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APPENDIX

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.neuroscience.2007.08.034.

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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.

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.

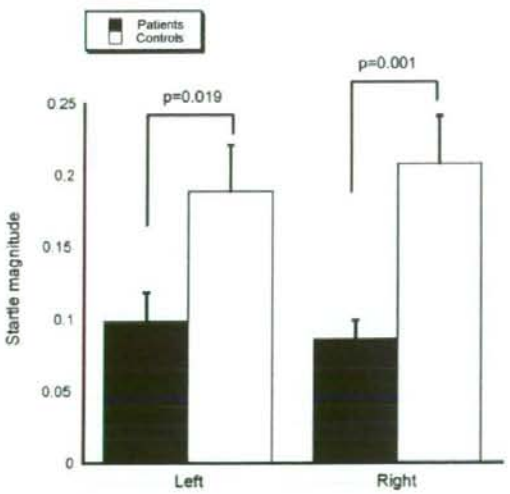

Fig. 1. Startle magnitude in patients and controls.

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

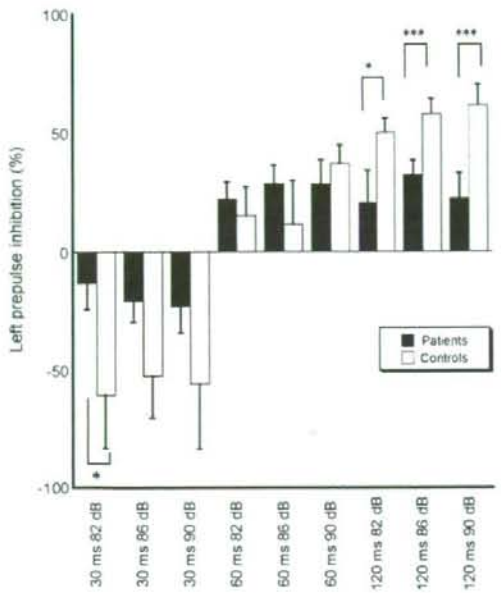

Fig. 2. Prepulse inhibition (PPI) in patients and controls. Records from the left side are shown. Error bars represent standard errors. $^{*}p < 0.10$, $^{***}p < 0.01$.

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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ORIGINAL ARTICLE

Pituitary adenylate cyclase-activating polypeptide is associated with schizophrenia

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

Introduction

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

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

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

Materials and methods

Subjects

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

Genetic analysis

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

Neuroimaging analysis

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

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

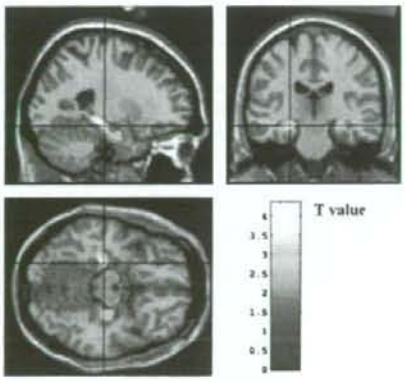
Statistical analyses were performed with SPM2, which implemented a general linear model. A hypothesis-driven regions of interest (ROIs) approach was used to investigate the hippocampus, using an ROI from the Wake Forest University PickAtlas.[17] Our hypothesis is that the PACAP genotype related to the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

Neurocognitive tests

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

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

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)	39.2 (10.5)	47.1(13.7)	34.6 (11.0)	37.3 (11.8)
Gender (M/F)	3/7	35/17	13/14	34/78
Education years (SD)	14.0 (2.6)	12.8 (3.3)	16.7 (2.8)	16.5 (3.1)
Full scale IQ (SD)	86.2 (21.5)	83.8 (18.8)	109.9 (13.4)	109.6 (11.6)
VPAI (SD)	13.3 (3.9)	9.0 (4.6)*	16.1 (2.7)	16.1 (2.3)

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

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

Animal study

All animal experiments were carried out in accordance with protocols approved by the Animal Research Committee of Osaka University and by the Ethics Review Committee for Animal Experimentation of the National Institute of Neuroscience. Generation of PACAP$^{-/-}$ mice by a gene targeting technique has been reported previously.[7] The null mutation was backcrossed onto the genetic background of Crlj:CD1 (Institute of Cancer Research) mice purchased from Charles River (Tokyo, Japan). All wild-type control mice and PACAP$^{-/-}$ mice (homozygous for the mutant PACAP gene) used in locomotor activity and PPI experiments were obtained from the intercross of heterozygous animals. C57BL/6J mice were purchased from Charles River and were allowed to acclimate in our animal facility for at least 5 days before initiation of experiments. Mice were housed in a temperature- (23 ± 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 ($30\times30\times30$ 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.

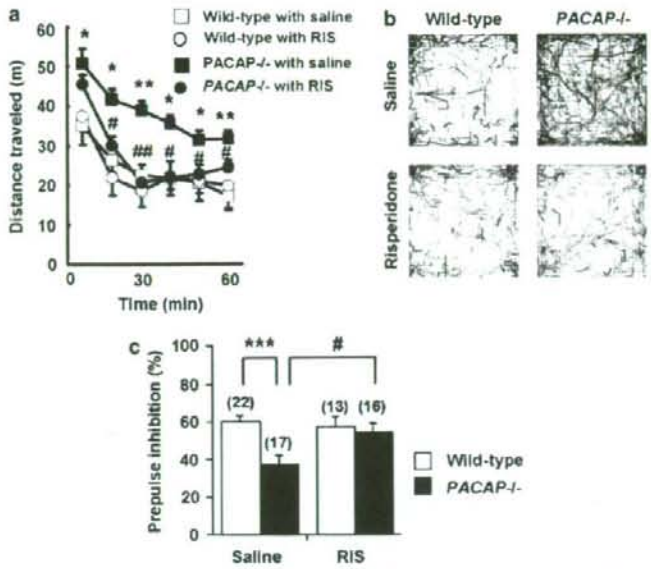


Figure 2 Hyperlocomotion and deficits in the PPI of PACAP$^{-/-}$ mice were normalized by risperidone treatment. (**a**) Locomotor activity in wild-type and PACAP$^{-/-}$ mice that received 0.1 mg/kg risperidone (RIS) or saline. $n = 6$ per group. (**b**) Representative locomotor patterns of saline- 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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Gene expression in the peripheral leukocytes and association analysis of PDLIM5 gene in schizophrenia

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Abstract

PDLIM5 modulates neuronal calcium signaling, co-localizes with synaptic vesicles of neurotransmitters and positive association between its gene and schizophrenia was reported but its relation is still ambiguous. The differential expression of the PDLIM5 gene both in the brain and in the lymphoblasts has been found in schizophrenia compared to control subjects. In this study, we measured the expression level of the PDLIM5 gene transcripts in the peripheral leukocytes from 19 medication-free and 21 chronically medicated schizophrenic patients as well as age- and sex-matched control subjects using a quantitative real-time PCR method. The mRNA levels of the PDLIM5 gene in the leukocytes of medication-free schizophrenic patients were significantly higher than those of control subjects. On the other hand, our group has previously shown that its mRNA expression in the leukocytes of medication-free major depressive patients was significantly lower compared with controls. There was no difference in the PDLIM5 mRNA levels between chronic schizophrenic patients with antipsychotic medication and their controls. Further, we failed to find any genetic association between the PDLIM5 gene and schizophrenia with six single nucleotide polymorphics (SNPs) of the PDLIM5 gene in Japanese subjects (279 subjects each) and there was no significant relation between PDLIM5 gene and schizophrenia with the haplotype analysis ($P = 0.48$), either. We suggest that the higher expression levels of the PDLIM5 mRNA in the peripheral leukocytes may be a candidate marker for medication-free schizophrenic patients.

Keywords: PDLIM5; Gene expression; Leukocytes; Association analysis; Schizophrenia

PDLIM5 is an intermediate protein that has been shown to regulate intracellular calcium levels by linking calcium channel and protein kinase C (PKC) [2,3,16]. PDLIM5 is ubiquitously expressed and its cellular localization in the brain is identical to Synapsin which is known to be involved in the neurotransmitter release [16]. The PDLIM5 gene lies on chromosome 4q22, a locus previously reported to be linked with schizophrenia [13,19]. While Kato et al. failed to find any association between the PDLIM5 gene and schizophrenia [15], Horiuchi et al. found a significant association between them [6]. It was reported that the expression level of PDLIM5 mRNA was significantly increased in the postmortem brain tissues of patients with schizophrenia, bipolar disorder and major depression, but was decreased in the immortalized lymphoblastoid cell lines derived from patients with schizophrenia and bipolar disorder [10,11]. Our group has recently shown that levels of mRNA expression in the peripheral leukocytes of the PDLIM5 gene were significantly lower in medication-free major depressive patients compared with controls [8].

The expressional alterations of genes in the peripheral blood lymphocytes and leukocytes have been reported to indicate the changes of the central nervous systems in schizophrenia and

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Table 1a
Demographic data for medication-free schizophrenic patients studied in PDLIM5 mRNA expression analysis ($N = 19$)

	Age (y.o)	Gender	Age at onset (years)	BPRS score	Family history of schizophrenia in first-degree relative
S1	25	M	22	64	+
S2	24	M	24	42	−
S3	24	M	24	31	−
S4	27	M	24	37	−
S5	36	M	36	34	−
S6	39	M	38	59	−
S7	27	M	26	58	−
S8	20	F	19	46	−
S9	23	F	23	48	−
S10	34	F	31	36	−
S11	47	F	47	30	−
S12	15	F	13	30	+
S13	26	F	21	100	−
S14	23	M	23	31	−
S15	28	M	25	63	−
S16	47	F	47	37	−
S17	37	F	21	36	−
S18	30	F	25	41	−
S19	45	F	43	36	+

The age (years old: y.o) represents the age of the subject when the leukocytes were drawn. M: male, F: female, '+' indicates that at least one of the first-degree relatives has schizophrenia.

major depressive disorder [7,8,9,17,21]. In this study, we measured the PDLIM5 mRNA expression levels in the peripheral leukocytes in unmedicated and medicated schizophrenic patients as well as in control subjects, using a quantitative real-time PCR method. In addition, we examined the genetic case-control study of the PDLIM5 gene with schizophrenia in Japanese subjects comprising of 279 patients with schizophrenia and 279 controls.

All patients and controls were biologically unrelated Japanese. The diagnosis of schizophrenia was made by at least two experienced psychiatrists according to DSM-IV criteria [1]. Clinical symptoms were evaluated by the Brief Psychiatric Rating Scale scores (BPRS) [20] when blood samples were taken. Age- and sex-matched controls were in good physical health without a history of any psychiatric or serious somatic diseases and taking any medication during the sample collection period. Probands who had first-degree relatives with psychiatric disorders were excluded from the control subjects.

For the measurement of expression levels of the PDLIM5 mRNA, the subjects consisted of 19 medication-free patients with schizophrenia (subject number S1–S19, Tables 1a and 1b) (14 first-episode and drug-naïve schizophrenic patients, 5 schizophrenic patients without antipsychotic treatment for at least 2 months; 9 males and 10 females, mean age: 30.4 ± 9.3), 19 age- and sex-matched controls (9 males and 10 females, mean age: 30.6 ± 8.6), 21 chronically treated patients with schizophrenia who were stably controlled under the same amount dosage of antipsychotics for at least 3 months (subject number S20–S40, Tables 2a and 2b) (13 males and 8 females, mean age: 47.7 ± 11.3) and 21 age- and sex-matched controls (mean age: 47.7 ± 11.1).

For the genetic association study, we used DNA samples from 279 in patients (189 male and 90 female; mean age: 51.3 ± 13.7 years) with schizophrenia from 13 psychiatric hospitals in the neighboring area of Tokushima Prefecture in Japan (population: about 820,000). Age- and sex-matched controls were selected from volunteers after assessing the psychiatric problems (189 male and 90 female; mean age: 51.4 ± 12.0) for the association and haplotype-based case-control study.

All subjects signed written informed consent to participate in the expression and genetic association studies approved by the institutional ethics committees.

Table 1b
PDLIM5 mRNA expression in medication-free schizophrenic ($N = 19$) and control subjects ($N = 19$)

	Male ($N = 9$)	Female ($N = 10$)	Total ($N = 19$)
Schizophrenia (S1–S19)			
Age	28.1 ± 5.6	32.4 ± 11.5	30.4 ± 9.3
The PDLIM5 mRNA expression before treatment	1.13 ± 0.3	1.29 ± 0.3	$1.21 \pm 0.3^{*}$
Control			
Age	27.6 ± 4.8	33.4 ± 10.4	30.6 ± 8.6
The PD1IM5 mRNA expression	0.95 ± 0.2	1.03 ± 0.4	1.00 ± 0.3

The mean PDLIM5 mRNA levels in the peripheral leukocytes from medication-free schizophrenia patients were significantly higher than those of age- and sex-matched controls (Mann–Whitney U test: $P = 0.023$); $^{*}P < 0.05$. No correlation between PDLIM5 mRNA levels and baseline BPRS scores were observed (Spearman's correlation efficient: $P = 0.38$).

Table 2a
Demographic data for chronic schizophrenic patients studied in PDLIM5 mRNA expression analysis ($N = 21$)

	Age (y.o)	Gender	Medication	BPRS Score
S20	57	M	QTP 75 mg, LP 150 mg, CP 300 mg	55
S21	56	M	Ris 6 mg	29
S22	56	M	Ris 5 mg, QTP 200 mg, sulpiride 150 mg	44
S23	60	M	Ris 8 mg, LP 20 mg	67
S24	57	M	HPD 9 mg, BPD 9 mg, propericyazine 60 mg	52
S25	40	M	Ris 12 mg	33
S26	46	M	Ris 6 mg, HPD 9 mg, sultopride 900 mg	49
S27	45	M	BPD 9 mg, clocaprarmine 75 mg	59
S28	31	M	BPD 2 mg, HPD 1 mg, LP 15 mg, Perospirone 24 mg	49
S29	49	F	Ris 6 mg, HPD 6 mg, CP 20 mg, HPD decanoate 150 mg	33
S30	53	F	HPD 2.25 mg, sulpride 150 mg	33
S31	65	F	HPD 4.5 mg, CP 37.5 mg	47
S32	51	F	Olz 10 mg	23
S33	43	F	Ris 6 mg, zotepine 50 mg	45
S34	54	F	Olz 20 mg, LP 50 mg	38
S35	54	M	Ris 12 mg, zotepine 150 mg timiperone 6 mg	42
Sc36	25	M	Ris 9 mg, perospirone 16 mg	39
Sc37	49	M	Ris 12 mg, LP 150 mg	54
Sc38	23	M	Ris 12 mg, LP 150 mg	38
Sc39	35	F	Olz 20 mg	33
Sc40	53	F	QTP 400 mg	27

The age (years old: y.o) represent the age of the subject when the leukocytes were drawn. M: male, F: female, Olz: olanzapine, Ris: risperidone, HPD: hapoperidol, BPD: bromperidol, LP: levom epromazine.

Total RNA was extracted from the peripheral leukocytes using the PAX gene Blood RNA kit (Qiagen, Tokyo, Japan) according to the manufacturer's recommendations. One microgram of total RNA was used for cDNA synthesis by QuantiTect Reverse Transcription Kit (Qiagen) after assessing RNA quality and quantity with NanoDrop (NanoDrop Technologies, DE, USA). Expression of the PDLIM5 gene transcript was quantified by real-time PCR with the TaqMan Gene Expression Assay (Applied Biosystems, CA, USA). Primers and probes (Hs00179051_m1) were purchased from Applied Biosystems as well as Horiuchi's group [6]. GAPDH gene expression was used as an internal control and measurement of threshold cycle (Ct) was performed in triplicate. Data were collected and analyzed with Sequence Detector Software version 2.1 (Applied Biosystems) and the standard curve method. Relative gene expression was calculated as the ratio of PDLIM5 to GAPDH gene and the mean of the three replicate measures was assigned to each individual. Almost all of blood samples were taken in the morning before lunch. The expression of the PDLIM5 mRNA was not changed among blood samples collected at several points during the day time or over several weeks in the same control subjects.

Genotyping was performed using commercially available TaqMan probes (C_2095059_10, C_16015055_20, C_3226622_10, C_16015313_10, C_1569781_10, C_11567561_10) with Applied Biosystems 7500 Fast Real Time PCR System according to the protocol recommended by the manufacturer (Applied Biosystems). We selected six single nucleotide polymorphic (SNP) markers for genotyping according to linkage disequilibrium (LD) and haplotype blocks in the PDLIM5 gene region [6]. Two SNPs (rs10008257, rs2433320) in the 5′-flanking region and four SNPs left in the genomic region are covered about 169-kb in the whole 214-kb of the PDLIM5 gene. The heterozygocities of four of these six SNPs, rs10008257, rs2433320, rs2433327 and rs2452600 in Japanese population are reported as 0.39, 0.18, 0.26 and

Table 2b
PDLIM5 mRNA expression in chronic treated schizophrenic ($N = 21$) and control subjects ($N = 21$)

	Male ($N = 13$)	Female ($N = 8$)	Total ($N = 21$)
Schizophirenia (S20–S40)			
Age	46.1 ± 12.7	50.4 ± 8.7	47.7 ± 11.3
The PDLIM5 mRNA expression	0.78 ± 0.2	0.93 ± 0.2	0.83 ± 0.2
Control			
Age	46.2 ± 12.3	50.1 ± 9.0	47.7 ± 11.1
The PDLIM5 mRNA expression	0.90 ± 0.3	1.14 ± 0.4	1.00 ± 0.3

The mean PDLIM5 mRNA levels in the peripheral leukocytes from schizophrenia patients who has been treated with antipsychotic drugs for many years were not different from controls' (Mann–Whitney U test: $P = 0.16$). No correlation between PDLIM5 mRNA levels and baseline BPRS scores were observed (Spearman's correlation efficient: $P = 0.82$).

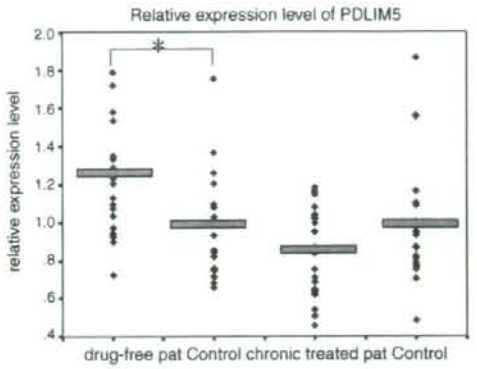

Fig. 1. Relative expression levels of PDLIM5 in the peripheral leukocytes in schizophrenic patients and control subjects. Compared with the normal control group, the mean PDLIM5 mRNA level in the leukocytes of medication-free schizophrenic patients ($N = 19$) was significantly higher (patients: 1.21 ± 0.29, controls: 1.00 ± 0.29, Mann–Whitney U test: $P = 0.023$). The mean PDLIM5 mRNA level in the leukocytes of chronic schizophrenic patients ($N = 21$) showed no significant difference compared with controls (patients: 0.83 ± 0.23, controls: 1.00 ± 0.32, Mann–Whitney U test: $P = 0.16$).

0.34, respectively. The heterozygocities of the other two SNPs, rs12641023 and rs14082, are not reported.

Statistical calculations were carried out using the SPSS Statistical Software Package 11.5 (SPSS, Tokyo, Japan). Expressional differences between patients and control subjects were calculated using the Mann–Whitney U test. Spearman correlation coefficients were used to evaluate the correlations between PDLIM5 mRNA levels and BPRS scores. Two-way ANOVA was performed to determine the independent and combined effects of age and the expression of PDLIM5 between groups. Allele and genotype frequencies of patients and control subjects were compared using Fisher's exact test. The SNPAlyze 3.2 Pro software (DYNACOM, Japan) was used to estimate haplotype frequencies, LD, and permutation P-values. Pair-wise linkage disequilibrium indices, D' and r2, were calculated in the control subjects. The criterion for significance was set at $P < 0.05$ for all tests. Data are presented as mean ± standard deviation.

Relative expression levels of PDLIM5 mRNA in 19 medication-free patients (S1–S19) were 1.21 ± 0.29 in the range of 0.73–1.79, while 1.00 ± 0.29 (range: 0.66–1.75) in healthy volunteers, showing a statistical difference (Mann–Whitney U test: $P = 0.023$, Fig. 1). Mean BPRS scores was 45.2 ± 17.4. No correlation between PDLIM5 mRNA levels and baseline BPRS scores were observed (Spearman's correlation efficient: $P = 0.38$). There was no significant expressional difference of PDLIM5 mRNA levels either between males and females or between genotypes of the single nucleotide polymorphism (rs2433320) both in patients with schizophrenia and in control subjects.

Relative PDLIM5 mRNA level was 0.83 ± 0.23 (0.46–1.18) in 21 chronically treated patients (S20–S40), while 1.00 ± 0.32 (0.49–1.87) in healthy volunteers, showing no significant statistical difference (Mann–Whitney U test: $P = 0.16$; Fig. 1). Mean chlorpromazine-equivalent doses were 932.1 ± 510.5 mg/day and mean duration of treatment was 23.5 ± 10.7 years and mean BPRS scores was 43.1 ± 10.8. No significant relationship between PDLIM5 mRNA levels and BPRS scores was observed (Spearman correlation efficient: $P = 0.71$). There was no significant expressional difference of PDLIM5 mRNA levels either between males and females or between genotypes of the single nucleotide polymorphism (rs2433320) both in patients with schizophrenia and in control subjects.

There were no significant deviations in all six SNPs from Hardy-Weinberg equilibrium in either patients or control subjects. Allele and genotype frequencies of the six SNPs are shown in Table 3. There were no associations between these SNPs and schizophrenia neither in the allelic frequency nor in the genotypic distributions. Although both rs2433320-rs2443327 and rs12641023-rs14082 were in a tight LD ($D' = 0.936$, 0.968, each), permutation test showed no significant difference in estimated frequencies of these haplotypes between the controls and patients (global permutation $P = 0.58$, 0.45, each, Table 4). Haplotypes of six SNPs were evaluated, but no significant difference was observed in frequencies of any estimated haplotype or in distributions of all estimated haplotypes between the controls and patients (global permutation $P = 0.48$).

The present study is the first report on the PDLIM5 gene expression in the peripheral leukocytes in schizophrenia. The mean PDLIM5 mRNA levels in the peripheral leukocytes from medication-free schizophrenia patients were significantly higher than those of age- and sex-matched controls. Altered mRNA expression in the peripheral lymphocytes could reflect the altered metabolism of brain cells [4]. Our result is consistent with the result of higher expression in the postmortem brains from schizophrenic patient but not with the result of lower expression in the lymphoblastoid cells derived from schizophrenic patients [10,11]. The differences of the mRNA expression between studies may be partly attributed to the difference in the materials. When using lymphoblastoid cells, the effect of virus infection or chromosomal alterations during culture must be taken into account [12]. On the other hand, the mRNA expression level of PDLIM5 gene was not significantly higher in chronically treated schizophrenics compared with that of controls. This finding in the chronic patients may be a consequence of pharmacological effects of antipsychotics or clinical improvement. This result suggests that expression of PDLIM5 mRNA may not be trait-oriented but state-related change. To confirm whether the expression of this gene is a state marker, a follow-up investigation is needed in the same patients before and after treatment.

The pathophysiological mechanism remains unknown, but we speculate that the higher expression of PDLIM5 is related with putatively elevated Ca^{2+} signaling in schizophrenia. It has been suggested that abnormalities in Ca^{2+} signaling was associated with molecular etiology of schizophrenia. Regulator of G protein signaling-4 (RGS4) and B-cell limphoma/leukaemia-2 gene (Bcl-2) which reduce free Ca^{2+} in a cell have been found to be down regulated in the temporal cortex of schizophrenic patients [14,18]. It was reported that there was high levels of free intracellular Ca^{2+} in platelets of schizophrenic patients

Table 3
Genetic studies of PDLIM5 gene with schizophrenia in case-control samples

Group	Genotype			n	Hardy-Weinberg equilibrium	P-value	Allele		P-value
ra1 0008257	A/A	A/G	G/G				A	G	
sch	42	127	105	274	0.823	0.471	211	337	0.804
cont	34	140	102	276	0.229		208	344	
rs2433320									
sch	7	75	197	279	0.858	0.601	89	469	0.871
cont	11	70	198	279	0.205		92	466	
rs2433327	T/T	T/C	C/C				T	C	0.833
sch	169	88	16	273	0.414	0.917	426	120	
cont	164	92	15	271	0.788		420	122	
rs2452600	T/T	T/C	C/C				T	C	
sch	54	125	96	275	0.306	0.232	233	317	0.080
cont	68	130	81	279	0.325		266	292	
rsl2641023	A/A	A/G	G/G				A	G	
sch	51	126	93	270	0.555	0.497	228	312	0.295
cont	42	131	103	276	0.924		215	337	
rs14082	A/A	A/G	G/G				A	G	
sch	58	124	91	273	0.243	0.302	240	306	0.141
cont	45	125	103	273	0.582		215	331	

sch: Schizophrenia, cont: control subjects. *P*-values are calculated by Fisher s exact test.

[22]. PDLIM5 regulates intracellular calcium levels by linking calcium channel and protein kinase C [2,3,16]. The levels of PDLIM5 might be up-regulated both in the brain and in the peripheral leukocytes in patients with schizophrenia in response to increased intracellular calcium levels. It has been demonstrated that antipsychotic drugs block IP3-induced release of Ca^{2+} [23] and Ca^{2+} dependence of PKC is well known [5]. So antipsychotic medication might normalize the up-regulation of PDLIM5 expression in schizophrenia by reducing Ca^{2+} signaling.

PDLIM5 may be involved in other mental disorders. Iwamoto et al. reported that expression level of PDLIM5 was significantly and commonly increased in the postmortem brain tissues of patients with schizophrenia, major depression and bipolar disorder [11]. However, we have already shown that mean PDLIM5 mRNA level in the peripheral leukocytes of medication-free patients with major depression was significantly lower than in control subjects [8]. Therefore, the higher expression of this gene in the peripheral leukocytes of medication-free patients with schizophrenia may be disease-specific and not due to non-specific stress of psychiatric condition. Further investigations of other psychiatric diseases including bipolar disorder are needed.

Table 4
Linkage disequilibrium (LD) indices (lower left are r2, upper right are *D'*)

	rs10008257	rs2433320	rs2443327	rs2452600	rs12641023	rs14082
rs10008257		0.37227	0.44147	0.28294	0.12734	0.15919
rs2433320	0.01632		0.9364	0.59209	0.37209	0.40839
rs2443327	0.03427	0.57218		0.54423	0.43945	0.45693
rs2452600	0.0447	0.05573	0.09541		0.19114	0.18089
rs12641023	0.00626	0.04284	0.08854	0.02152		0.99645
rs14082	0.01002	0.05068	0.09508	0.01918	0.93062	

Horiuchi et al. reported that there were significant association between polymorphisms (rs2433320 and rs2433322) of PDLIM5 gene and schizophrenia. Their group also showed that the different alleles of the rs2433320 showed different DNA–protein complexes on electrophoreic mobility shift assay and GA heterozygotic genotype might have higher transcriptional activity in schizophrenia [6]. However, our result showed that there was not significant association between schizophrenia and six polymorphisms of PDLIM5 gene, including rs2433320, and this result is consistent with a previous study with a large number of subjects ($n = 562$) [15]. In addition, neither patients nor controls showed a significant difference of the PDLIM5 mRNA expression in the peripheral leukocytes between GG and GA genotypes of this SNP in our subjects although the typeII error was not denied.

In conclusion, our investigation revealed that the mean PDLIM5 mRNA levels in medication-free schizophrenic patients were significantly higher compared to those in controls and the chronic schizophrenic patients with antipsychotic treatment for many years showed almost the same expression levels as healthy control levels. There were no associations between schizophrenia and PDLIM5 gene. These results suggest that the higher expression levels of PDLIM5 mRNA in the leukocytes may be a candidate marker for medication-free schizophrenic patients. Further studies are necessary to confirm the present results.

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