=431$)			CON ($n=572$)			Genotypic	SCZ	CON	Allelic	OR
SNP IDs	Position[a]	M/m	gene	M/M	M/m	m/m	M/M	M/m	m/m	*p* ($\chi 2$)	MAF		*p* ($\chi 2$)	(95% CI)
rs493172	128388089	C/G	intron1	346	77	3	451	116	3	0.63 (0.9)	0.10	0.11	0.49 (0.5)	0.90 (0.67–1.21)
rs10893947	128375634	G/A	intron1	122	217	88	177	294	94	0.25 (2.8)	0.46	0.43	0.14 (2.2)	1.15 (0.96–1.37)
rs2276027	128355514	T/C	intron8	241	158	27	303	229	36	0.57 (1.1)	0.25	0.27	0.42 (0.7)	0.92 (0.75–1.13)
rs3796668	128349062	A/C	intron11	186	182	62	206	292	72	**0.020 (7.8)**	0.36	0.38	0.22 (1.5)	0.89 (0.74–1.07)
rs581258	128348083	A/G	exon12	293	125	8	373	171	17	0.46 (1.6)	0.17	0.18	0.32 (1.0)	0.89 (0.70–1.12)
rs3740829	128344366	A/G	exon13	375	50	2	513	54	1	0.37 (2.0)	0.06	0.05	0.18 (1.8)	1.30 (0.89–1.91)
rs546239	128340968	A/G	3′	325	91	9	402	149	12	0.18 (3.4)	0.13	0.15	0.11 (2.6)	0.81 (0.63–1.05)
rs2298599	128340162	G/A	3′	167	184	76	219	296	53	**0.00015 (17.6)**	0.39	0.35	0.07 (3.3)	1.18 (0.99–1.42)

SCZ: patients with schizophrenia, CON: controls, M: major allele, m: minor allele, MAF: minor allele frequency, OR: odds ratio, 95%CI: 95% confidence interval.
[a]db SNP build 129.
All of the alleles are represented according to the minus strand DNA sequence. Numbers of genotypes were represented as genotype counts. *P* values<0.05 are in boldface and underlined.
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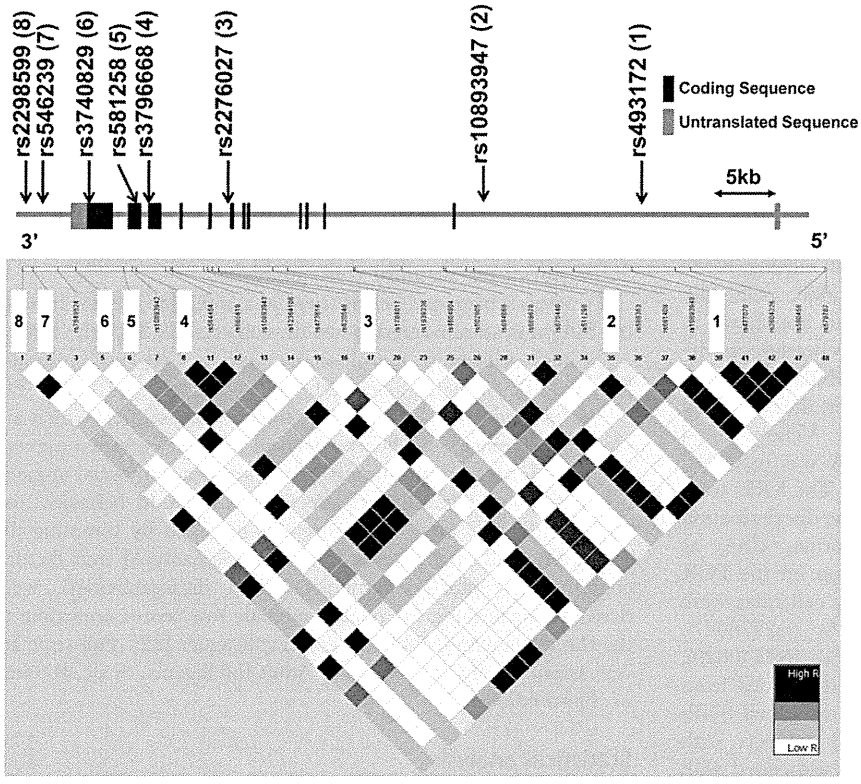

Figure 1. The genomic structure of *p250GAP* and linkage disequilibrium of the *p250GAP* in the HapMap JPT. The genomic structure of *p250GAP* is based on an entry in the Entrez Gene database (National Center for Biotechnology Information). The locations of the SNPs analyzed in this study are indicated by arrows. The numbers indicated in parentheses refer to the numbering of the SNPs in the linkage disequilibrium (LD) diagram. The distances of the exons-introns and the intermarkers are drawn to scale. The LDs between the pairwise SNPs are shown using the r^2 value at the bottom of the map of the gene structure for the HapMap JPT samples. High levels of LD are represented by black (r^2) coloring, with increasing color intensity shown by the color bars.
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fit. The allelic and genotypic distributions of *p250GAP* polymorphisms between the patients and the controls were analyzed using χ^2 tests.

Pairwise linkage disequilibrium (LD) analyses, expressed by r^2, were applied to detect the intermarker relationships in each group using the Haploview 4.2 software (http://www.broad.mit.edu/mpg/haploview/contact.php). Haplotype frequencies were estimated using the maximum likelihood method with the genotyping data. We used the expectation-maximization algorithm from the SNPAlyze V5.1.1 Pro software. Rare haplotypes, detected in less than 3% of the patients and the controls, were excluded from the haplotypic association analysis, as previously described [42,43]. Using a 2×2 contingency table approach, we performed 10,000 permutations of significance tests to determine empirical significance. We used a 2- to 8-window fashion analysis. We applied Bonferroni corrections in allelic and genotypic association analyses (eight tests) and in haplotypic association analyses (28 independent global tests).

The effects of the *p250GAP* genotype on the total score and on the three factors of the SPQ were analyzed by a one-way analysis of variance (ANOVA). To control confounding factors, the effect of the *p250GAP* genotype on the significance factor of the SPQ was analyzed by a one-way analysis of covariance (ANCOVA). Age, sex and education years were used as covariates because the SPQ total score and the three factors were correlated with these confounding factors in a previous study [44]. Standardized effect sizes were calculated using Cohen's *d* method (http://www.uccs.edu/faculty/lbecker). All *p* values are two tailed, and statistical significance was defined as $p<0.05$.

Results

Genetic association analysis

Our study size of 431 patients with schizophrenia and 572 controls had sufficient power (>80%) to detect a genetic effect at ORs of 1.30 or larger when the allele frequency was 0.35. The genotype and allele frequencies of the eight tagging SNPs located in the *p250GAP* gene and flanking regions are summarized in Table 1. We found significant differences in genotype frequencies between the patients and the controls in rs3796668 ($\chi^2=7.8$, $p=0.020$) and rs2298599 ($\chi^2=17.6$, $p=0.00015$). No allelic or genotypic associations were observed with schizophrenia for any other SNPs ($p>0.05$). The major genotype frequency of rs3796668 was significantly higher in the patients with schizophrenia (43%) than in the controls (36%) ($\chi^2=5.2$, $p=0.023$), but no differences were observed in the frequencies of the minor or heterozygous genotypes of rs3796668 ($p>0.05$). The minor genotype frequency of rs2298599 was higher in the patients with schizophrenia (18%) than in the controls (9%) ($\chi^2=15.5$, $p=0.000083$), but no differences were observed in the frequencies of the major or heterozygous genotypes of rs2298599 ($p>0.05$). The evidence for genotypic association of rs2298599 remained significant after a Bonferroni correction for multiple tests (corrected $p=0.0012$). Genomic sequencing data for rs2298599 for each individual were in agreement with genotyping data using the TaqMan methods. Haplotype analysis showed a marginally significant association with schizophrenia in the rs3740829- rs546239- rs2298599 haplotype ($\chi^2=7.9$, global $p=0.049$) (Table S2). However, the

Table 2. Demographic variables for subjects included in the SPQ analysis.

Variables	Total ($n=180$)	G carrier ($n=159$)	A/A ($n=21$)	p values	(z)
Age (years)	36.6±11.5	36.5±11.5	37.5±11.8	0.69	0.40
Sex (male/female)[a]	87/93	77/82	10/11	0.94	<0.01
Education (years)	15.4±2.4	15.6±2.4	14.4±2.0	**0.041**	−2.05
Full scale IQ	109.0±12.0	109.0±12.2	108.8±11.2	0.75	−0.33

Means ± SD are shown. P values<0.05 are in boldface and underlined.
[a]χ^2 test.
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association did not survive correction for multiple testing ($p>0.05$ after Bonferroni correction).

The LD relationships between the investigated markers are provided in Figure S1. The LD pattern observed in our controls was similar to our patients and the JPT HapMap samples; however, it was different from the LD pattern of the Utah residents with Northern and Western European ancestry from the CEPH collection (CEU) HapMap samples.

In silico genotype-expression analysis

We examined an association between the rs2298599 and the expression levels of the *p250GAP* gene in immortalized lymphoblasts derived from 45 HapMap JPT subjects using WGAViewer software (http://compute1.lsrc.duke.edu/softwares/WGAViewer). However, *in silico* analysis revealed that there was no significant association between the SNP and the *p250GAP* expression in the immortalized lymphoblasts ($p=0.28$).

Impact of *p250GAP* genotype on schizotypal personality traits

We examined a possible association between the *p250GAP* genotype of rs2298599 and schizotypal personality traits in healthy subjects. Compared to controls, patients with schizophrenia were significantly more likely to carry the rs2298599 A/A genotype. Therefore, these analyses focused on a comparison of homozygous risk A/A genotype carriers versus homozygous carriers of one or two copies of the G allele (a combined G/G and G/A genotype group), under a recessive inheritance model of the risk A/A genotype. Demographic variables, age, sex, and full-scale IQ were not significantly different between the genotype groups, except for years of education ($z=-2.05$, $p=0.041$) (Table 2). We first examined the possible effect of *p250GAP* rs2298599 on the total SPQ score and found a significant effect of the genotype ($F_{1,178}=4.08$, $p=0.045$) (Table 3). Then, we investigated the genotype effects on the three SPQ factors, cognitive/perceptual, interpersonal and disorganization. A significant genotype effect was observed on the interpersonal factor ($F_{1,178}=5.85$, $p=0.017$), but no significant genotype effects were observed on the cognitive/perceptual or disorganization factors ($p>0.1$). The effect of genotype on the interpersonal factor remained significant after adjusting for confounding factors ($F_{1,175}=4.71$, $p=0.031$). Subjects with the risk A/A genotype of rs2298599 showed higher scores on schizotypal traits, particularly the interpersonal factor, compared with subjects with the G allele (Figure 2). The effect sizes of the total score and interpersonal factor were 0.41 and 0.47, respectively. When the two genotypes were divided into opposite two genotype groups (homozygous carriers of one or two copies of the A allele versus homozygous G/G genotype carriers) under a dominant model of inheritance, there was no significant difference in scores between A carriers and individuals with G/G genotype ($p>0.05$, Table S3).

Discussion

This study is the first investigation of the association of the *p250GAP* gene with schizophrenia. In this study, we first provided evidence that a genetic variant of the *p250GAP* gene was associated with the risk for schizophrenia. The frequency of individuals with the rs2298599 risk A/A genotype was higher in patients with schizophrenia than in the controls. Second, we indicated that the risk genotype of the *p250GAP* gene was associated with high schizotypal personality traits, particularly the interpersonal factor, in healthy subjects. Individuals with the rs2298599 risk A/A genotype scored higher on schizotypal personality traits and the interpersonal factor than did individuals with non-risk genotypes. These findings suggest that the *p250GAP* gene may be related to the risk for schizophrenia and the schizotypal personality traits.

Rs2298599 is situated within the relatively large LD block, which includes the *p250GAP* and the *P53AIP1* (OMIM 605426) genes (Figure S2). The SNP is located 2.9 kb downstream of the *p250GAP* gene and located 22.1 kb upstream of the *P53AIP1* gene. To confirm whether a significant association signal of rs2298599 with schizophrenia is attributed to the *p250GAP* gene, we checked strength of LDs in the genomic region (±50 kb) around rs2298599 using HapMap data (JPT, Chr 11: 128,290,162..128,390,161). Seven SNPs was related to rs2298599 with the criteria of r^2 greater than 0.8. Of the seven SNPs, five SNPs were located 5′ upstream from rs2298599 and four SNPs were included in the *p250GAP* gene. Two SNPs were located 5.2 and 7.9 kb downstream from rs2298599. These findings suggest that our association signal could be attributed to the *p250GAP* but not the *P53AIP1* gene. However, the *P53AIP1* may be a susceptibility gene for schizophrenia. Future

Table 3. Association of the *p250GAP* gene risk variant with the schizotypal personality traits.

SPQ	Total	G carrier	A/A		Genotype effect		
Variables	($n=180$)	($n=159$)	($n=21$)	Cohen's d	$F_{1,178}$	p values	η^2
Total score	10.7±8.9	10.3±8.4	14.4±11.4	−0.41	4.08	**0.045**	0.02
Cognitive/perceptual	3.3±3.8	3.2±3.7	4.0±4.5	−0.19	0.94	0.33	0.01
Interpersonal	5.0±4.5	4.7±4.1	7.2±6.3	−0.47	5.85	**0.017**	0.03
Disorganization	3.1±3.3	3.0±3.3	4.1±3.8	−0.31	2.13	0.15	0.01

SPQ: Schizotypal Personality Questionnaire. Means ± SD are shown. The effect sizes are typically categorized as small ($d=0.20$, $\eta^2=0.01$), medium ($d=0.50$, $\eta^2=0.06$) or large ($d=0.80$, $\eta^2=0.14$). Significant p values are shown in boldface and underlined.
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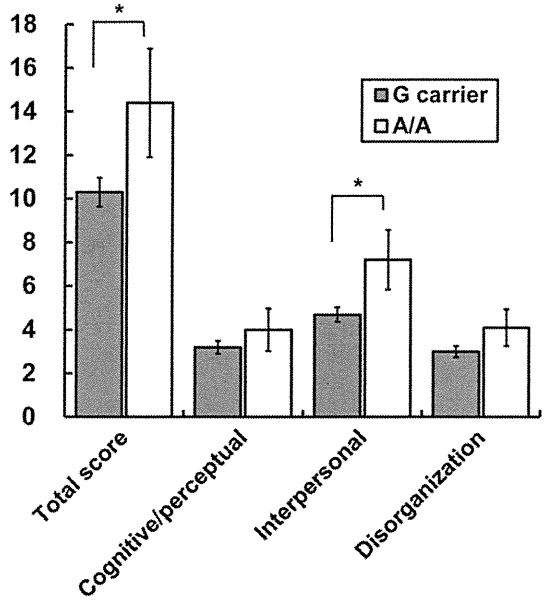

Figure 2. The association between the risk-associated *p250GAP* genotype and SPQ total score and the three factors. The gray bars represent individuals who are G-carriers (G/G and G/A genotypes) of rs2298599. The white bars represent individuals with the A/A genotype of the SNP. Error bars represent standard errors of the mean. * $p<0.05$.
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studies are required to investigate the association between the *P53AIP1* and schizophrenia.

Although significant associations between the *p250GAP* gene and schizophrenia were observed in this study, no experimental evidence has indicated that the rs2298599 SNP of *p250GAP* is functional. To define a possibly functional SNP associated with the disease, followed by evaluation of altered function caused by the relevant SNP, may be able to narrow down the region of the association observed with the rs2298599. For example, gene expression analyses at either mRNA or protein levels of the *p250GAP* gene using postmortem or lymphoblast samples may be an alternative approach. We examined an association between the rs2298599 and *p250GAP* expression in immortalized lymphoblasts. However, *in silico* analysis revealed that the SNP might not be related to *p250GAP* mRNA expression in a Japanese population. A future biological study of the function of rs2298599 or *p250GAP* gene is required to verify our results.

MicroRNAs (miRs) regulate cellular fate by controlling the stability or translation of the mRNA transcripts. The miR132, located on 17p12.3, controls p250GAP protein levels and regulates neuronal morphogenesis by decreasing the levels of p250GAP [45]. The miR132 target sequence in the *p250GAP* 3′UTR, AACAGTCCACTGTCCAGCAGAGG, is conserved across vertebrate evolution. We performed a mutation search of the genomic region (the target sequence of miR132±250 bp) in the *p250GAP* gene using 48 patients with schizophrenia to evaluate the presence of a genetic variant in this region. However, we had no polymorphisms in our sequence data. This result suggests that the miR132 target sequence in the *p250GAP* might not play a major role in risk for schizophrenia.

Several molecular genetic studies have investigated the influences of susceptibility genes for schizophrenia on schizotypal personality traits. These studies have reported associations between the *COMT* [46,47,48], *NRG1* [49], *DTNBP1* [50,51], *RGS4* [52], *DAAO* [51] and *ZNF804A* [35] genes and schizotypal components. Risk alleles or haplotypes of schizophrenia were correlated with high scores on schizotypal personality traits. Of these genes, the *COMT*, *NRG1*, *DTNBP1*, *DAAO* and *RGS4* genes, as well as the *p250GAP* gene, are directly or indirectly responsible for NMDA receptor-mediated glutamate transmission or signaling via glutamate receptors [53]. However, involvement of the glutamate NMDA receptors in SPD is still unknown. Further research will need to clarify the relationship between the glutamate NMDA receptors and SPD.

The interpretation of our results has several limitations. We found a significant association of the *p250GAP* gene with schizophrenia using 431 patients with schizophrenia and 572 controls. Our sample sizes had sufficient power (>0.80) to detect the effects of ORs of 1.30 or larger. Because our results were based on a relatively small sample to detect the effects of ORs of 1.30 or fewer, a future replication study using larger sample sizes is needed to confirm our findings. Our positive results might have been derived from a sample bias due to population stratification and non-age-matched samples, although the Japanese are a relatively homogeneous population. We used schizotypal personality traits as a phenotype of interest. As the assessment of the personality traits was based on a self-reported questionnaire, it was not an objective measurement. Importantly, to be included in the SPQ analysis, subjects were not required to meet criteria for SPD. We had hypothesized that schizotypal personality trait is a continuous measure of the genetic liability to schizophrenia. G allele carriers had marginally higher years of education and lower scores on schizotypal traits than did subjects with the risk A/A genotype. In a previous study, years of education had significant inverse effects on the total SPQ score and the three factor scores, indicating that the SPQ scores decreased with increased years of education [44]. The educational level difference between the genotype groups may have affected the genotype effects on the schizotypal personality trait. However, our results remained significant after adjusting for years of education.

In this study, we proposed *p250GAP* as a new candidate gene for susceptibility to schizophrenia. The association between the *p250GAP* gene and schizophrenia might partially explain the relationship between the hypofunction of the glutamate NMDA receptor and schizophrenia. Future studies are required to confirm the association between the *p250GAP* gene and schizophrenia in other populations.

Supporting Information

Figure S1 Linkage disequilibrium pattern of eight SNPs in the patient, control, HapMap JPT and CEU groups. The linkage disequilibriums (LDs) between the pairwise SNPs are shown using the r^2 value separately for the patients with schizophrenia, the controls, the HapMap JPT samples and the HapMap CEU samples. High levels of LD (r^2) are represented by black coloring, and increasing color intensity from 0 to 100 is shown by the color bars. The numbers (from 1 to 8) in the boxes refer to the eight tagging SNPs; rs493172 (1), rs10893947 (2), rs2276027 (3), rs3796668 (4), rs581258 (5), rs3740829 (6), rs546239 (7) and rs2298599 (8).
(TIF)

Figure S2 Linkage disequilibrium in the genomic region (±50 kb) around rs2298599 SNP in HapMap JPT. LD structure is based on an entry in the HapMap data release 24/PhaseII Nov 08, on NCBI B36 assembly, dbSNP b126 (JPT, Chr 11: 128,290,162..128,390,161). The LD structure between the pairwise SNPs is shown using the r^2 value. High levels of LD are represented by black (r^2) coloring, with increasing color intensity.
(TIF)

Table S1 Selected tagging SNPs in the *p250GAP* gene and its flanking regions.
(DOC)

Table S2 Haplotype analysis of the *p250GAP* gene between patients with schizophrenia and the controls.
(DOC)

Table S3 Association of the *p250GAP* gene variant with schizotypal personality traits under dominant model of inheritance.
(DOC)

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Author Contributions

Conceived and designed the experiments: KO RH TN TY MK MT. Performed the experiments: TO YY HY MF SU. Analyzed the data: KO RH TN MI HK. Contributed reagents/materials/analysis tools: TO YY HY MF SU MI HK TY MK MT. Wrote the paper: KO RH TN.

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Effect of Xenon on Excitatory and Inhibitory Transmission in Rat Spinal Ventral Horn Neurons

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ABSTRACT

Background: The minimum alveolar concentration is determined in the spinal cord rather than in the brain. Xenon inhibits glutamatergic excitatory synaptic transmission in the dorsal horn neurons. However, its actions in the ventral horn neurons have not been investigated.

Methods: The effects of 50 or 75% xenon on excitatory and inhibitory synaptic transmission were examined in the spinal lamina IX neurons of neonatal rats by using a whole cell patch clamp technique.

Results: Fifty percent xenon inhibited the α-amino-3-hydroxy-5-methyl-4-isoxazole-4-propionic acid-induced currents (amplitudes = 72 ± 9% and integrated area = 73 ± 13% of the control values), and α-amino-3-hydroxy-5-methyl-4-isoxazole-4-propionic acid receptor-mediated electrically evoked excitatory postsynaptic currents (amplitudes = 69 ± 13% of the control values). Seventy-five percent xenon similarly inhibited α-amino-3-hydroxy-5-methyl-4-isoxazole-4-propionic acid-induced currents. However, xenon had no effect on the *N*-methyl-D-aspartate-induced currents or *N*-methyl-D-aspartate receptor-mediated electrically evoked excitatory postsynaptic currents. Xenon decreased the amplitude, but not the frequency, of miniature excitatory postsynaptic currents. There were no discernible effects on the currents induced by γ-aminobutyric acid or glycine or on miniature inhibitory postsynaptic currents.

Conclusions: Xenon inhibits α-amino-3-hydroxy-5-methyl-4-isoxazole-4-propionic acid receptor-mediated glutamatergic excitatory transmission in the spinal lamina IX neurons *via* a postsynaptic mechanism. In contrast, there are no substantial effects on *N*-methyl-D-aspartate receptor-mediated or inhibitory synaptic transmission. The suppressive effects on excitatory synaptic transmission in the ventral horn neurons partly account for the mechanism behind xenon's ability to produce immobility in response to noxious stimuli and to determine the minimum alveolar concentration.

What We Already Know about This Topic

- The site of action of anesthetics to prevent movement during surgery largely resides in the spinal cord
- Xenon produces anesthesia in part by reducing *N*-methyl-D-aspartate (NMDA) receptor and α-amino-3-hydroxy-5-methyl-4-isoxazole-4-propionic acid (AMPA) receptor activation, but whether it does so in the ventral horn is not known

What This Article Tells Us That Is New

- In the lamina IX neurons of rat spinal cord, xenon inhibits excitation of AMPA receptors but not NDMA receptors
- The immobilizing effects of xenon may be through an unexpected mechanism, reduction in excitation of AMPA receptors

XENON, as an inhalational anesthetic, combines profound anesthetic and analgesic properties with a low side-effect profile, and if used with an elaborate low-flow delivery system, it has the potential to become a main-line anesthetic.[1,2] Significant progress has been made toward elucidating how xenon produces anesthesia, and it has been revealed that xenon suppresses the excitatory synaptic transmission in the central nervous system.[3–8] A study on cultures of rat hippocampal neurons showed that xenon inhibited *N*-methyl-D-aspartate (NMDA) receptor-mediated synaptic transmission but not α-amino-3-hydroxy-5-methyl-4-isoxazole-4-propionic acid (AMPA) receptor-mediated synaptic transmission.[4] In later studies, xenon blocked both NMDA receptor-mediated and AMPA receptor-mediated synaptic transmission in cultures of mouse embryonic cortical neurons,[5] rodent amygdala,[6] prefrontal cortex,[7] and spinal cord dorsal horn.[7,8] By contrast, xenon had no effect on inhibitory synaptic transmission.[6–8]

Originally, the brain was considered to be the principal effect site of anesthetics and the determiner of the minimum alveolar concentration (MAC) for volatile anesthetics, a common means to express the strength or potency of anesthetics.

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However, the MAC merely refers to the anesthetic concentration needed to prevent movement in response to noxious stimuli[9] and is caused by anesthetic actions in the spinal cord, rather than in the brain.[10,11]

In the spinal cord, anesthetic actions might result from a combination of a reduction of the sensory transmission of nociceptive signals within the spinal dorsal horn and a suppression of the neuronal activity in the spinal ventral horn.[12] We reported previously that 50% xenon inhibited both NMDA receptor-medicated and AMPA receptor-mediated glutamatergic excitatory transmission *via* a postsynaptic mechanism in the spinal dorsal horn[8]; however, xenon's actions in the spinal ventral horn have not yet been clarified. Therefore, the current study investigated the effects of xenon on spinal ventral horn neurons.

Materials and Methods

All experimental procedures involving the use of animals were approved by the Animal Care and Use Committee at Niigata University Graduate School of Medical and Dental Sciences (Niigata, Japan).

Preparation of the Spinal Cord Slices

Neonatal Wistar rats (200 rats, 6–14 days old) were anesthetized with urethane (1.5 g/kg, intraperitoneal). Dorsal laminectomy was performed, and the lumbosacral segment of the spinal cord was removed.[13] The rats were killed immediately by exsanguination. Each spinal cord was placed in preoxygenated ice-cold artificial cerebrospinal fluid containing 117 mM NaCl, 3.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11.5 mM D-glucose. After all the ventral and dorsal roots were cut, the pia-arachnoid membrane was removed. The spinal cord was mounted on the metal stage of a microslicer (DTK-1500; Dosaka, Kyoto, Japan) and cut into 500-μm transverse slices. Each spinal cord slice was transferred to a recording chamber and placed on the stage of an upright microscope equipped with an infrared-differential interference contrast system (E600FN; Nikon, Tokyo, Japan). The slice was fixed with an anchor and superfused at 4–6 ml/min with artificial cerebrospinal fluid equilibrated with a gas mixture of 95% O_2 and 5% CO_2 (pH = 7.4) and maintained at 36°C using a temperature controller (TC-324B; Warner Instruments, Hamden, CT).

Patch Clamp Recordings from Spinal Lamina IX Neurons

Lamina regions were identified under low magnification (×5 objective lens), and individual neurons were identified using a ×40 objective lens under an infrared-differential interference contrast microscope and monitored by a charge-coupled device camera (C2400–79H; Hamamatsu Photonics, Hamamatsu, Japan) on a video screen. Whole cell patch clamp recordings were made from the large lamina IX neurons (size, 15–25 μm).[13] In a previous study, these neurons were identified as motoneurons by fluorescence labeling with Evans blue dye injected into the rat hind limb the day before sacrifice.[14] Whole cell patch pipettes were constructed from borosilicate glass capillaries (1.5 mm OD; World Precision Instruments, Sarasota, FL). The resistance of a typical patch pipette was 4–8 MΩ when filled with internal solution. The patch pipette solutions contained 110 mM Cs_2SO_4, 5 mM tetraethylammonium, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM EGTA, 5 mM HEPES, and 5 mM ATP-Mg.

Signals were amplified by an Axopatch 200B amplifier (Molecular Devices, Union City, CA), filtered at 2 kHz, and digitized at 5 kHz. All experiments were performed in voltage-clamp mode at a holding potential of −70 mV for recording exogenously applied AMPA-induced currents or AMPA receptor-mediated electrically evoked excitatory postsynaptic current (EPSCs) and miniature EPSCs. Exogenously applied NMDA-induced currents and NMDA receptor-mediated electrically evoked EPSCs were recorded at +40 mV. Inhibitory currents induced by exogenous γ-aminobutyric acid (GABA) or glycine, as well as miniature inhibitory postsynaptic current (IPSCs), were recorded at 0 mV. Electrically evoked EPSCs were elicited by focal stimulation of the deep dorsal horn with a concentric bipolar tungsten electrode (diameter = 10 μm). This stimulation area was identified visually under low magnification (×5 objective lens). Stimulus parameters were as follows: intensity, 1–5 V; duration, 0.05 ms; and frequency, 0.1 Hz. Electrically evoked EPSCs that displayed a constant latency and lack of failures with high frequency stimulation (10 Hz) were classified as monosynaptic. Data were stored and analyzed using a pCLAMP 9.2 data acquisition program (Molecular Devices) and Mini-analysis 6.0.7 (Synaptosoft, Leonia, NJ). Each peak current was measured before and after xenon treatment and expressed as (posttreatment/pretreatment) × 100 (as percentages).

Drug Application

Xenon was acquired from TG Showa (Tokyo, Japan). AMPA, NMDA, GABA, glycine, bicuculline, strychnine, 6-cyano-7-nitroquinoxaline-2,3-dione disodium, and D, L-2-amino-5-phosphonopentanoic acid were acquired from Sigma–Aldrich (St. Louis, MO). Tetrodotoxin was acquired from Wako (Osaka, Japan). Drugs were applied to the whole slice by perfusion *via* a three-way stopcock without changing the perfusion rate or temperature. The volume of the recording chamber was approximately 1.2 ml. The drugs reached the recording chamber within 15 s after the stopcock was opened, and the drugs were completely washed out within 30 s after the stopcock was closed. Xenon was mixed in a 1:1 ratio with a prefabricated gas containing 90% O_2 and 10% CO_2, so that the composition of the gas mixture was 50% xenon, 45% O_2, and 5% CO_2. For the additional experiments, xenon was mixed in a 3:1 ratio with a prefabricated gas containing 80% O_2 and 20% CO_2, so that the composition of the gas mixture was 75% xenon, 20% O_2, and 5% CO_2. Xenon was delivered through an ultrathin polyethyl-

ene tube and applied by bubbling through the perfusing solution saturated with the gas mixture for 30 min.

Statistical Analyses

Numerical data are represented as means ± SD. Statistical significance was determined as $P < 0.01$ using the Student paired t test or the Kolmogorov-Smirnov test, as appropriate. When referring to electrophysiological data, "n" indicates the number of neurons studied. The continuous curve for the concentration-response relationship of AMPA, NMDA, GABA, and glycine was drawn according to the Hill equation:

$$y = \frac{y_{max}x^n}{x^n - k^n}$$

where x is the agonist concentration, y is the relative amplitude of agonist-induced current (%), and y_{max} is the maximal value of y. The term k is the half-maximal effective concentration (EC_{50}; μM or mM), and n is the slope of the curve (Hill coefficient).

Results

EC_{50} of AMPA, NMDA, GABA, and Glycine on Spinal Lamina IX Neurons

Because there was no database available regarding the proper concentration of the agonists for the experiments in the spinal lamina IX neurons, the concentration-response curve was obtained for the agonists AMPA, NMDA, GABA, and glycine on the spinal lamina IX neurons (fig. 1). Exogenous application of AMPA elicited an inward current in neurons at −70 mV. Exogenous application of NMDA at +40 mV or of GABA or glycine at 0 mV elicited an outward current. Each agonist was applied by perfusion for 15 s and induced a current in a concentration-dependent manner. The EC_{50} values for AMPA, NMDA, GABA, and glycine on the spinal lamina IX neurons were as follows: AMPA, 7.0 μM; NMDA, 62.8 μM; GABA, 0.1 mM; and glycine, 0.2 mM. The Hill coefficient values were as follows: AMPA, 1.6; NMDA, 1.7; GABA, 1.1; glycine, 1.3. We set the concentration of the agonists as follows: AMPA, 10 μM; NMDA, 50 μM; GABA, 0.5 mM; glycine, 0.5 mM.

To confirm that these exogenous agonist-induced currents were postsynaptic phenomena, we examined these currents in the presence of tetrodotoxin (1 μM) to remove any possible influence of presynaptic neurons. Tetrodotoxin did not affect the amplitudes or the integrated area: AMPA (amplitude = 105 ± 12% of control values, $P = 0.36$; area = 106 ± 13% of control values, $P = 0.31$, n = 6), NMDA (amplitude = 95 ± 13% of control values, $P = 0.29$; area = 102 ± 23% of control values, $P = 0.84$, n = 9), GABA (amplitude = 108 ± 13% of control values, $P = 0.21$; area = 113 ± 12% of control values, $P = 0.05$, n = 5), glycine (amplitude = 110 ± 14% of control values, $P = 0.10$; area = 105 ± 18% of control values, $P = 0.50$, n = 6). Therefore, the following experiments to examine the effects of xenon on exogenous agonist-induced currents were performed in the absence of tetrodotoxin.

Effects of Xenon on Exogenous AMPA- and NMDA-induced Currents

Exogenous application of AMPA (10 μM, 15 s) elicited an inward current in the spinal lamina IX neurons at −70 mV (fig. 2A and D). Preapplication of 50% xenon for 5 min reversibly reduced the peak amplitudes of the AMPA-induced currents to 72 ± 9% of the control values (n = 7, $P < 0.01$; fig. 2B) and the integrated area to 73 ± 13% of the control values (n = 7, $P < 0.01$; fig. 2C). Exogenous application of NMDA (50 μM, 15 s) induced an outward current at +40 mV (fig. 3A); 50% xenon did not change the peak amplitudes (99 ± 13% of control values, n = 6, $P = 0.91$) or the integrated area (99 ± 22% of control values, n = 6, $P = 0.91$) of NMDA-induced currents.

Seventy-five percent xenon reversibly reduced the peak amplitudes of the AMPA-induced currents to 67 ± 12% of the control values (n = 10, $P < 0.01$; fig. 2E) and the integrated area to 62 ± 12% of the control values (n = 10, $P < 0.01$; fig. 2F). However, 75% xenon did not change the peak amplitudes (103 ± 7% of control values, n = 10, $P = 0.30$) or the integrated area (100 ± 14% of control values, n = 10, $P = 0.97$) of NMDA-induced currents.

Effects of Xenon on AMPA and NMDA Receptor-mediated Electrically Evoked EPSCs

To examine the effects of xenon on synaptic transmission, electrically evoked EPSCs were elicited by focal stimulation of the deep dorsal horn. Evoked EPSCs can be divided into different groups. EPSCs that displayed a constant latency and lack of failure with high frequency stimulation (10 Hz) were classified as monosynaptic, whereas EPSCs that displayed variable latencies and failures were considered polysynaptic (fig. 4, A–C). We investigated the effects of xenon on evoked monosynaptic EPSCs.

α-Amino-3-hydroxy-5-methyl-4-isoxazole-4-propionic acid receptor-mediated electrically evoked EPSCs were isolated pharmacologically with D,L-2-amino-5-phosphonopentanoic acid (50 μM), bicuculline (10 μM), and strychnine (2 μM) at −70 mV. Figure 5A shows the representative average traces of AMPA receptor-mediated evoked EPSCs before, during, and after xenon application. Fifty percent xenon reversibly reduced the peak amplitudes of AMPA receptor-mediated evoked EPSCs to 69 ± 13% of the control values (n = 9, $P < 0.01$). These evoked EPSCs were almost completely abolished by the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione disodium (10 μM, 10 ± 3% of the control values, n = 9, $P < 0.01$; fig. 5, A–C), suggesting that the evoked EPSCs were mediated by AMPA receptor.

N-methyl-D-aspartate receptor-mediated electrically evoked EPSCs were isolated pharmacologically with 6-cyano-7-nitroquinoxaline-2,3-dione disodium (10 μM), bicuculline (10

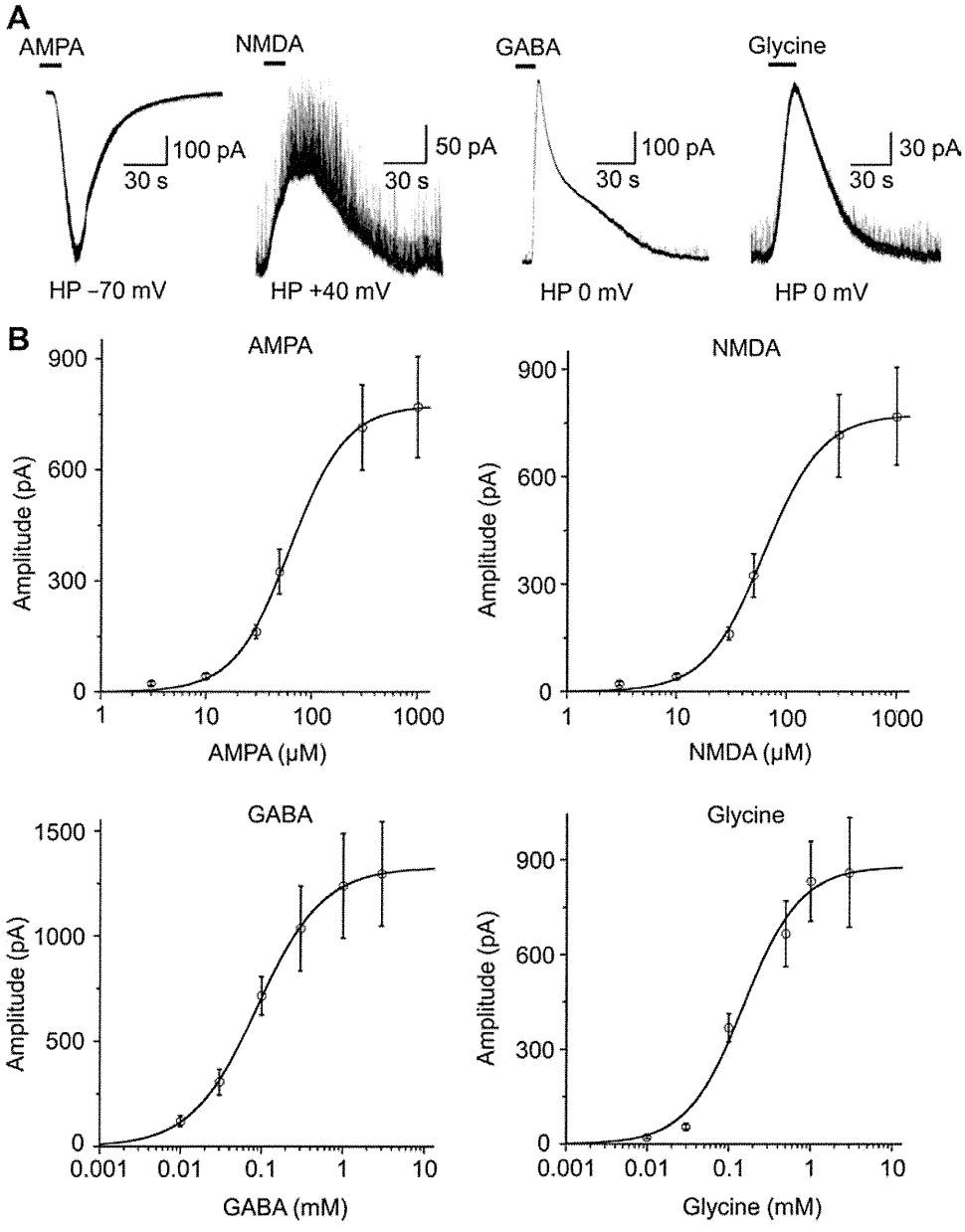

Fig. 1. Representative traces of agonist-induced currents and the concentration-response curve for each agonist. In this figure and subsequent figures, the *horizontal bars* in the recording charts indicate the duration of drug superfusion. Each agonist was applied for 15 s. Exogenous application of α-amino-3-hydroxy-5-methyl-4-isoxazole-4-propionic acid (AMPA) elicited an inward current in the spinal lamina IX neurons at −70 mV. Exogenous application of *N*-methyl-D-aspartate (NMDA) at +40 mV, γ-aminobutyric acid (GABA) at 0 mV, and glycine at 0 mV elicited an outward current (*A*). Peak amplitudes of the induced currents at various concentrations are plotted at the logarithm of their concentrations. Each *point with vertical bars* represents the mean value and SD. The continuous curves are drawn according to the Hill plot with each EC_{50} value and Hill coefficient. The EC_{50} values for AMPA, NMDA, GABA, and glycine on the spinal lamina IX neurons were as follows: AMPA 7.0 μM, NMDA 62.8 μM, GABA 0.1 mM, glycine 0.2 mM. Hill coefficient values for AMPA, NMDA, GABA, and glycine were as follows: AMPA 1.6, NMDA 1.7, GABA 1.1, glycine 1.3 (*B*). HP = holding potential.

μM), and strychnine (2 μM) at +40 mV. Figure 5D shows the representative average traces of NMDA receptor-mediated evoked EPSCs before and during xenon application. The peak amplitudes of NMDA receptor-mediated evoked EPSCs were not affected by 50% xenon (98 ± 3% of the control values, n = 6, $P = 0.09$). These evoked EPSCs were almost completely abolished by the NMDA receptor antagonist D,L-2-amino-5-phosphonopentanoic acid (50 μM, 7 ± 3% of the control values, n = 6, $P < 0.01$; fig. 5, D–F), suggesting that the evoked EPSCs were mediated by NMDA receptor.

Effects of Xenon on Miniature EPSCs

To determine the site of the inhibitory action of xenon, we examined the effects of xenon on miniature EPSCs in the

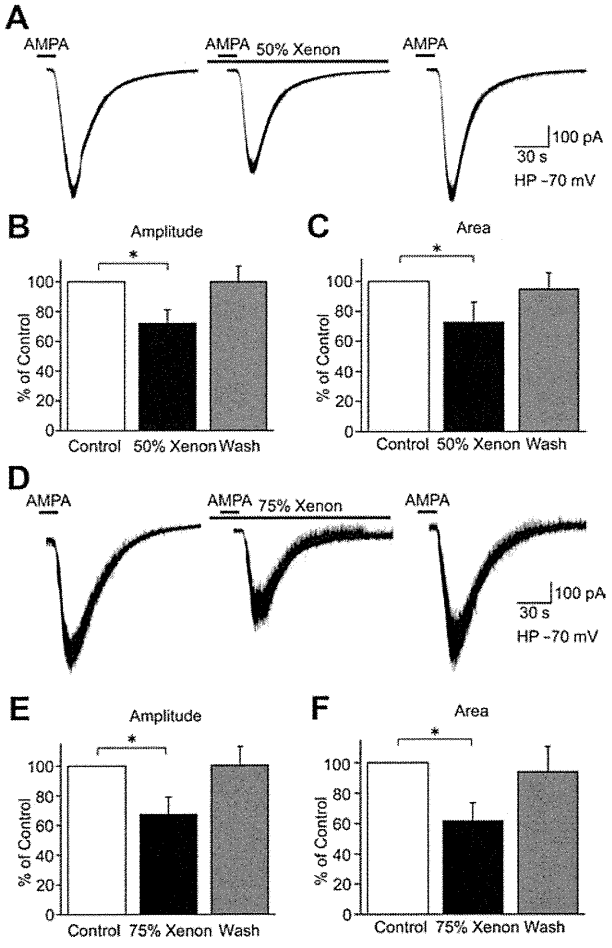

Fig. 2. Xenon inhibited exogenous α-amino-3-hydroxy-5-methyl-4-isoxazole-4-propionic acid (AMPA)-induced currents. Representative traces showing AMPA (10 μM)-induced currents in the spinal lamina IX neurons at −70 mV, which were reversibly reduced by 50% xenon (*A*). Fifty percent xenon decreased the peak amplitudes of AMPA-induced currents to 72 ± 9% of control values and the integrated area to 73 ± 13% of control values, respectively (*B, C*). Representative traces showing AMPA-induced currents in the spinal lamina IX neurons at −70 mV, which were reversibly reduced by 75% xenon (*D*). Seventy-five percent xenon decreased the peak amplitudes of AMPA-induced currents to 67 ± 12% of control values and the integrated area to 62 ± 12% of control values (*E, F*). * $P < 0.01$. HP = holding potential.

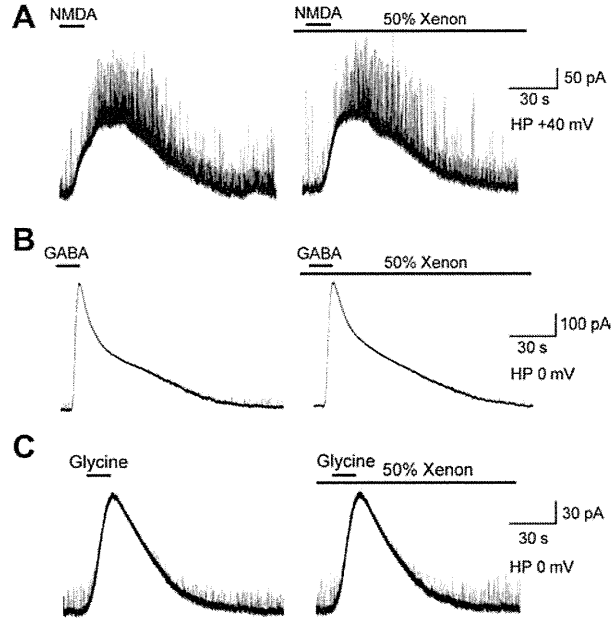

Fig. 3. Xenon did not affect exogenous *N*-methyl-D-aspartate (NMDA)-, γ-aminobutyric acid (GABA)-, and glycine-induced currents. Representative traces showing NMDA (50 μM)-induced currents in the spinal lamina IX neurons at + 40 mV. Fifty percent xenon did not change the peak amplitudes and the integrated area of NMDA-induced currents (*A*). Representative traces showing current induced by GABA (0.5 mM) in the spinal lamina IX neurons at 0 mV. Fifty percent xenon did not change the peak amplitudes and the integrated area of GABA-induced currents (*B*). Representative traces showing currents induced by glycine (0.5 mM) in the spinal lamina IX neurons at 0 mV. Fifty percent xenon did not change the peak amplitudes and the integrated area of glycine-induced currents (*C*). HP = holding potential.

presence of tetrodotoxin (1 μM) at −70 mV. Fifty percent xenon decreased the mean amplitudes of the miniature EPSCs to 82 ± 14% of the control values (n = 9, $P < 0.01$), whereas the mean frequencies of miniature EPSCs remained unchanged (102 ± 40% of the control values, n = 9, $P = 0.91$, fig. 6A). Figure 6B shows the effects of xenon on cumulative distributions of miniature EPSC amplitudes and interevent intervals. Although xenon increased the proportion of miniature EPSCs with a significantly smaller amplitude compared with that of the control ($P < 0.01$), it had no effect on the cumulative distribution of the interevent intervals of miniature EPSCs ($P = 0.39$). The decay time of miniature EPSCs was reversibly prolonged by xenon (111 ± 25% of the control values, n = 9, $P = 0.22$); however, this change was not statistically significant.

Effects of Xenon on Exogenous GABA- and Glycine-induced Currents and on Miniature IPSCs

Exogenous application of GABA (0.5 mM, 15 s) and glycine (0.5 mM, 15 s) elicited an outward current at 0 mV (fig. 3, B and C). Fifty percent xenon did not change the peak amplitudes (99 ± 9% of the control values, n = 10, $P = 0.76$) or the integrated area (101 ± 19% of the control values, n = 10, $P = 0.87$) of GABA-induced currents. Similarly, xenon did not change the peak amplitudes (105 ± 13% of the control values, n = 9, $P = 0.29$) or the integrated area (108 ± 12% of the control values, n = 9, $P = 0.07$) of glycine-induced currents.

We also examined the effects of xenon on miniature IPSCs in the presence of tetrodotoxin (1 μM) at 0 mV. Fifty percent xenon did not change the mean amplitudes (100 ± 32% of the control values, n = 9, $P = 0.97$; fig. 7A) or the mean frequencies (100 ± 20% of the control values, n = 9, $P = 0.95$) of miniature IPSCs. Xenon had no effect on the cumulative distributions of miniature IPSC amplitudes ($P = 0.15$; fig. 7B) or interevent intervals ($P = 0.61$).

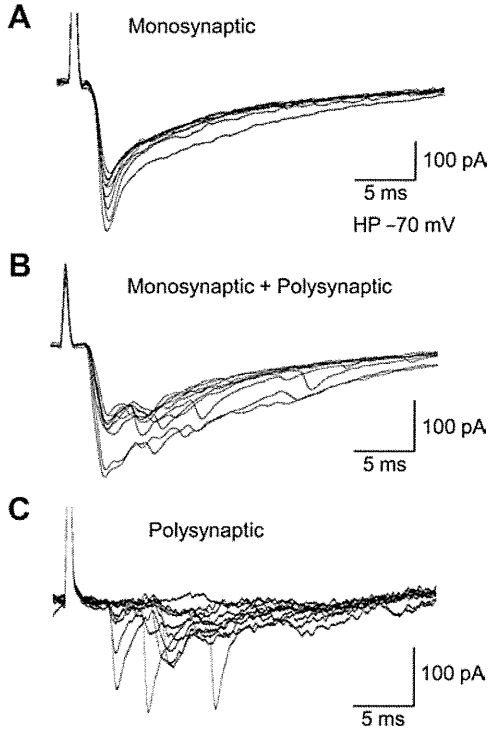

Fig. 4. Various patterns of excitatory postsynaptic currents (EPSCs) elicited by focal stimulation at a frequency of 10 Hz. Monosynaptic evoked EPSCs (*A*). Both the monosynaptic- and polysynaptic-evoked EPSCs (*B*). Polysynaptic evoked EPSCs (*C*). These evoked EPSCs were observed in different neurons. HP = holding potential.

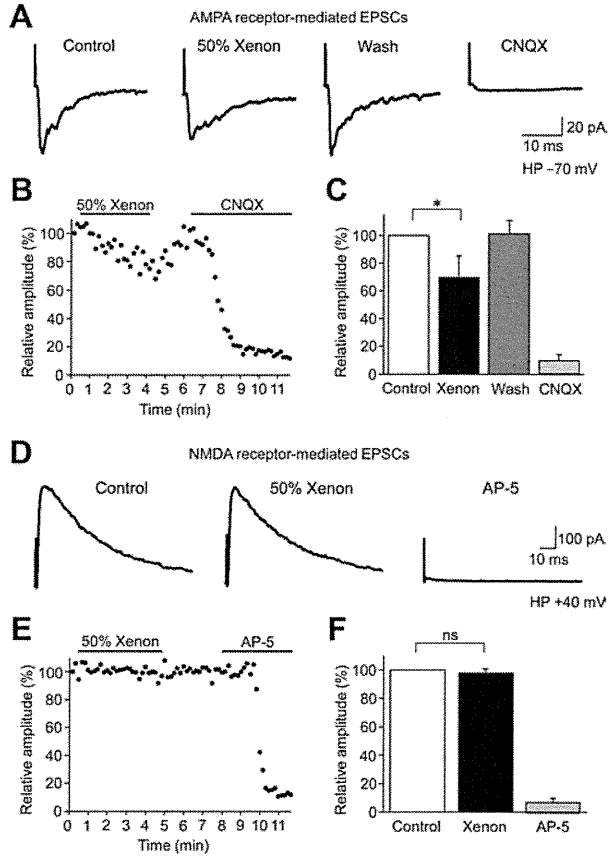

Fig. 5. Xenon reversibly decreased the amplitudes of α-amino-3-hydroxy-5-methyl-4-isoxazole-4-propionic acid (AMPA) receptor-mediated electrically evoked excitatory postsynaptic currents (EPSCs) but not *N*-methyl-D-aspartate (NMDA) receptor-mediated electrically evoked EPSCs in the spinal lamina IX neurons. Averaged traces of six consecutive AMPA receptor-mediated evoked EPSCs at −70 mV before, during, and after xenon (*A*). Time course of the relative peak amplitudes of AMPA receptor-mediated evoked EPSCs before, during, and after xenon (*B*). Summaries of the suppressive effects of xenon and 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX) on AMPA receptor-mediated evoked EPSCs relative to control value (*C*). Averaged traces of six consecutive NMDA receptor-mediated evoked EPSCs at +40 mV before and during xenon (*D*). Time course of the relative peak amplitudes of NMDA receptor-mediated evoked EPSCs before and during xenon (*E*). Summaries of the suppressive effects of xenon and D,L-2-amino-5-phosphonopentanoic acid (AP-5) on NMDA receptor-mediated evoked EPSCs relative to control value (*F*). * $P < 0.01$. The time scales of electrically evoked EPSCs are 1,000 times as small as those of exogenous agonist-induced currents. HP = holding potential; ns = not significant.

Discussion

Several lines of evidence suggest that xenon suppresses excitatory synaptic transmission in central nervous system areas such as the brain and spinal cord dorsal horn.[3–8] However, its actions in the spinal ventral horn have not been clarified. Thus, we investigated the effects of xenon on excitatory and inhibitory synaptic transmission in the spinal ventral horn neurons.

In the current study, 50 and 75% xenon inhibited both the peak amplitudes and the integrated area of exogenous AMPA-induced currents, as well as the peak amplitudes of AMPA receptor-mediated evoked EPSCs. In addition, 50% xenon decreased the mean amplitudes of miniature EPSCs, but the mean frequency of miniature EPSCs remained unchanged. However, 50 and 75% xenon had no substantial effect on exogenous NMDA-induced currents or NMDA receptor-mediated evoked EPSCs. In addition, 50% xenon had no effect on currents induced by GABA or glycine or on miniature IPSCs. Taken together, these findings suggest that xenon acts on both synaptic and extrasynaptic AMPA receptors and inhibits AMPA receptor-mediated glutamatergic excitatory transmission *via* a postsynaptic mechanism in the spinal lamina IX neurons.

However, several studies have demonstrated that xenon depressed both NMDA and AMPA receptor-mediated glutamatergic excitatory synaptic transmission postsynaptically in the amygdala,[6] prefrontal cortex,[7] and substantia gelatinosa,[7,8] whereas xenon did not affect inhibitory synaptic transmission. This discrepancy between our results and those of previous studies could be attributed to a different composition of NMDA receptors expressed in these neurons.

The NMDA receptor has been proposed as a major target for xenon.[4,15,16] This receptor is composed of subunits from at least two families: NR1 and NR2. The NR1 subunit is essential for the function of NMDA receptors and is ex-