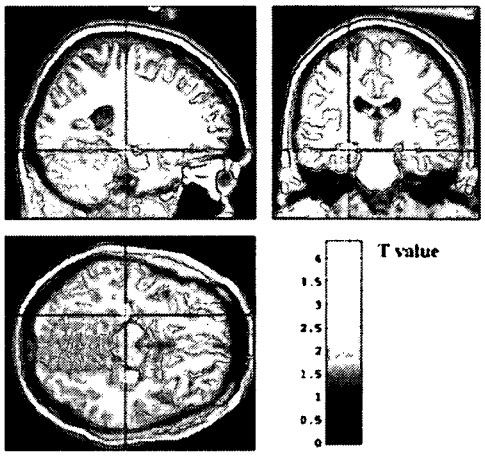
verbal paired associates II, visual paired associates I and visual paired associates II) and the general intelligence IQ (from full scale of the Wechsler Adult Intelligence Scale, revised edition, WAIS-R), were performed by some of the subjects recruited at National Center of Neurology and Psychiatry.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

a



b

	Schizophrenia (n=62)		Control (n=139)	
Variables	A/A, A/G (n=10)	G/G (n=52)	A/A, A/G (n=27)	G/G (n=112)
Age (SD)	39.1 (10.5)	47.1(13.7)	34.6 (11.0)	37.3 (11.8)
Gender (M/F)	3/7	35/17	13/14	34/78
Education years (SD)	14.0 (2.6)	12.8 (3.3)	16.7 (2.8)	16.5 (3.1)
Full scale IQ (SD)	86.2 (21.5)	83.8 (18.8)	109.9 (13.4)	109.6 (11.6)
VPA1 (SD)	13.3 (3.9)	9.0 (4.6)*	16.1 (2.7)	16.1 (2.3)

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

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

Animal study

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

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

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

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

Results

Genetic analysis

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

Intermediate phenotype

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

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

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

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

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

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

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

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

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

Animal study

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

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

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

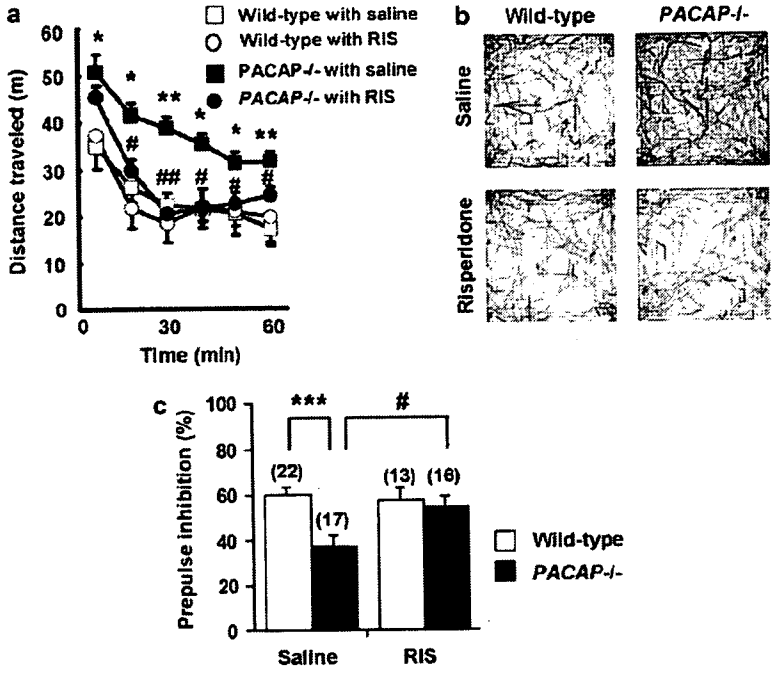


Figure 2 Hyperlocomotion and deficits in the PPI of PACAP$^{-/-}$ mice were normalized by risperidone treatment. (**a**) Locomotor activity in wild-type and PACAP$^{-/-}$ mice that received 0.1 mg/kg risperidone (RIS) or saline. $n = 6$ per group. (**b**) Representative locomotor patterns of saline-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

Discussion

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

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

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

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

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

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ORIGINAL ARTICLE

Epigenetic aberration of the human *REELIN* gene in psychiatric disorders

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Epigenetic genome modifications such as DNA methylation appear to be involved in various diseases. Here, we suggest that the levels of DNA methylation at the *BssH*II methylation-sensitive restriction enzyme sites in the human *REELIN* (*RELN*) gene in the forebrain vary among individuals. Interestingly, although a statistically significant correlation between the levels of DNA methylation in *RELN* and age was detected in healthy individuals, no such correlations were seen in either schizophrenic or bipolar patients. In addition, reverse correlations between DNA methylation levels and *RELN* expression were also detected in postmortem brain RNA and on *in vitro* assay. These data suggest the possibility that epigenetic aberration from the normal DNA methylation status of *RELN* may confer susceptibility to psychiatric disorders.
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Keywords: epigenetics; DNA methylation; REELIN; promoter activity; human postmortem forebrains

Introduction

DNA methylation is an epigenetic modification on the mammalian genome, and cytosine residues in CpG dinucleotides appear to be favorable targets for methylation, resulting in C^mpG. The process of DNA methylation and resultant C^mpG appear to be involved in tissue-specific gene expression, chromatin modification and genomic imprinting, which play essential roles in development, cell proliferation and differentiation.[1,2] DNA methylation is also involved in various diseases, including cancer,[3] and may be associated with psychotic disorders such as schizophrenia.[4–8] To better understand the implications of DNA methylation in disease susceptibility, it is necessary to examine both the qualitative and quantitative status of DNA methylation within the genome.

In this study we developed a method by which relative levels of DNA methylation at methylation-sensitive restriction enzyme sites within the genome can be determined. The method depends on methylation-sensitive restriction enzymes and real-time polymerase chain reaction (real-time PCR). We investigated this method in order to determine any associations between DNA methylation and psychotic disorders by measuring the levels of DNA methylation in genomic DNA from schizophrenic, bipolar and healthy subjects using samples kindly provided by the Stanley Medical Research Institute.

The results presented here suggest that the levels of DNA methylation at methylation-sensitive restriction enzyme sites in the human *REELIN* (*RELN*) gene vary among individuals, and that a possible correlation between the levels of DNA methylation in *RELN* and age occurs in healthy individuals, but not in either schizophrenic or bipolar patients. The present study suggests the possible association between epigenetic aberration in DNA methylation in *RELN* and psychiatric disorders.

Materials and methods

DNA and RNA samples

All genomic DNA and RNA samples examined here were extracted from human postmortem forebrains and were kindly provided by the Stanley Medical Research Institute. Samples were blinded (coded) during our experiments (determination of DNA methylation and examination of expression levels), and after reporting all experimental data to the Institute, we obtained information regarding the diagnoses of the coded subjects, and then analyzed our typing data using subject information (Table 1).

Determination of relative DNA methylation levels

Genomic DNA (0.5 μg) was digested with *EcoR*I and *BssH*II (methylation-sensitive restriction enzyme) at 37°C overnight, collected by ethanol precipitation, and dissolved in 50 μl of TE. Digested DNA (60 ng/

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Table 1 Data of the subjects examined in this study

	Schizophrenia (n = *35*)	*Bipolar* (n = *35*)	*Control* (n = *35*)
Gender	26 males, nine females	17 males, 18 females	26 males, nine females
Age range (years at death)	19–59	19–64	31–60
Mean age (years)	43	46	45
Age at disease onset (years)			
⩽15	5	4	NA
16–20	14	9	NA
21–25	9	9	NA
26–30	3	4	NA
⩾31	4	9	NA
Lifetime alcohol use			
Little or no	10	4	18
Social	7	8	12
Moderate (past or present)	6	9	3
Heavy (past or present)	12	13	2
No information	0	1	0
Lifetime drug use			
Little or no	14	11	30
Social	4	4	4
Moderate (past or present)	6	9	1
Heavy (past or present)	9	11	0
No information	2	0	0
Suicide victims	7	15	0
Postmortem interval (hours)	9–80	12–84	9–58

Abbreviation: NA, not applicable.

test) was used as a template and was examined by means of real-time PCR using the following PCR primer sets:

For amplification of the control region of *RELN*;
RC-F1, 5′-GAACAGTCCGGCGAAGAGAG-3′
RC-R1, 5′-CAGAGCCTCATCTGTAGAGGATTT-3′
For the test region of *RELN*, we carried out real-time PCR with a *RELN* probe that we designed:
RC-F3, 5′-CGGCGTCTCCAAAACTGAATGA-3′
RC-R3, 5′-GTGGGGTTGCCCGCAATATGCAG-3′
RELN probe, 5′-(FAM)-CTAGCGCTGTTGCTGGGGGC GACGCTG-(TAMRA)-3′

Real-time PCR was carried out using the ABI PRISM 7000 or 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA) with SYBR GREEN PCR Master Mix (for control region) or TaqMan Universal PCR Master Mix (for test region) according to the manufacturers' instructions and was repeated at least three times. Two individuals' samples were examined as controls for every PCR in order to normalize test data.

Cell culture
NTera2D1 cells were grown as described previously.[9] Cells ($\sim 7 \times 10^4/cm^2$) were treated with 10^{-5} M all-*trans*-retinoic acid (RA) (SIGMA-ALDRICH, St Louis, MO, USA) in Dulbecco's modified eagle medium (SIGMA-ALDRICH) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen) for 3 weeks and reseeded ($\sim 5 \times 10^4/cm^2$), and further culture was carried out in the absence of RA.

Reverse transcription (RT)-(real-time) PCR
Total RNAs isolated from the same postmortem forebrain specimens were also provided by the Stanley Medical Research Institute, and each RNA sample ($\sim$0.5. μg) was examined by means of reverse transcription (RT) – real-time PCR (RT-PCR) with TaqMan Gene Expression Assays (Applied Biosystems) using TaqMan probes specific for *RELN* (assay ID: Hs00192449_m1) and *S18 rRNA* (assay ID: Hs99999901_s1) as a control. The assay RT-PCR) was carried out using the ABI PRISM 7000 or 7300 sequence detection system (Applied Biosystems) with TaqMan One Step RT-PCR Master Mix Reagents kit (Applied Biosystems) according to the manufacturer's instructions and was repeated at least three times.

Construction of reporter plasmid and partial methylation
In order to construct a reporter plasmid carrying a putative *RELN* promoter linked to the *Photinus luciferase* reporter gene, the *RELN* promoter region from positions −525 to +333 relative to the *RELN*

transcription start site was amplified by PCR with human genomic DNA and the following PCR primer set:

RELN-532F; 5′-GTTCTAGATCTTCCCAGGAAAAAC AGGGCACACTG-3′
RELN + misR; 5′-AATATCCATGGTGGCGAGCACCTC GCCCTGC-3′

The resultant PCR product was digested with *Bgl*II and *Nco*I and was subjected to ligation with pGL3-control treated with the same restriction enzymes. The resultant plasmid possessing the putative human *RELN* promoter was designated 'pRELN–Luc'.

Partial methylation was carried out as follows: ten micrograms of the pRELN–Luc plasmid was treated with 4 U of CpG methylase (M. Sss I) (New England BioLabs, Inc., Beverly, MA, USA) in the presence of 2×, 1× and 0.2× provided *S*-adenosyl methionine at 37°C for 6, 3 and 2 h, respectively. The treated plasmids were purified with a Wizard SV Gel and PCR clean-up system (Promega, Madison, WI, USA), and aliquots of the purified plasmids were subjected to digestion with *BamH*I and *BssH*II followed by real time PCR (1.25 ng each/test) for determination of DNA methylation levels. The PCR primer sets used were as follows:

For amplification of control region in the vector;
GL3-F1044, 5′-TTTGATATGTGGATTTCGAG-3′
GL3-R1194, 5′-ATCGTATTTGTCAATCAGAG-3′

For test region in the partially methylated *RELN*, the same PCR primers, RC-F3 and RC-R3, and the *RELN* probe described above were used. Real-time PCR and analyses of methylation levels were also carried out as described above.

Transfection

The day before transfection, NTera2D1 cells treated with RA for 3 weeks were trypsinized, diluted with fresh medium without RA and antibiotics, and seeded into 24-well culture plates (approximately 0.5×10^5 cells/well). Cotransfection of partially methylated pRELN–Luc plasmid with phRL–TK plasmid (Promega) as a control was carried out using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Before cotransfection, the culture medium was replaced with 0.4 ml of OPTI-MEM I (Invitrogen), and to each well, 0.2 μg of

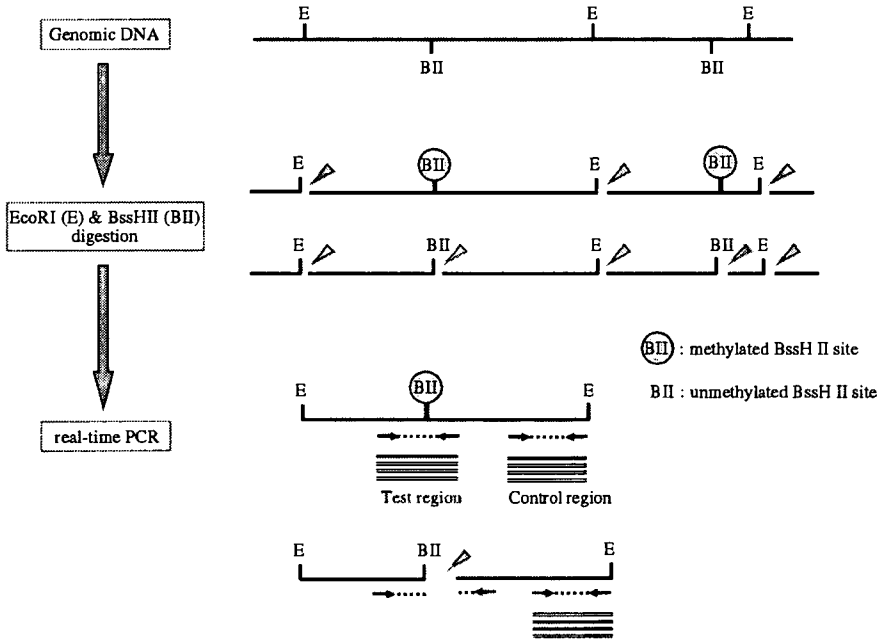

Figure 1 Outline of typing strategy for determination of DNA methylation status in the genome. Genomic DNA was digested with *Eco*RI and *BssH*II (methylation-sensitive restriction enzyme), and was subjected to real-time PCR such that a target region carrying *BssH*II site(s) of interest could be amplified. In addition, a region adjacent to the target region, where no *BssH*II sites were present, was also amplified and used as a control to normalize the amplified target region. When the *BssH*II site of interest in the target region was methylated, digestion of the site with the enzyme was inhibited, resulting in successful PCR amplification of the target region during real-time PCR. In contrast, when the *BssH*II site was not methylated, complete digestion of the site by the enzyme occurred, resulting in little or no PCR amplification. Accordingly, the level of DNA methylation at the *BssH*II site of interest in the target region is quantitatively reflected by the efficiency of real-time PCR amplification, and the observed amount of target region was subjected to normalization against that of the control region. A schematic representation is shown. *Eco*RI, unmethylated *BssH*II and methylated *BssH*II sites are indicated by 'E', 'BII', and 'BII', respectively. Arrow heads indicate restriction enzymes. PCR primers and amplified products are indicated by arrows and bars, respectively.

pRELN–Luc plasmid and 0.05 μg of phRL–TK plasmid were applied. Cells were incubated for 4 h at 37°C. After the 4-h incubation, 1 ml of the fresh culture medium without RA and antibiotics was added, and further incubation at 37°C was carried out. Forty-eight hours after transfection, cell lysate was prepared and expression levels of luciferase were examined by the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Results and discussion

Typing strategy for determination of DNA methylation status

Figure 1 shows our typing strategy (method) for determination of DNA methylation status in the genome. Briefly, genomic DNA is digested with a methylation-sensitive restriction enzyme, and is then subjected to real-time PCR such that a target region carrying methylation-sensitive restriction enzyme site of interest can be amplified. In addition, regions adjacent to the target region, where no restriction enzyme sites are present, are also amplified and used as controls for normalization of the amplified target region. Compared with the method with bisulfite-modification of genomic DNA, the present method appears to have little or no bias from insufficient chemical modification of genomic DNA and biased PCR amplification with such a modified DNA in determination of methylation levels of target genes (or regions). Furthermore, this method, unlike a conventional Southern blot analysis with methylation-sensitive restriction enzymes, does not need a large amount of genomic DNA. Although the present system for determination of methylation levels appears to have the benefits that are not in conventional methods, the system profoundly depends upon methylation-sensitive restriction enzymes, by which investigation is usually restricted to the restriction enzyme sites; accordingly, this may be a major drawback of this method.

By using the method we investigated the levels of DNA methylation in genomic DNA from schizophrenic, bipolar and healthy subjects to determine any associations between DNA methylation and psychotic disorders. The genomic DNA examined in this study was extracted from human postmortem forebrains of schizophrenic, bipolar and healthy individuals (35 samples each) (Table 1), and was kindly provided by the Stanley Medical Research Institute.

DNA methylation status in RELN

We examined the human *REELIN* (*RELN*) gene, as its putative promoter region (including exon 1) includes a GC-rich sequence containing several methylation-sensitive restriction enzyme sites,[5] and because possible associations between this gene and psychotic disorders have been reported.[10–14] The methylation status at the *BssH*II sites (GCGCGC) in the promoter region was investigated using the above-described

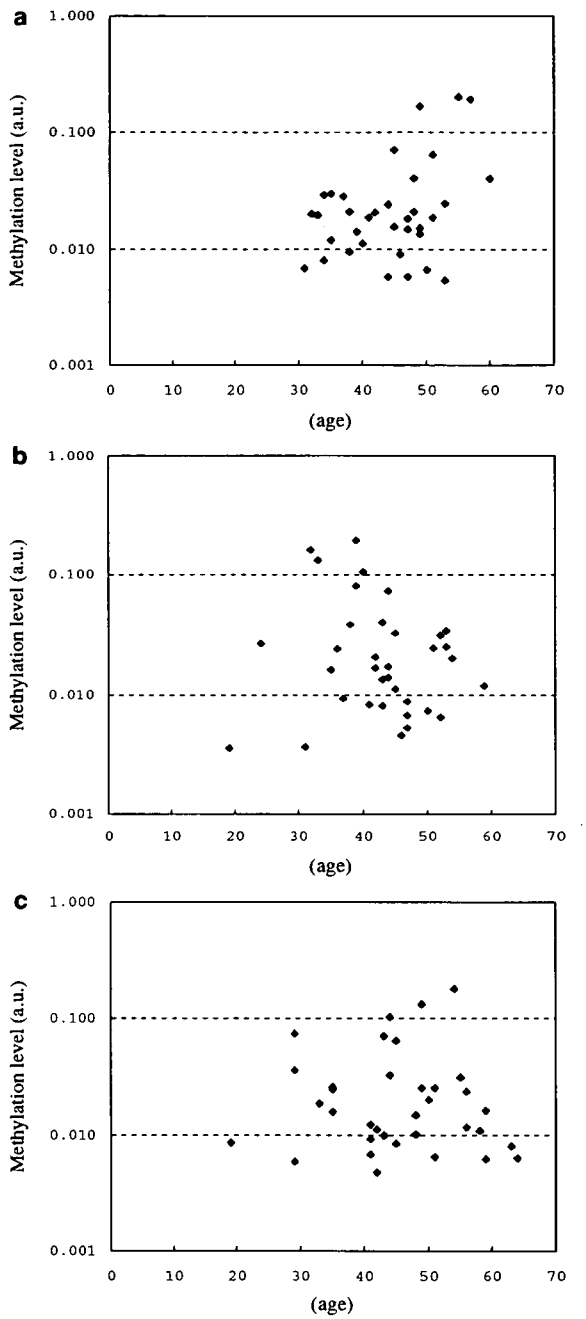

Figure 2 Scatter graphs of DNA methylation levels in *RELN* and age. All genomic DNA samples (35 samples each from healthy (**a**), schizophrenic (**b**) and bipolar (**c**) individuals) extracted from human postmortem forebrains were kindly provided by the Stanley Medical Research Institute. Relative levels of DNA methylation at *BssH*II sites in *RELN* were examined as described in Figure 1. Data regarding the levels of the DNA methylation are averages of three independent examinations and are given in arbitrary methylation units (a.u.). Tests for equal variance were carried out to determine whether there were any correlations between DNA methylation levels and aging.

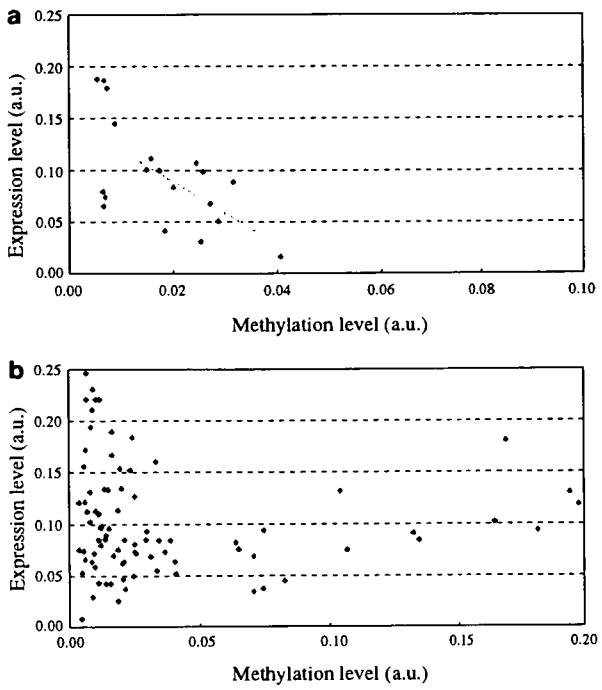

Figure 3 Relationship between DNA methylation levels and expression of *RELN*. Levels of expression of *RELN* and *18S rRNA* (control) among RNA samples extracted from the same postmortem forebrains as described in Figure 2 were examined by means of RT-PCR with TaqMan Gene Expression Assays (Applied Biosystems). Levels of expression of *RELN* were normalized against those of the control (18S rRNA). Data for DNA methylation and RNA expression are averages of three independent examinations and are given in arbitrary (methylation or expression) units (a.u.). Data for samples prepared within 18 h of death or beyond 18 h after death are shown in **a** and **b**, respectively. Tests for equal variance were performed to determine whether there were any correlations between the levels of DNA methylation and expression.

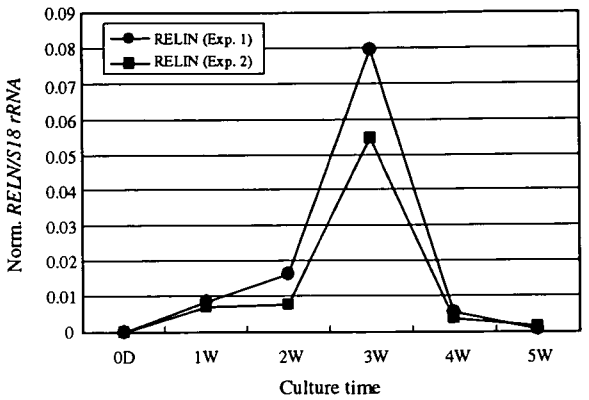

Figure 4 Expression profiles of *RELN* during neuronal differentiation of NTera2D1 cells. To investigate the endogenous expression of *RELN* during neuronal differentiation of NTera2D1 cells, expression profile analysis was carried out. Cells were treated with 10^{-5} M all-RA for 3 weeks and reseeded ($\sim 5 \times 10^4/cm^2$), and further culture was carried out in the absence of RA. Total RNA was extracted from cells before RA treatment (0 day: 0 D) and cells at the indicated time point after RA treatment (1 week ~ 5 weeks: 1 W ~ 5 W). Extracted RNAs were subjected to RT-PCR with TaqMan Gene Expression Assay as in Figure 3. Levels of *RELN* expression were normalized against those of *S18 rRNA*. Expression profile analysis was repeated twice independently (Exp.1 and Exp. 2). The results indicate that the expression of endogenous *RELN* is induced by RA treatment and that *RELN* expression peaks at around 3 weeks after RA treatment. Accordingly, we decided to use NTera2D1 cells treated with RA for 3 weeks host cells in the transient expression assay using partially methylated pRELN–Luc plasmids (Figure 5).

method. The results indicated that the relative levels of the DNA methylation varied among the schizophrenic, bipolar and healthy individuals. Because differences in the average levels of DNA methylation among the three groups did not reach statistical significance, further analyses stratified by age, gender, age at disease onset, life time alcohol and drug use and suicide status (Table 1) were carried out In this series of analyses, we identified an intriguing association; when DNA methylation levels and subject age were plotted in scatter graphs (Figure 2), a correlation between DNA methylation and age was seen in healthy individuals ($r = 0.436$, $P < 0.01$). It should be noted that no such correlation was seen in either schizophrenic or bipolar patients. In addition, it was observed that several patients under the age of 50 had higher levels of DNA methylation >0.03 (a.u.) at the level of DNA methylation shown in Figure 2: nine schizophrenia (25.7%) and seven bipolar (20.0%) cases met these criteria, but only three healthy individuals (8.6%) met these criteria. The difference in percentages between healthy individuals and either schizophrenic or bipolar patients, however, did not reach statistical significance owing to the small number of samples examined in this study. As for the other stratification analyses, we could not see their significant associations with the DNA methylation status in *RELN*.

Grayson *et al.* showed significant association of methylated cytosine at positions −139(CpApG) and −134(CpTpG) in *RELN* with schizophrenia by means of bisulfite-modification of genomic DNA followed by PCR amplification and sequence determination.[4] Our present study focused on the *BssH*II sites at positions +131, +227 and +229 in *RELN*. Therefore, different results between the previous and present studies may be attributable to the different positions examined.

Reverse correlation between the levels of DNA methylation and expression of the RELN *gene*

A previous study with completely methylated *RELN* promoter by CpG methylase (M.Sss I) suggested that DNA methylation of the *RELN* promoter *in vitro* decreased its promoter activity;[5] but correlations between DNA methylation levels in *RELN* and *RELN*

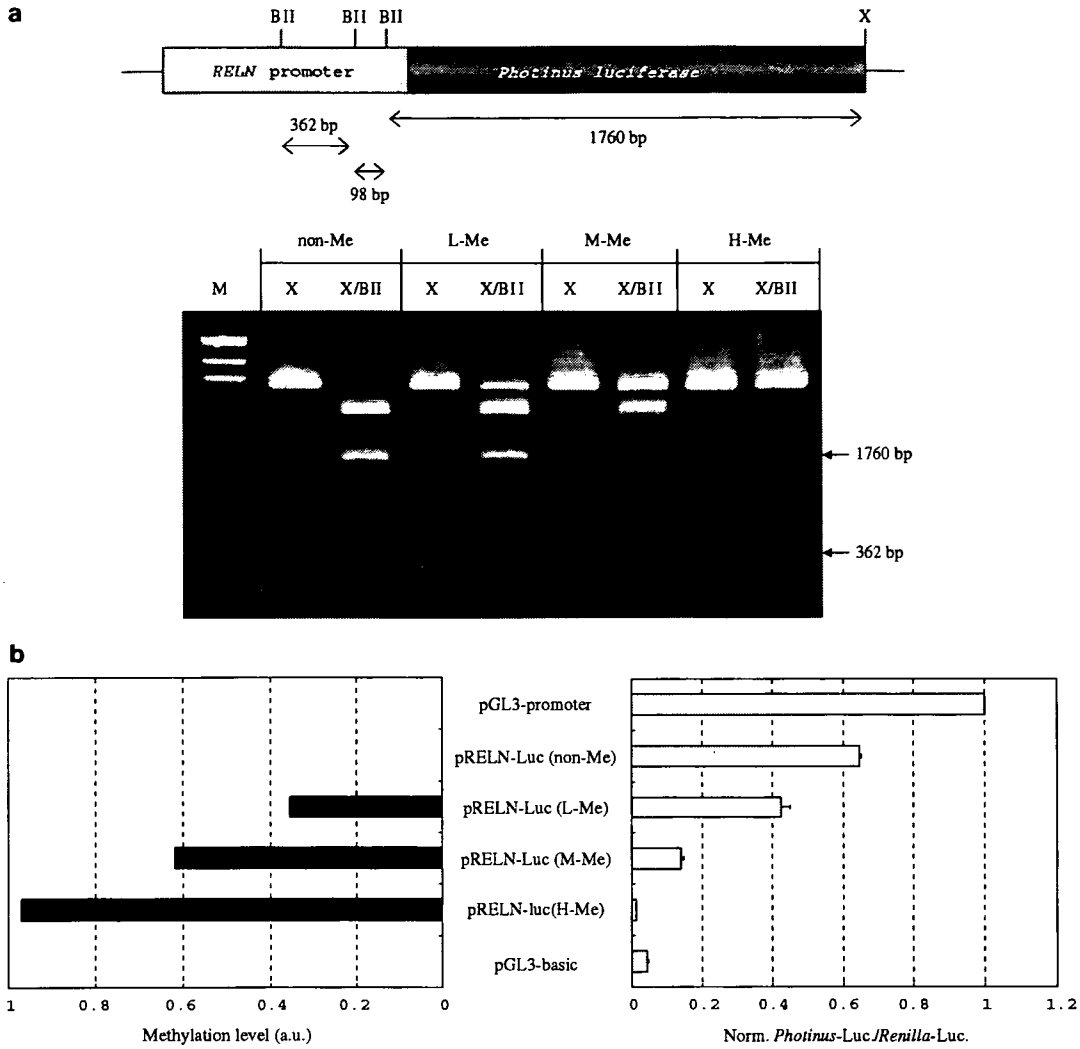

Figure 5 Reverse correlation between DNA methylation levels and transcriptional expression of *RELN*. (**a**) Digestion profiles of partially methylated plasmids with restriction enzymes. The pRELN–Luc plasmid carrying *Photinus luciferase* driven by the human *RELN* promoter was partially methylated using a CpG methylase (M. Sss I), after which the levels of DNA methylation were determined as in Figure 1. The resultant plasmids with various levels of DNA methylation, high (H-Me)-, moderate (M-Me)- and low (L-Me)-level methylated pRELN–Luc, were further confirmed by restriction enzyme digestion with *BssH* II (BII) and *Xba* I (X) followed by agarose gel electrophoresis. A schematic restriction enzyme map of pRELN–Luc is shown. Non-methylated plasmids and size markers (*Hind*III-cut λ DNA) are indicated by 'non-Me' and 'M', respectively. Note that the data also indicate that our typing method properly worked. (**b**) Reverse correlation between the levels of DNA methylation and expression of *RELN*. pRELN–Luc plasmids with various levels of DNA methylation were subjected to transfection together with phRL–TK encoding *Renilla luciferase* (control) into human teratocarcimona NTera2D1 cells treated with RA for 3 weeks, and in which endogenous *RELN* was being expressed (Figure 4). In addition, unmethylated SV40 promoter (in pGL3-Promoter) and no promoter (in pGL3-Basic) were also examined as positive and negative controls, respectively. Forty-eight hours after transfection, luciferase activities were examined. Test (*Photinus*) luciferase activity normalized against control (*Renilla*) luciferase activity and is given in arbitrary units (a.u.). Levels of DNA methylation are indicated as in Figure 2 and are given in arbitrary methylation units (a.u.).

expression remained unanswered. It is of interest and importance to determine whether there is any association between DNA methylation levels and expression in *RELN*. Total RNAs isolated from the same postmortem forebrains were also provided by the Stanley Medical Research Institute, and the expression levels of *RELN* and *18S rRNA* (control) were examined by means of RT-PCR. In the samples prepared within 18 h of death (eight schizophrenia and four bipolar cases, and seven healthy controls), a reverse correlation between DNA methylation and *RELN* expression was detected ($r = -0.63$, $P < 0.01$), but no such correlation was seen in the samples prepared beyond 18 h after death (Figure 3). These

observations suggest that the level of DNA methylation in *RELN* likely influences its expression; and the observations also suggest the possibility that the postmortem DNA and/or RNA might undergo substantial degradation within 18 h of death. To address the possibility, more extensive studies must be carried out.

To further evaluate the correlation between DNA methylation levels and expression in *RELN*, we constructed a reporter plasmid carrying the *RELN* promoter region linked to the *Photinus luciferase* gene. The reporter plasmid was subjected to partial methylation with CpG methylase (M.Sss I), after which the level of DNA methylation was determined using the above-described method. The resultant reporter plasmids with various levels of DNA methylation (Figure 5a) and phRL–TK carrying *Renilla luciferase* (control) were cotransfected into NTera2D1 cells where endogenous *RELN* was expressed (Figure 4), that is, in which transacting (transcription) factors necessary for the proper *RELN* expression could occur. After incubation, luciferase expression levels in the transfected cells were examined. As shown in Figure 5b, *Photinus luciferase* expression decreased with increased levels of DNA methylation in the *RELN* promoter; thus, the results indicate a reverse correlation between the levels of DNA methylation and expression of the *RELN* gene. Together with the results shown in Figure 3a, the data strongly suggest that the level of DNA methylation in *RELN* is significantly associated with its expression. The previous study using the *RELN* promoters which were completely methylated *in vitro* with various bacterial methylases suggested that DNA methylation participated in downregulation of *RELN* expression.[5] Therefore, it is most likely that DNA methylation in *RELN* is a key element functioning in regulation of the expression of *RELN*.

Our present data appear to be compatible with those of the previous studies using bisulfite-modification of genomic DNA,[4,6] both suggesting the aberrant DNA methylation status of *RELN* in psychiatric disorders. In addition, recent studies also indicated that the mouse *DNA methyltransferase 1* (*Dnmt1*) gene knockdown was accompanied by increased expression of *Reln*[15] and that protracted administration of L-methionine, a precursor of the methyl donor *S*-adenosyl-methionine in Dnmt1 catalytic activity, into mice led to decreased *Reln* expression,[7] suggesting possible inhibitions of the expression of *Reln* involving DNA methylation *in vivo*. Altogether, it is conceivable that *RELN* may undergo various levels of epigenetic modifications of DNA methylation, and that aberrant DNA methylation status in *RELN* leading to aberrant expression may confer susceptibility to psychiatric disorders.

Genetic and epigenetic factors conferring susceptibility to psychiatric disorders

The human *RELN* gene is mapped to chromosome 7q22, where few associations with human genetic diseases, except for a possible association with Finnish schizophrenia,[16] have been reported, whereas significant reductions in *RELN* transcripts and polypeptides in the brains of patients with psychiatric disorders have been repeatedly observed.[10–14] The present study indicates the possible association between epigenetic aberration in DNA methylation in *RELN* and psychiatric disorders; such aberration is probably undetectable using conventional genetic analyses. It is conceivable that even though genes were negative for associations with diseases on conventional genetic analysis, genes such as *RELN* may be involved in disease susceptibility through aberrant epigenetic modifications. Therefore, to identify the culprits conferring susceptibility to diseases, extensive studies focusing on both genetic factors and epigenetic factors are required.

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Prepulse inhibition of acoustic startle in Japanese patients with chronic schizophrenia

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Abstract

Prepulse inhibition (PPI) of acoustic startle reflex has been suggested as a neurophysiologic measure of information processing abnormalities in schizophrenia. However, there has been little information on PPI and related measures in Asian patients with schizophrenia. We examined startle response to acoustic stimuli, its habituation, and PPI in 20 Japanese patients with chronic schizophrenia under antipsychotic medication and 16 healthy controls matched for age and sex. We measured PPI with 115 dB of pulse (40 ms), 82, 86, or 90 dB of prepulse (20 ms) and 30, 60, or 120 ms of lead interval (LI). The startle response to pulse alone trials was significantly smaller in schizophrenics than in controls, which may be due, at least in part, to medication. There was no significant difference in habituation of startle response during the test session between the two groups. PPI differed significantly between the two groups when LI was 120 ms. No significant relationship was found on startle response or PPI with age of onset, number of previous admission, medication dosages, or symptom scores assessed with the Positive and Negative Syndrome Scale (PANSS). Our results confirm impaired PPI in chronic schizophrenia patients compared with controls in Japanese.
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Keywords: Acoustic startle response; Prepulse inhibition; Habituation; Schizophrenia; Japanese; Parameters

1. Introduction

Prepulse inhibition (PPI) of acoustic startle reflex has been suggested as a neurophysiologic measure of information processing abnormalities in schizophrenia (reviewed by Cadenhead and Braff, 1999; Braff et al., 2001a,b). This deficit of PPI may reflect a biological correlate of sensory flooding and cognitive fragmentation in individuals with schizophrenia. Furthermore, PPI shows substantial heritability (Anokhin et al., 2003), and it has been considered to be a reliable intermediate phenotype of sensorimotor gating deficits in schizophrenia that could be useful in genetic studies as well as diagnostic tests (Braff and Light, 2005). However, PPI is substantially dependent on measurement parameters such as sound pressure of prepulse and lead interval (LI) between pulse and prepulse (Blumenthal, 1999; Braff et al., 2001a,b). Moreover, a recent study has suggested ethnic differences in startle magnitude and PPI between Caucasian and Asian subjects (Swerdlow et al., 2005), indicating the possible importance of determining optimal test parameters in Asian subjects. To our knowledge, however, there has been little information on PPI and related measures from Asian populations, and no published data have been thus far available on whether PPI is impaired in Asian patients with schizophrenia.

The aims of the study were to examine startle response to acoustic stimuli, its habituation during the test session, and PPI

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in Japanese patients with chronic schizophrenia in comparison with healthy controls. We attempted to determine optimal parameters such as sound pressure of prepulse and LI in our sample. Furthermore, we examined the possible relationships of the deficits in PPI with clinical characteristics of the patients.

2. Subjects and methods

2.1. Subjects

Subjects were 20 patients with schizophrenia who were under treatment at the National Center of Neurology and Psychiatry Musashi Hospital, Tokyo, Japan. Consensus diagnosis according to the Diagnostic and Statistical Manual of Mental Disorders 4th ed. (DSM-IV; American Psychiatric Association, 1994) was made by at least two psychiatrists for each patient based on detailed interviews and medical records. All the patients were clinically stable on a stable dose of antipsychotic medication for at least 3 months prior to PPI test. Symptom severity of the patients was assessed with the Positive and Negative Syndrome Scale (PANSS) (Kay et al., 1987) by a single psychiatrist (H.K.) at the time of PPI test. Sixteen healthy volunteers served as controls. They were asked if they have had been to psychiatric or psychosomatic medicine clinic for any mental health problem. Individuals who had a current or past history of contact to such clinics or those who had a family history of psychosis (i.e., presence of individuals with current or past contact to psychiatric services for schizophrenia spectrum disorders, depressive disorder or bipolar disorder within the first degree relatives) were not enrolled in the study. The absence of current or past history of major psychiatric illnesses was further confirmed by using the structured interview of the Japanese version of the Mini-International Neuropsychiatric Interview (MINI, Otsubo et al., 2005; Sheehan et al., 1998). All the subjects had no difficulty in hearing, which was confirmed by an interview. Age and sex distributions were not significantly different between the patients and controls. All the patients and controls were biologically unrelated Japanese who resided in the same geographical area (western part of Tokyo metropolitan area). After description of the study, written informed consent was obtained from every subject. The study protocol was reviewed and approved by the ethics committee of the National Center of Neurology and Psychiatry, Japan.

2.2. Measurement of startle response and its prepulse inhibition

Startle reflex to acoustic stimuli was measured by using the Startle Reflex Test Unit for Humans (O'Hara Medical Co., Tokyo, Japan). The room for the measurement was completely sound-proofed and electrically shielded. Subjects refrained from smoking for at least 20 min prior to testing. They were seated comfortably in a couch. They were instructed to be awake and stare at a fixed point. Small electrodes (6 mm in diameter) with gel paste ("Gelaid", Nihon Kohden, Tokyo, Japan) were placed below both eyes over the orbicularis oculi muscle after polishing and cleaning skin surface with skin preparation gel for bioelectrical measurement ("skinPure" by Nihon Kohden, Tokyo, Japan) and 70% isopropyl alcohol for disinfection, and ground electrodes were placed behind ears over the mastoid. Broadband white noise (50–24,000 Hz) of 70 dB was presented as the background noise which was continuously presented afterwards throughout a session. Acoustic startle stimuli of the broadband white noise were presented through headphones. During the initial 5 min of each session, the background noise alone was given for acclimation. In total, 72 trials of startle response were carried out in a session. These trials consisted of three blocks. In the first block, startle response to pulse (sound pressure: 115 dB; duration: 40 ms) alone was recorded for six times. In the second block, startle response to the same pulse with or without prepulse (sound pressure: 82, 86, or 90 dB; duration: 20 ms; LI [onset to onset]: 30, 60, or 120 ms) was measured six times for each condition. The differential conditions of trials were presented in a pseudo-random order; however, the order was the same for all the subjects. In the final block, startle response to pulse alone was again measured for six times (to see habituation of response to pulse alone). Inter-trial intervals (15 s on average, range 10–20 s) were randomly changed. The entire session lasted approximately 30 min. The eye-blink component was measured using electromyographic (EMG) records. The system recorded 1052 epochs of EMG for 600 ms starting 200 ms prior to the onset of prepulse or pulse (for pulse alone trials). EMG activity was low (250 Hz) and high (90 Hz) pass filtered. Startle response was quantified as the peak of EMG waves, observed during 20–120 ms after the onset of pulse stimulus, which were rectified and smoothed by software using a moving average method with a time constant of 10 ms. All recordings were screened to exclude spontaneous eye-blink that was observed immediately before the acoustic stimuli. Eye-blinks observed in EMG during the period 200 ms before the index prepulse/pulse to 20 ms after the onset of pulse were considered to be spontaneous eye-blinks.

We obtained measures of (1) startle response to pulse alone trials in the first block, (2) habituation (%) of startle response during the session calculated by the formula ([1 − mean startle magnitude in block 3/mean startle magnitude in block 1] × 100), and (3) PPI (%) under the formula ([1 − mean startle response with prepulse trials)/mean startle response to pulse alone trials in the second block] × 100).

2.3. Statistical analysis

All the statistical analyses were performed with the SPSS ver11 (SPSS Japan, Tokyo, Japan). *t*-Test and chi-square tests (Fisher's exact test when appropriate) were used to compare means and categorical proportions, respectively. PPI measures with differential parameters were examined with ANOVA with repeated measures on trial parameters. Pearson's correlation was employed to see possible correlation between PPI and clinical characteristics. All *p*-values reported are two-tailed. Statistical significance was considered when *p*-value was < 0.05.

3. Results

Clinical and demographic characteristics of the subjects are presented in Table 1.

3.1. Startle response and habituation

Startle responses in the first block are illustrated in Fig. 1. Mean startle magnitude was significantly reduced in patients than in controls for both left ($t = -2.5$, d.f. = 34, $p = 0.019$) and right ($t = -3.7$, d.f. = 34, $p = 0.001$) sides (Fig. 1). We defined *a priori* the non-responders to the startle stimuli as the smallest 20 percentile in the total subjects; their average value of left and right startle magnitude was <0.05 (digital unit). Five patients and two controls were non-responders. There was no significant difference in any of the clinical characteristics listed in Table 1 between the 5 non-responders and 15 responders in the patient group. Analyses for habituation and PPI were performed in the responders (15 patients and 14 controls).

With respect to habituation of startle response, there was no significant difference between patients and controls for either left (70.0 ± 23.0% in patients and 65.9 ± 19.7% in controls; $t = 0.5$, d.f. = 27, $p = 0.61$) or right (64.2 ± 27.4% in patients and 65.4 ± 22.5% in controls; $t = -0.1$, d.f. = 27, $p = 0.90$) side.

3.2. Prepulse inhibition

PPI (%) measured in nine conditions (three sound pressures by three LIs) in patients and controls are presented in Fig. 2 (data on right PPI are not shown because left and right PPI were essentially similar). Right PPI measures of one patient were not well recorded for unknown reasons and thus excluded from the analysis. We examined the possible effects of side, LI, prepulse

Table 1
Characteristics of the study subjects (mean ± S.D.)

	Patients	Controls	Significance
Number of subjects	20	16	
Male/female	12/8	9/7	$\chi^2 = 0.1$, d.f. = 1, $p = 0.82$
Age (years) mean (range)	42 ± 9 (22–55)	41 ± 13 (20–72)	$t = 0.4$, d.f. = 34, $p = 0.72$
Current smoker/non-smoker	7/13	4/12	$\chi^2 = 0.4$, d.f. = 1, $p = 0.52$
Handedness right/left	20/0	15/1	$p = 0.44$[a]
Out-/inpatients	17/3	–	
Age of onset (years)	21 ± 6	–	
Number of hospitalization	2.0 ± 1.8		
Family history positive/negative[b]	5/15		
Number of medicated patients (%)		–	
Antipsychotics	20 (100%)		
Antiparkinsonian drugs	17 (85%)		
Anxiolytics/hypnotics	15 (75%)		
Medication dosage (mg/day)			
Antipsychotics[c]	852 ± 654		
Antiparkinsonian drugs[d]	3.0 ± 1.9		
Anxiolytics/hypnotics[e]	6.4 ± 5.4	–	
PANSS			
Total score	64.5 ± 16.0	–	
Positive syndrome	13.2 ± 7.6	–	
Negative syndrome	21.8 ± 7.1	–	
General psychopathology	29.6 ± 7.7	–	

[a] Fisher's exact probability.
[b] Positive family history: at least one relative with schizophrenia within the second degree relatives.
[c] Equivalent to chlorpromazine.
[d] Equivalent to biperiden.
[e] Equivalent to diazepam.

intensity (within-subjects factors), sex, smoking, and case–control status (between-subjects factors) on PPI, controlling for age as a covariate by using ANOVA with repeated measures on trial parameters. There was a highly significant effect of LI on PPI ($F = 6.6$, d.f. = 2, 40, $p = 0.003$); however, there was no significant effect of side ($F = 0.3$, d.f. = 1, 20, $p = 0.58$) or prepulse intensity ($F = 0.7$, d.f. = 2, 40, $p = 0.50$). In addition, there was a significant interaction between LI and case–control status ($F = 3.5$, d.f. = 2, 40, $p = 0.039$), suggesting significant differences in PPI between cases and controls depending on LI. No other significant interaction was detected. As shown in Fig. 2, PPI markedly differed depending on parameters, particularly on LI. When the LI was 30 ms, both patients and controls showed augmented startle response (i.e., facilitation), resulting in PPI values of both sides below zero. Although controls showed more facilitation than patients, any of differences did not reach statistical significance. Only when sound pressure was 82 dB, left response showed a statistical trend towards greater facilitation in controls than in patients. When the LI was 60 ms, there was no significant difference in PPI at any sound pressure of prepulse between patients and controls. When the LI was 120 ms, in contrast, all differences reached or approached statistical significance. When the sound pressure of prepulse was 90 dB, highly significant differences in PPI were observed for both left ($t = -2.8$, d.f. = 27, $p = 0.009$) and right ($t = -3.0$, d.f. = 26, $p = 0.006$) sides between patients and controls.

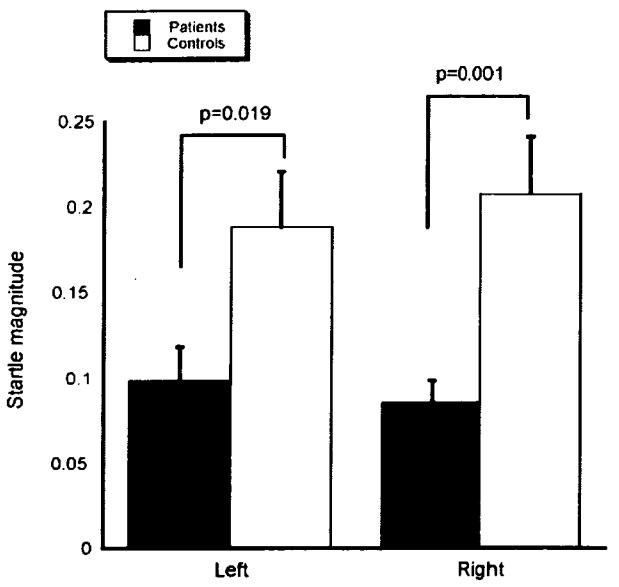

Fig. 1. Startle magnitude in patients and controls.

3.3. Relationship of startle response and PPI with demographic and clinical variables

We examined whether startle response in the first block and PPI had any relationship with demographic and clinical variables within the patients, excluding the non-responders. As

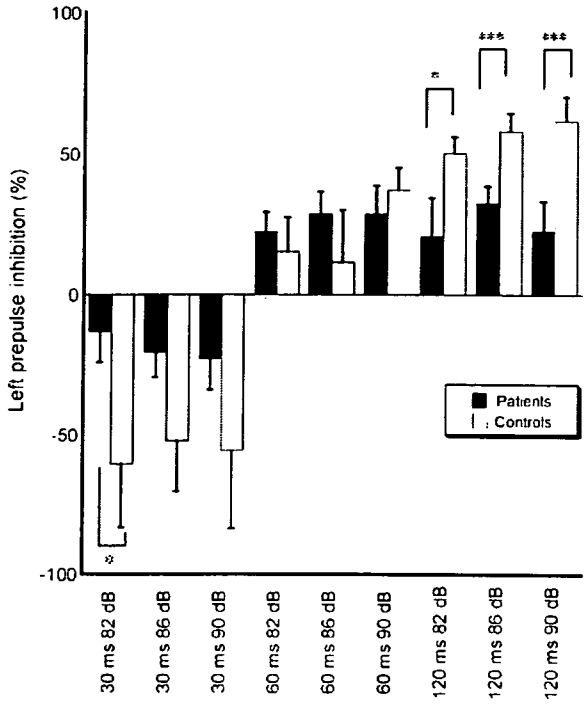

Fig. 2. Prepulse inhibition (PPI) in patients and controls. Records from the left side are shown. Error bars represent standard errors. $*p < 0.10$, $***p < 0.01$.

described above, the greatest statistical differences in PPI between patients and controls were obtained when LI was 120 ms and prepulse 90 dB. Thus PPI values with these parameters were used in the analysis. There was no significant correlation of startle response or PPI for either side with age, age of onset, number of previous admission, any of medication dosages, or any of PANSS scores.

4. Discussion

To our knowledge, the present study is the first report on PPI in Asian (Japanese) patients with schizophrenia. Our main findings were reduced startle response in the initial pulse alone trials and decreased PPI under certain conditions of test parameters in patients with schizophrenia, compared with controls. We found significant differences in PPI between the two groups when LI was 120 ms, but not 30 or 60 ms. With respect to habituation of startle response, no significant difference was found between the two groups in our sample. No significant relationship between clinical variables and PPI was detected in our patients. Although we recorded both left and right sides, there was no substantial difference between the sides for any measure.

4.1. Startle response

We observed substantially reduced startle response in patients with schizophrenia than in controls. In contrast to our finding, the majority of previous studies did not report such a difference in startle response in pulse alone trial (Braff et al., 1978, 1992, 1999, 2001a,b, 2005; Cadenhead et al., 2000; E.J. Duncan et al., 2003; E. Duncan et al., 2003; Ford et al., 1999; Geyer and Braff, 1982; Grillon et al., 1992; Kumari et al., 2002, 2004, 2005a,b; Leumann et al., 2002; Ludewig et al., 2002, 2003; Ludewig and Vollenweider, 2002; Mackeprang et al., 2002; Parwani et al., 2000; Perry et al., 2001, 2004; Swerdlow et al., 2006; Weike et al., 1999; Wynn et al., 2004), although some studies reported a significantly reduced startle magnitude in patients with schizophrenia (Quednow et al., 2006; Meincke et al., 2004). As pointed out by Meincke et al. (2004), it is likely that the observed lower startle response in our patients was attributable, at least in part, to medication. The majority of our patients (75%) received anxiolytics and/or hypnotics, most of which were benzodiazepines. This high rate of prescribing benzodiazepines is not unique to our patients; a relatively high proportion of patients with schizophrenia are co-prescribed with benzodiazepines in Japan, compared with other countries (Bitter et al., 2003). Benzodiazepines have been shown to reduce startle magnitude (Schachinger et al., 1999; Rodriguez-Fornells et al., 1999; Abduljawad et al., 2001). Although we failed to find a significant correlation between daily doses of such drugs (equivalent to diazepam) and startle response (data not shown), this failure is not surprising, given that the patients received differential drugs with differential effects and metabolism rate, and that time lag between drug intake and measurement of startle was not controlled for. Recently, Quednow et al. (2006) also found markedly and significantly reduced startle response in their patients with schizophrenia, compared with controls, at both pre- and post-treatment periods with antipsychotics of amisulpride or olanzapine. In their study, some benzodiazepines were allowed for adjunctive treatment; however, these substances were discontinued 24 h before measurement of startle response, indicating that there is a possibility that reduced startle response occurs in patients with schizophrenia even when effects of benzodiazepines are minimal. Quednow et al. (2006) stated that reduced startle response reflects the "hyporeactivity" in schizophrenia. Further studies controlling for medication status are required to draw any conclusion as to whether startle response at pulse alone trial is altered in schizophrenia.

4.2. Habituation

We failed to find a significant difference in habituation between the patients and controls, which is in line with the majority of previous studies (Braff et al., 2001a,b; Cadenhead et al., 2000; Kumari et al., 2002, 2004, 2005a,b; Leumann et al., 2002; Ludewig et al., 2002; Ludewig and Vollenweider, 2002; Mackeprang et al., 2002; Oranje et al., 2002; Perry et al., 2001, 2004; Swerdlow et al., 2006; Wynn et al., 2004). However, some other studies found reduced habituation in schizophrenia (Braff et al., 1992; Geyer and Braff, 1982; Ludewig et al., 2003; Parwani et al., 2000). Since the majority of the previous studies did not find altered habituation in schizophrenia, the difference in habituation between schizophrenics and controls might be, if any, small, and our sample size might have been too small to detect such a small difference (i.e., type II error).

4.3. Prepulse inhibition

PPI differed markedly depending on LI and intensity of prepulse. When LI was 30 ms, facilitation rather than inhibition of startle response was observed for both patients and controls with no significant difference between the two groups. The facilitated response was likely to result from summation of prepulse and pulse stimuli because of the very short LI. However, the majority of previous studies did not report such facilitated response even when LI was 30 ms (Braff et al., 1978, 1992, 2005; Cadenhead et al., 2000; E.J. Duncan et al., 2003; E. Duncan et al., 2003; Kumari et al., 1999, 2000, 2004, 2005a; Ludewig et al., 2002, 2003; Ludewig and Vollenweider, 2002; Leumann et al., 2002; Mackeprang et al., 2002; Meincke et al., 2004; Parwani et al., 2000; Perry et al., 2001, 2002, 2004; Swerdlow et al., 2005, 2006), although a few studies reported facilitated response only in patients with schizophrenia treated with typical antipsychotics (Kumari et al., 2002, 2005b). The discrepancy between previous studies and ours may be due to ethnic difference; however, this possibility was not supported by Swerdlow et al. (2005) who examined PPI in Asian healthy subjects. Thus there may be some unknown differences in the test procedures between previous studies and ours. We used the Startle Reflex Test Unit for Humans (O'Hara Medical Co., Tokyo, Japan) for recording startle responses. Although this apparatus has been made to be essentially similar to previously used "standard apparatuses (e.g., EMG-SR-LAB; San Diego Instruments, San Diego, California)", there may be some unknown differences between the former and the latter. To elucidate such differences, it is necessary to compare results obtained by the two apparatuses in the same subjects in the same test procedure.

We could not detect any difference in PPI between the patients and controls when LI was 30 or 60 ms; however, we found significant differences in PPI when LI was 120 ms. When LI was 120 ms and prepulse 90 dB, PPI values were highest and the difference between the patients and controls became most significant for both left and right sides, suggesting that the best condition for PPI among the examined conditions might be 120 ms of LI and 90 dB of prepulse in order to discriminate patients and controls in our sample. In the literature, LI values that could discriminate schizophrenics and controls differ across studies. Consistent with our result, many studies reported significantly lower PPI in schizophrenics or a subpopulation of schizophrenics compared with controls when LI was 120 ms (Braff et al., 1992, 2001a,b, 2005; Kumari et al., 1999, 2000; Mackeprang et al., 2002; Oranje et al., 2002; Parwani et al., 2000; Perry et al., 2001, 2002, 2004; Quednow et al., 2006; Weike et al., 1999), while others did not find significant differences (Braff et al., 1978, 1999; Cadenhead et al., 2000; E.J. Duncan et al., 2003; E. Duncan et al., 2003; Grillon et al., 1992; Kumari et al., 2002; Ludewig et al., 2002, 2003; Leumann et al., 2002; Swerdlow et al., 2006; Wynn et al., 2004). In several studies, 60 ms of LI was superior to 120 ms to detect differences between schizophrenia patients and controls (Braff et al., 1978; Kumari et al., 2002; Ludewig et al., 2002, 2003; Leumann et al., 2002; Swerdlow et al., 2006), while in other studies 120 ms was superior to 60 ms (Braff et al., 2005; Parwani et al., 2000). To our knowledge, there was only one study (Cadenhead et al., 2000) that reported 30 ms of LI was superior to other LI values. Taken together, although our results were in favor of 120 ms of LI to discriminate schizophrenics and controls, 60 ms of LI should also be used in the test session.

PPI has been reported to be associated with several clinical characteristics such as severity of positive (Braff et al., 1999; Weike et al., 1999) or negative (Braff et al., 1999) symptoms, thought disorder (Meincke et al., 2004; Perry and Braff, 1994), and age of onset (Kumari et al., 2000). In our sample, however, we could not detect any significant correlation between PPI and clinical variables. Since the present sample size was relatively small, further studies in a larger sample may be necessary to detect such relationships.

5. Conclusions

Our results suggest that startle response in the pulse alone trial was reduced in Japanese patients with schizophrenia compared with controls that may be due, at least in part, to medications of the patients. We confirmed that PPI was reduced in Japanese patients with chronic schizophrenia under stable medication when LI between pulse and prepulse was 120 ms. No apparent relationship was found between PPI and clinical characteristics.

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Failure to confirm an association between the *PLXNA2* gene and schizophrenia in a Japanese population

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Abstract

Plexins are receptors for multiple classes of semaphorins, either alone or in combination with neuropilins. Plexins participate in many cellular events that include axonal repulsion, axonal attraction, cell migration, axon pruning, and synaptic plasticity. *PLXNA2* maps to chromosome 1q32. Several linkage studies reported schizophrenia susceptibility loci in the 1q22–42 region. A recent study reported that intronic single nucleotide polymorphisms (SNPs) of *PLXNA2* were associated with schizophrenia in a European American population. We attempted to replicate this finding in a Japanese sample of 336 patients with schizophrenia and 304 controls. In addition, we examined 3 non-synonymous SNPs (Arg5Gln, GLn57Arg, and Ala267Thr) in *PLXNA2*. Genotyping was performed by the TaqMan allelic discrimination assay. There was no significant difference in genotype or allele distribution of either the 4 intronic SNPs or the 3 non-synonymous SNPs between patients and controls. Furthermore, haplotype-based analyses did not provide evidence for an association. These results suggest that *PLXNA2* may not play a major role in the development of schizophrenia in our Japanese sample.
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Keywords: Association; Haplotype; Plexin A2; Schizophrenia; Single nucleotide polymorphism (SNP)

1. Introduction

Plexin was originally identified as a neuronal cell surface molecule that has been identified in *Xenopus* (Ohta et al., 1995; Takagi et al., 1987). To date, plexins have been identified in both invertebrate and vertebrate (Fujii et al., 2002; Kameyama et al., 1996a,b; Maestrini et al., 1996; Suto et al., 2003; Tamagnone et al., 1999; Winberg et al., 1998). Plexins were shown to act as receptors for multiple classes of semaphorins either alone or in a complex together with neuropilins (reviewed in Yazdani and Terman, 2006). Plexin A families and neuropilins form a stable complex as functional receptors for class 3 semaphorins (Takahashi et al., 1999). For example, semaphorin 3A (Sema3A) binds to neuropilin-1 (Nrp1) and activates plexin A1 (Plxna1) or plexin A2 (Plxna2) to transduce a repulsive axon guidance signal (Takahashi and Strittmatter, 2001). Many studies of plexins have concentrated on its roles in nervous development (reviewed in Kruger et al., 2005; Waimey and Cheng, 2006; Halloran and Wolman, 2006). In addition to their roles in axon guidance, semaphorin–plexin signaling has been known to play important roles in axon pruning (Liu et al., 2005; Bagri et al., 2003). Moreover, recent studies suggest that some semaphorins and their receptors might be involved in modulation of synaptic structure (Godenschwege et al., 2002; Morita et al., 2006; Bouzioukh et al., 2006; Waimey and Cheng, 2006).

Growing evidence has suggested that schizophrenia has neurodevelopmental abnormalities that might occur early in life

Abbreviations: SNP; single nucleotide polymorphism; DSM-IV; 4th edition of the Diagnostic and Statistical Manual of Mental Disorders.

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