

Fig. 2. Comparison of HVA contents in the cortex (CX,  $n=5$ ), hippocampus (HIP,  $n=5$ ) and hypothalamus (HT,  $n=9$ ) of WT (open columns) and Sdy (closed columns) mice. HVA in the each sample was determined by HPLC–ECD. Each column represents the mean  $\pm$  S.E.M.

The DA contents were significantly reduced in three brain regions of Sdy mice compared to WT mice (Fig. 1); CX (WT:  $0.16 \pm 0.01$  nmol/g versus Sdy:  $0.09 \pm 0.01$  nmol/g,  $p=0.001$ ), HIP (WT:  $0.10 \pm 0.01$  nmol/g versus Sdy:  $0.06 \pm 0.00$  nmol/g,  $p=0.0001$ ), HT (WT:  $2.75 \pm 0.08$  nmol/g versus Sdy:  $2.38 \pm 0.04$  nmol/g,  $p=0.004$ ). In contrast, no significant difference was detected in HVA contents between Sdy mice and WT mice (Fig. 2). Western blot analysis revealed that there were no significant differences in the amount of TH in MB between both strains (Fig. 3). The ratio of HVA to DA, which is an index of DA turnover, was significantly higher in two brain regions of Sdy mice compared to WT mice (Fig. 4); in the CX (WT:  $1.28 \pm 0.02$  versus Sdy:  $1.96 \pm 0.11$ ,  $p=0.0002$ ), HIP (WT:  $1.57 \pm 0.22$  versus Sdy:  $2.82 \pm 0.29$ ,  $p=0.006$ ).

Schizophrenia is a highly complicated mental illness and the illness is complexly intertwined with many related factors. However, the mechanisms of schizophrenia such as the onset of this disease, susceptibility genes, and related neuronal systems have been gradually revealed. Therefore, studies using

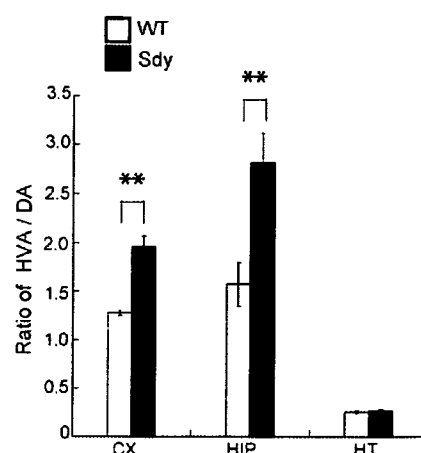


Fig. 4. Comparison of the ratios of HVA/DA in the cortex (CX,  $n=5$ ), hippocampus (HIP,  $n=5$ ) and hypothalamus (HT,  $n=9$ ) of WT (open columns) and Sdy (closed columns) mice. Each column represents the mean  $\pm$  S.E.M.  $**p < 0.01$  compared to WT mice ( $t$ -test).

Sdy mice would be able to throw light on the understanding of schizophrenia.

In the present study, DA contents in the CX and HIP of Sdy mice were significantly lower compared to WT mice ( $p=0.001$ ) but no difference was seen in TH in the MB, which contain the cell bodies of the dopaminergic neurons, between WT and Sdy. This finding demonstrates that the TH in MB may not contribute to the DA levels in CX and HVA of Sdy mice. No differences were observed in HVA contents in these areas, thus the DA turnover ratio was enhanced in these specific regions ( $p=0.0002$ ). These changes may be attributed to genetic absence of dysbindin in Sdy mice. However, there is a possibility that several genes are mutated and the results which were observed in the present study may be attributed to other gene mutations, not dysbindin. As the CX and HIP are thought to be the brain regions related to the onset of schizophrenia, these findings suggest that Sdy mice may be a useful animal model for the study of schizophrenic symptoms.

It is well known that the administration of phencyclidine (PCP), a non-competitive antagonist of the NMDA receptor, induces schizophrenia-like hyperlocomotion, and PCP-injected

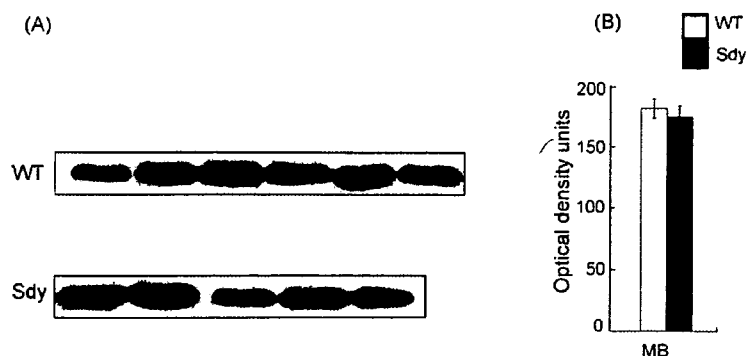


Fig. 3. (A) Western blot of TH in MB of WT ( $n=6$ ) and Sdy ( $n=5$ ) mice. (B) Quantitative data were obtained by densitometry of the band corresponding to TH. Data represent mean  $\pm$  S.E.M.

rodents are regarded as a model of schizophrenia [20]. In PCP-administered rodents, DA turnover is increased because extracellular DA as well as DA metabolite levels are upregulated, accompanied by the reduction of DA reuptake; the administration of PCP triggers a notable increase of the DA metabolite contents in the mesolimbic and mesocortical sites [5,20,28,33]. These findings correspond with the present data that DA turnover was increased in the CX and HIP of Sdy mice.

In the present study, the CX region was cut out with the limbic system including the amygdala. The amygdala is one of the main targets of the mesolimbic DA pathway and receives the dopaminergic input from the lateral VTA and medial half of the SN [8]. The D<sub>1</sub> and D<sub>2</sub> receptors are highly expressed in the amygdala [15], and systemic injections of D<sub>2</sub> agonists and antagonists modulate emotional state [22]. These findings indicate a potential function of the dopaminergic neurons in the amygdala to influence the emotional modulation mediated by D<sub>1</sub> and D<sub>2</sub> receptors.

Moreover, the amygdala is known to have a relationship with the deficit in processing of emotion in schizophrenia. Schizophrenic patients show functional abnormalities of the amygdala; the amygdala is not activated in response to emotional stimuli like sad, aversive, and threatening [26]. Experimental stimulations or lesions of the limbic system in humans induce several schizophrenic symptoms, for example, stimulation of the amygdala causes fear, confusion, automatism, and impairment in performing skilled acts [32]. In addition to these findings, the amygdaloid volume itself in schizophrenia patients were smaller compared to normal subjects [10].

The possibility that abnormality in the HIP may contribute to schizophrenia has been intensively explored. The HIP is a temporal lobe structure, which is vital for the encoding and recall of episodic memory. Postmortem [29] as well as magnetic resonance imaging studies [27] suggest a reduction of HIP volume in schizophrenia compared to normal subjects. The HIP receives dopaminergic innervation which comes from both the substantia nigra and VTA [25], and both D<sub>1</sub> and D<sub>2</sub> receptor mRNAs are found in this area [1,16]. The DA uptake site [17] and DA metabolizing enzyme catechol-*O*-methyl transferase (COMT) [14] are present in the HIP. In addition to the evidence, it is well known that the hippocampal DA enhances learning and memory functions [13] and the dopaminergic system affects long-term potentiation, a form of synaptic plasticity thought to encode long-term memory [13].

The low DA contents and the high DA turnover in the CX and HIP of Sdy mice in the present study suggest that the DA transmission in these areas is altered in the Sdy mice and in turn this abnormality of the DA system may contribute to the expression of schizophrenic symptoms. DA contents in the HT of Sdy mice were significantly low compared to WT mice, while no differences were seen in HVA contents and DA turnover. This may reflect the changes of the dopaminergic tone in the HT of Sdy mice. The hypothalamic DA neuron modulates proopiomelanocortin (POMC)-derived melanocortin peptides activity such as  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) [9]. The release of melanocortin peptides derived from POMC from glandular cells of the intermediate pituitary are under the inhibitory con-

trol of the dopaminergic systems of the HT [3,9]. Therefore, Sdy mice in which DA content was lower than WT mice in the HT may have alternation in their melanocortin peptides balance.  $\alpha$ -MSH is related to the parameters of learning, attention, memory, feeding behavior, body weight, organ weight, regulation of energy balance and body temperature, and variety of immunomodulating in several species [3,9,19,23], suggesting that these phenotypic patterns in Sdy mice may be altered.

The DA neurons originating from the tubero-infundibular tract in the HT also have important effects in the modulation of thyroid-releasing hormone (TSH) secretion in human and rats [35]. Because the reduction of the DA contents in the HT of Sdy mice may reflect alternation in the tubero-infundibular dopaminergic systems, the TSH secretion mechanism in the pituitary may be affected.

In conclusion, DA turnover is increased in the CX including the limbic system and the HIP of Sdy mice. The Sdy mouse is a useful candidate animal model for understanding the pathogenic mechanism of 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 polymorphisms (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.

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**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. Proband 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:  $30.4 \pm 9.3$ ), 19 age- and sex-matched controls (9 males and 10 females, mean age:  $30.6 \pm 8.6$ ), 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:  $47.7 \pm 11.3$ ) and 21 age- and sex-matched controls (mean age:  $47.7 \pm 11.1$ ).

For the genetic association study, we used DNA samples from 279 in patients (189 male and 90 female; mean age:  $51.3 \pm 13.7$  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:  $51.4 \pm 12.0$ ) 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   | $28.1 \pm 5.6$ | $32.4 \pm 11.5$ | $30.4 \pm 9.3$   |
| The PDLIM5 mRNA expression before treatment | $1.13 \pm 0.3$ | $1.29 \pm 0.3$  | $1.21 \pm 0.3^*$ |
| Control                                     |                |                 |                  |
| Age   | $27.6 \pm 4.8$ | $33.4 \pm 10.4$ | $30.6 \pm 8.6$   |
| The PDLIM5 mRNA expression                  | $0.95 \pm 0.2$ | $1.03 \pm 0.4$  | $1.00 \pm 0.3$   |

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 coefficient:  $P = 0.38$ ).

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<br>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, clozapramine 75 mg                       | 59         |
| S28  | 31        | M      | BPD 2 mg, HPD 1 mg, LP 15 mg<br>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, sulpiride 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 morn-

ing 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) |
|----------------------------|-------------|--------------|--------------|
| Schizophrenia (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 coefficient:  $P=0.82$ ).

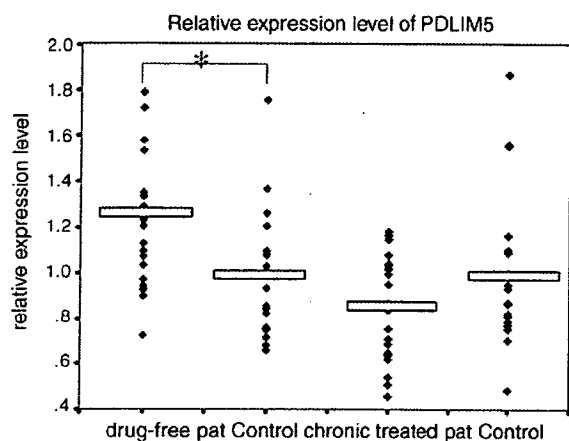


Fig. 1. Relative expression levels of PDLIM5 in the peripheral leukocytes in schizophrenic patients and control subjects. Compared with the normal control group, the mean PDLIM5 mRNA level in the leukocytes of medication-free schizophrenic patients ( $N=19$ ) was significantly higher (patients:  $1.21 \pm 0.29$ , controls:  $1.00 \pm 0.29$ , 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:  $0.83 \pm 0.23$ , controls:  $1.00 \pm 0.32$ , 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  $r^2$ , were calculated in the control subjects. The criterion for significance was set at  $P < 0.05$  for all tests. Data are presented as mean  $\pm$  standard deviation.

Relative expression levels of PDLIM5 mRNA in 19 medication-free patients (S1–S19) were  $1.21 \pm 0.29$  in the range of 0.73–1.79, while  $1.00 \pm 0.29$  (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  $45.2 \pm 17.4$ . No correlation between PDLIM5 mRNA levels and baseline BPRS scores were observed (Spearman's correlation coefficient:  $P=0.38$ ). 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  $0.83 \pm 0.23$  (0.46–1.18) in 21 chronically treated patients (S20–S40), while  $1.00 \pm 0.32$  (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  $932.1 \pm 510.5$  mg/day and mean duration of treatment was  $23.5 \pm 10.7$  years and mean BPRS scores was  $43.1 \pm 10.8$ . No significant relationship between PDLIM5 mRNA levels and BPRS scores was observed (Spearman correlation coefficient:  $P=0.71$ ). 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 lymphoma/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 |
|-------------|----------|-----|-----|-----|----------------------------|---------|--------|-----|---------|
| ral 0008257 | A/A      | A/G | G/G |     |                            |         | A      | G   |         |
|             | sch      | 42  | 127 | 105 | 274                        | 0.823   | 211    | 337 | 0.804   |
|             | cont     | 34  | 140 | 102 | 276                        | 0.229   | 208    | 344 |         |
| rs2433320   |          |     |     |     |                            |         |        |     |         |
|             | sch      | 7   | 75  | 197 | 279                        | 0.858   | 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   | 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   | 233    | 317 | 0.080   |
|             | cont     | 68  | 130 | 81  | 279                        | 0.325   | 266    | 292 |         |
| rs12641023  | A/A      | A/G | G/G |     |                            |         | A      | G   |         |
|             | sch      | 51  | 126 | 93  | 270                        | 0.555   | 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   | 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<sup>2+</sup> [23] and Ca<sup>2+</sup> dependence of PKC is well known [5]. So antipsychotic medication might normalize the up-regulation of PDLIM5 expression in schizophrenia by reducing Ca<sup>2+</sup> 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 r<sup>2</sup>, 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    | 0.50709   | 0.37209    | 0.40839 |
| rs2443327  | 0.03427    | 0.57719   |           | 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   |            | 0.98845 |
| 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 electrophoretic 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 type II 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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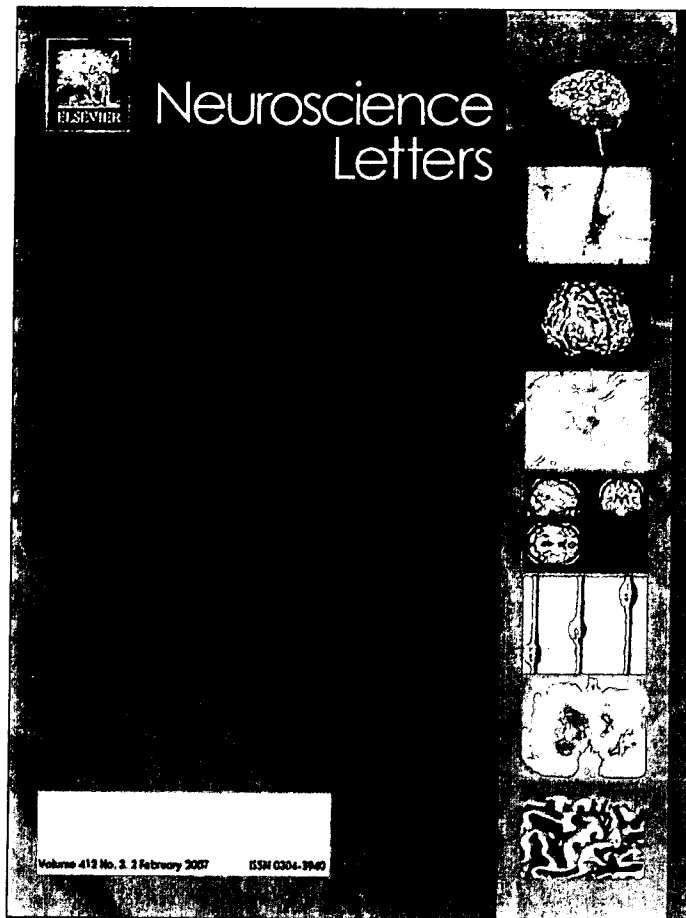


Akita Hospital, Fujii Hospital, Hosogi Unity Hospital, Jounan Hospital, Jousei Hospital, Kawauchi Hospital, Nankai hospital, Sea Gull hospital, Taoka East Hospital, Tokushima University Hospital, Tokushima Prefectural Central Hospital and Yu-ai Hospital). The authors would like to thank Mrs. Akemi Okada and Mrs. Kumiko Kikuchi for their technical assistance. This work was supported by a Health and Labor Science Research Grant from the Japanese Ministry of Health, Labor and Welfare, a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology and a Grant-in-Aid for Scientific Research from the 21st Century COE program, Human Nutritional Science on Stress Control, Tokushima, Japan.

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## A new gain-of-function allele in chimpanzee tryptophan hydroxylase 2 and the comparison of its enzyme activity with that in humans and rats

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### Abstract

Tryptophan hydroxylase 2 (TPH2) is a rate-limiting enzyme of neuronal serotonin biosynthesis. Recently, two single nucleotide polymorphisms (SNPs) at the exon 11 coding region that resulted in amino acid substitutions in the C-terminal domain have been reported to affect enzyme activity in humans and mice. We determined 175 base-pair sequences of the exon 11 region in nine primate species from all recognized lineages. All nucleotide sequence substitutions were synonymous, with the exception of one adenine (A) to guanine (G) substitution at the 1404th position in the open reading frame (ORF). This substitution leads to a glutamine (Q) to arginine (R) amino acid substitution at the 468th position within chimpanzee sequences. The frequency of the G allele was 0.24 among 66 chimpanzees. Therefore, it is a novel SNP observed in chimpanzees, and we have named these two alleles as *ch468Q* and *ch468R*, respectively. When expressed in HeLa cells, *ch468R* caused an approximate 20% increase in enzyme function during L-5-hydroxytryptophan (5HTP) production ( $P < 0.001$ ). We also surveyed the interspecies difference in enzyme activity among human, chimpanzee, and rat. Although the rat showed an identical amino acid sequence at the C-terminal region as those of human and *ch468Q*, the rat enzyme was more active than those of human or chimpanzee ( $P < 0.001$ ), indicating the importance of substitutions in other regions. Our findings on the chimpanzee SNP will be a useful genetic marker in understanding the individual difference in the serotonin-related behavior.

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**Keywords:** TPH2; SNP; 5HTP; Chimpanzee; Serotonin

Serotonin synthesis is mediated by the rate-limiting enzyme tryptophan hydroxylase (TPH), which catalyzes the pterin-dependent hydroxylation of L-tryptophan to L-5-hydroxytryptophan (5HTP). Additionally, this reaction is the first step in the synthesis of melatonin, which is involved in the regulation of mammalian reproduction and circadian rhythms [27]. The TPH belongs to the family of tetrahydrobiopterin-dependent aromatic amino acid hydroxylases, which includes phenylalanine hydroxylase (PAH) and tyrosine hydroxylase (TH) [14]. These enzymes

share similar protein organization composed of a regulatory N-terminal domain, a catalytic domain, and a short C-terminal oligomerization domain (Fig. 1a) [6–8]. Although the precise manner is often unique for each hydroxylase, they can be activated by similar mechanisms [4,9,10].

Recently, TPH isoform 2 was discovered to be preferentially located in brain dorsal raphe [23,28] and in the peripheral myenteric neurons in the gut [4]. The discovery of TPH2 has renewed great interest in studying the role of this enzyme in the neurochemical function of serotonin. To date, over 500 single nucleotide polymorphisms (SNPs) have been identified in the TPH2 gene in humans and mice, but coding non-synonymous SNPs are only six [3,33]. A number of recent studies have

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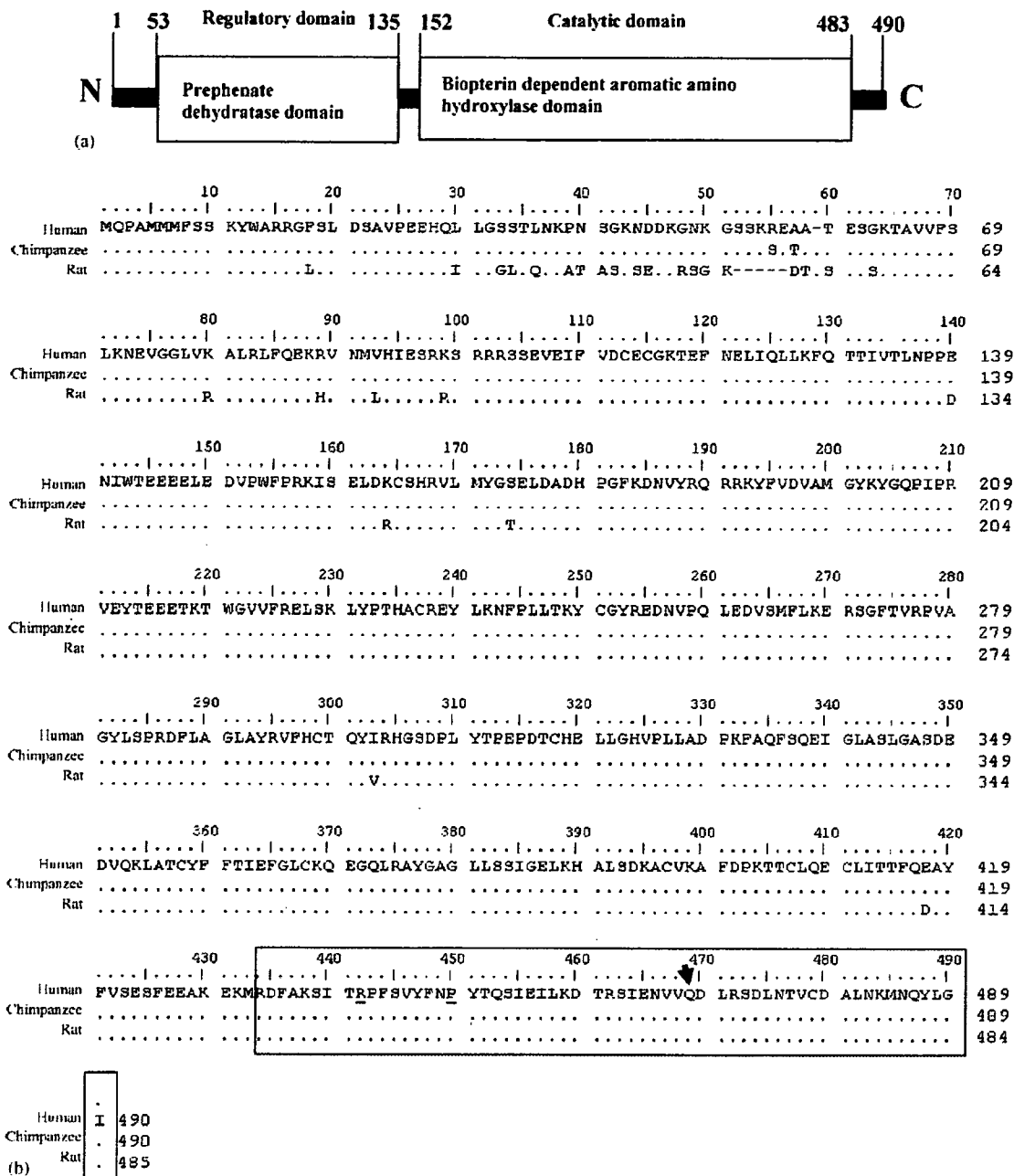


Fig. 1. The schematic TPH2 structure and amino acid sequence alignment. (a) The schematic TPH2 structure including two functional domains. The numbers indicate the amino acid position based on the human coding region. (b) The amino acid alignments of three mammals. The sequenced region (corresponding exon 11 coding region) of primate species is boxed and the chimpanzee-specific Q468R polymorphic site is indicated by an arrowhead. Polymorphic sites in humans (R441H) and mice (P447R) are underlined. Accession numbers of human, chimpanzee, and rat are NP\_775489, XP\_522470, and NP\_776211, respectively.

already reported the association between genetic polymorphisms and affective disorder and suicidal tendency [3,9,24,34]. Additionally, a number of interesting differences in the properties of TPH1 and TPH2 have been revealed, suggesting that these enzymes may have different catalytic control mechanisms or are differentially regulated at the post-translational level [19].

In human and mouse studies, SNPs in TPH2 have been suggested to alter enzyme function [31,32]. The human TPH2 contained an SNP that replaced a wild-type arginine with a histidine (R441H). Expression of the mutant TPH2 in PC12 cells

resulted in an approximate 80% decrease in serotonin levels as compared to the wild-type. R441H mutants were identified in unipolar major depression patients. In the mouse TPH2, a wild-type proline has been reported to be replaced by arginine (P447R mutant). Expression of mutant TPH2 revealed an approximate 55% decrease in serotonin levels as compared to wild-type. The mutant P447R homozygous inbred strains (BALB/c and DBA/2J) of mice showed 50–70% decrease in the rate of serotonin synthesis in the brain when compared to the wild-type homozygous strains (C57BL/6 and 129X1/SvJ). Interestingly,

the mice from these strains display significantly different aggressive behavior [16] and responses to antidepressants [2,18]. These results raised the possibility that similar mutations in nonhuman primates may affect their brain serotonin levels.

In general, the nucleotides and amino acids similarities among the TPH2 sequences of human, chimpanzee, and rat that were downloaded from GenBank database were >80% and >90%, respectively. The C-terminal region (highlighted by a box) was identical among the major allele of the species except for a proline to arginine replacement in mouse and for an arginine to histidine replacement in human (Fig. 1a and b).

The close genetic, physiological, and behavioral similarities between humans and nonhuman primates provide the basis for a comparative analysis of the genetic and environmental factors underlying both normative and pathological outcomes in behavioral development [1]. In this study, our primary purpose was to identify a new polymorphic loci in chimpanzees neurotransmitter related genes. Therefore, we sequenced the exon 11 coding region that corresponds to the polymorphic regions in humans and mice for several primate species.

Genomic DNA was extracted from the peripheral blood or buccal mucous membrane obtained from humans (healthy Japanese subjects,  $n=10$ ; informed consent was obtained in accordance with the guidelines of Gifu University), chimpanzees (*Pan troglodytes*,  $n=66$ ), gorillas (*Gorilla gorilla*,  $n=10$ ), orangutans (*Pongo pygmaeus*,  $n=10$ ), agile gibbons (*Hyllobates agilis*,  $n=5$ ), Japanese macaques (*Macaca fuscata*,  $n=4$ ), mandrill (*Papio sphinx*,  $n=1$ ), common marmosets (*Callithrix jacchus*,  $n=3$ ), tarsier (*Tarsius bancanus*,  $n=1$ ), and galago (*Galago crassicaudatus*,  $n=1$ ). Thus, all primate lineages were covered. Most of the nonhuman primate samples were obtained from the Primate Research Institute of Kyoto University and Sanwa Kagaku Kenkyusho Co. Ltd.

We determined the sequences in 10 individuals of chimpanzees and each one individual for the other species. PCR was performed for the amplification of a 295 bp sequence including 175 bp of the exon 11 coding region. Ten microliters of the reaction mixture containing 20 ng DNA, 0.5  $\mu\text{M}$  of each primer, 0.5 U *LA Taq* polymerase, GC buffer I (TaKaRa, Shiga, Japan), and 400  $\mu\text{M}$  of each dNTP was used. The primer sequences employed were 5'-TTCTGTTTATCTGCAGG-GACT-3' (TPH2F) and 5'-TTAGCCAAGCCATGACACAG-3' (TPH2R), corresponding to intron 10 and 3'UTR of the human TPH2 genomic sequence. After an initial incubation at 95 °C for 2 min, PCR amplification was performed for 35 cycles consisting of 95 °C for 30 s, 60 °C for 1 min and 74 °C for 2 min; this was followed by a final extension at 74 °C for 10 min. The PCR products were purified by using a PCR purification kit (Roche, Mannheim, Germany), followed by sequencing of both strands at least twice by using an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, CA) according to manufacturer's instructions.

The nucleotide sequences of the C-terminal domain (exon 11 coding region of TPH2) in the nine primate species are shown in DDBJ database with accession numbers AB68316–AB68324. A total of 23 nucleotide substitutions were observed. All substitutions were silent, except one substitution within chimpanzees.

An adenine (A) at the 1404th position was substituted by guanine (G), and this change caused a replacement of the 468th glutamine (CAG, *ch468Q*) by arginine (CGG, *ch468R*). Both chimpanzee sequences contained one substitution synonymous with that of human sequence at the 1468th position.

Genotyping for the chimpanzee SNP for remaining 56 individuals was performed by using a PCR-RFLP method. After amplification using TPH2F and TPH2R primers, 1  $\mu\text{l}$  of the PCR product was incubated at 37 °C overnight in the reaction mixture having a total volume of 10  $\mu\text{l}$ , containing 1  $\times$  NEBuffer 4 with 1.5 U of *HpyCH4V* (New England BioLabs, Beverly, MA). The products were subsequently separated by electrophoresis on a 2.0% agarose gel.

The allelic frequency of G allele among 66 chimpanzees was 0.24. The genotypic frequencies of G/G, A/A, and G/A were 0.091, 0.606, and 0.303, respectively. The presence of Hardy–Weinberg equilibrium was examined by using the Chi-square test for goodness of fit. The observed genotypic frequency distribution well accorded with the expectation under the Hardy–Weinberg equilibrium (Chi-square test,  $P=0.364$ ). To confirm the chimpanzee specificity of the SNP, we also conducted the SNP genotyping in each of the 10 humans, 10 gorillas, 10 orangutans, five agile gibbons, four Japanese macaques, and three common marmosets, but we could not detect the same substitution. If these species have the SNP site in the same frequency as chimpanzee, then it should be detected by the possibility of 99.9% in 10 individuals [15]. For confirmation, we need to survey larger sample size and diverse population.

For elucidating the functional effect of chimpanzee SNP, the human full-length ORF was amplified from the human brain hippocampus cDNA library (TaKaRa, Shiga, Japan) by using the primers 5'-CCCTGCTGCAGAGAAAGAAT-3' (TPH2Fm) and 5'-AGATCATGCTGGCAACAACA-3' (TPH2Rm), and was subsequently ligated into a human TPH2/TOPO TA cloning vector (Invitrogen, Carlsbad, CA). The chimpanzee amino acid sequence differed from that of the humans at only three positions. Of these, the two positions – C164A (R54S) and G170A (A56T) – were common in all the chimpanzees surveyed, but the A1404G (Q468R) substitution was polymorphic within chimpanzees. Therefore, two types of chimpanzee TPH2 ORFs were obtained by the mutagenesis of human ORF. The following are the oligonucleotides used to generate the *ch468Q* and *ch468R* (lowercase underlined letters represent new codons introduced by site-directed mutagenesis): 5'-AGCAAAagtGAAactGCTACCGAAAG-3' for R54S and A56T, and 5'-ATGTGGTCgcgGACCTTCGCAG-3' for Q468R.

The rat ORF was amplified by 5'-TCCCCGCGGTTCGA-AACCatgcagcccgcaatgatgat-3' (ratTPH2Fm) and 5'-GGAC-TAGTCTAGAtcaaatcccaaatattgttcatt-3' (ratTPH2Rm). The ratTPH2Fm included *Csp45I* restriction sites and a ribosome-binding sequence (ACC) flanking the 5' region of the TPH2 ORF, and the ratTPH2Rm included the *XbaI* restriction sites flanking the 3' region of the rat TPH2 ORF.

The human and chimpanzee inserts were obtained by digestion with *KpnI* and *NotI*, the recognition sites of which were located on the TOPO TA cloning vector, and the rat insert

was obtained by digestion with *Csp451* and *XbaI*. The inserts were then ligated with the Gateway® pENTR™11 entry vector (Invitrogen, Carlsbad, CA) digested with the same restriction enzymes. The inserts were transferred from the entry vector to the Gateway™ pDEST™ 12.2 destination vector (Invitrogen, Carlsbad, CA) by an LR reaction with Gateway® LR Clonase™ II Enzyme Mix (Invitrogen, Carlsbad, CA).

Transfection of pDEST12.2/TPH2 ORFs to HeLa cells was performed using jet PEI® (PolyPlus-Transfection, Illkirch, France) essentially according to the manufacturer's instructions. HeLa cells ( $2 \times 10^5$  cells/3.5 cm dish) were plated 28 h prior to the treatment. The cells were transfected with 1.7 µg DNA (1.5 µg of TPH2/pDEST12.2 and 0.2 µg of β-GAL/pCMV-SPORT) plus 3.4 µl jet PEI for 48 h.

After transfection of the expression vectors for 48 h, HeLa cells were collected and the TPH activity was determined essentially as described previously [11]. Cells in the monolayer culture were collected in PBS(-) "Ca/Mg-free PBS" and then subjected twice to freezing in liquid nitrogen and thawing on water. The disrupted cells were pre-incubated for 15 min at 30 °C in 0.1 M Tris-HCl (pH 8.0) containing 30 mM DTT, 50 µM Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>, and 4 mg/ml catalase in a total volume of 100 µl. Subsequently, 50 µl of another cocktail was added to yield a final reaction mixture of 250 µM tryptophan, 400 µM 6R-tetrahydrobiopterin, 500 µM NADH, 1 mM NSD-1015, 2 mg/ml catalase, and 50 µg/ml dihydropteridine reductase in 0.1 M K-phosphate buffer (pH 6.9). The enzyme reaction was allowed to proceed for 10 min at 30 °C and was then terminated by adding 1 M perchloric acid.

The 5HTP formed was measured using a high performance liquid chromatography (HPLC) system equipped with a fluorescence monitor (JASCO model, FP920) set at excitation and emission wave lengths of 302 nm and 350 nm, respectively. The solid phase was ODS (4.6 mm × 250 mm, JASCO, Finepak SIL-C18T5), the mobile phase was a 100:5:7 mixture of 40 mM sodium acetate (adjusted to pH 3.5 with formic acid): acetonitrile: methanol and the flow rate was 1 ml/min [10]. To correct the enzyme activity, we employed the β-galactosidase enzyme assay system (Promega, Madison, WI) according to manufacturer's instructions.

Statistical analysis of enzyme activity was performed with one-way ANOVA followed by Tukey post hoc comparison. All *P*-values reported were two tailed. Statistical significance was defined at *P* < 0.05.

We measured the TPH2 enzyme activity to estimate the production of L-5-hydroxytryptophan (5HTP) in *ch468Q*, *ch468R*, human, and rat. The results of the enzyme activity assay were  $49.40 \pm 0.45$  pmole/10 min/β-gal mU in *ch468Q*,  $64.36 \pm 1.67$  pmole/10 min/β-gal mU in *ch468R*,  $50.11 \pm 1.72$  pmole/10 min/β-gal mU in human, and  $65.32 \pm 0.99$  pmole/10 min/β-gal mU in rat (Fig. 2). The *ch468R* and rat isoform showed significantly higher activity than *ch468Q* and human isoform (ANOVA, *P* < 0.05).

The sequences of the exon 11 coding region were well conserved among human and nonhuman primate species, except one missense substitution at A1404G leading to Q468R in chimpanzees. The enzyme activity assay indicated that the capacity

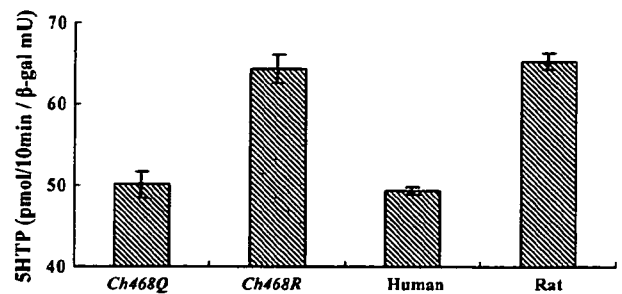


Fig. 2. Inter- and intra-specific comparisons of TPH2 activity. The TPH2 activity assay was conducted *in vitro* with disrupted cells of transfected HeLa cells and expressed as pmoles of 5-hydroxytryptophan (5HTP) per 10 min per β-gal mU. All data are presented as mean ± S.D.

of L-5-hydroxytryptophan biosynthesis was the same in *ch468Q* and human TPH2, whereas it was significantly high in the case of *ch468R*. If there were no significant changes in TPH2 expression levels, it could be a new gain-of-function allele. Although the molecular mechanism by which the *Q468R* mutation enhances catalytic function was not elucidated in the present study, replacement of the non-polar glutamine by the positively charged arginine could suggest the influence of the conformation of the catalytic site of the enzyme in some way [26].

This is the first comparative study on human and rat enzyme activity. In mice, *in vitro* 5HTP synthesis corresponded to their brain serotonin levels [31]. Therefore, although the difference in the activity was produced by an *in vitro* analysis, the high level of rat TPH2 activity probably implies the genetically higher serotonin concentration in serotonergic neurons of rodents than in human and nonhuman primates, if there were no significant differences in the amount of TPH2 protein.

TPH2, similar to all other monoamine-oxygenases, assembles into tetramers [29,30], and the tetramerization domain is present mostly within a 24-residue α-helix in the extreme C-terminal region of the enzymes [4,5]. The region of exon 11 is a part of the biopterin-dependent aromatic amino acid hydroxylase domain and also contains the tetramerization domain. The chimpanzee Q468R substitution is located on the tetramerization domain of the C-terminal. Therefore, the chimpanzee SNP may influence tetramer formation.

This result implies that low enzyme activity in humans and chimpanzees may result in low serotonin concentrations in the brain and may influence differences in the behavioral traits among chimpanzees as observed in mice [31,32]. Neurotransmitters such as dopamine and serotonin have been the prime target for understanding the biological basis of animal behaviors and interactions among animal groups. Primate social colonies are very sophisticated, and social interactions of chimpanzees are an interesting target for anthropologists. However, few genetic markers were reported for understanding primate behavioral traits [1,22].

In this study, we discovered a functional genetic marker for understanding the relationship between serotonin and chimpanzee behaviors including social dominance or aggression. The previous primate studies on the relationship between the serotonergic system and social dominance and aggression were

performed using vervet monkeys and rhesus macaques. Male vervet monkeys with a high rank within a group's social dominance hierarchy demonstrate elevated levels of serotonin in the blood and higher levels of the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) in the cerebrospinal fluid (CSF) [21]. When drugs that increase serotonin transmission were administered to an individual, they acquired a higher dominance status in the colony [25]. Conversely, low CSF 5-HIAA levels were associated with increased ratings of aggression and risk-taking behavior in adolescent male rhesus macaques [19,20]. It is unclear, however, how measurements of whole blood 5-HT or CSF levels of 5-HIAA that relate to the actual functional turnover of 5-HT in discrete regions of the brain are associated with social behavior [17]. Our results might present a potential molecular mechanism for the elevated biosynthesis of 5HTP function in chimpanzee dominance-related behavioral traits.

To elucidate the total neurochemical mechanism for serotonin function, we may also need to conduct a survey on the other serotonin-related genes. Among such genes, tandem repeat polymorphisms in the 5' promoter regions of serotonin transporter (5HTT) or monoamine oxidase A (MAOA) genes, which are associated with personality traits in humans, have been surveyed in chimpanzees and have shown less or low frequencies polymorphism [12,13]. Therefore, the TPH2 polymorphism reported in this study is particularly important in individual differences in the behaviors among chimpanzees. In future studies, detection of the levels of 5-HIAA in CSF in chimpanzees may facilitate the understanding total serotonin function in the brain. Also, we are searching polymorphic markers relating several serotonin receptor genes in chimpanzees.

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## Progressive changes of white matter integrity in schizophrenia revealed by diffusion tensor imaging

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### Abstract

Recent magnetic resonance imaging (MRI) studies using diffusion tensor imaging (DTI) have suggested reduced fractional anisotropy (FA) in the white matter (WM) of the brain in patients with schizophrenia. We tried to examine whether such reduction in FA exists and whether such changes in FA progress in an age-dependent manner in a Japanese sample of chronic schizophrenia. FA values were compared between 42 patients with chronic schizophrenia and 42 controls matched for age and gender, by using DTI with voxel-by-voxel and region-of-interest analyses. Correlations of FA values with age and duration of illness were examined. Patients with schizophrenia showed lower FA values, compared to controls, in the widespread WM areas including the uncinate fasciculi and cingulum bundles. A significant group-by-age interaction was found for FA in the WM, i.e., age-related reduction of FA was more pronounced in schizophrenics than in controls. A significant negative correlation between FA and duration of illness was also found in the WM. Our data confirmed decreased FA in schizophrenics, compared to controls in the widespread WM areas. Such decreased FA values in schizophrenia might be attributable, at least in part, to progressive changes after the onset of the illness.

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**Keywords:** Magnetic resonance imaging (MRI); DTI; Fractional anisotropy (FA); Aging

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## 1. Introduction

Diffusion tensor imaging (DTI) (Basser et al., 1994), a newly developed method to estimate the white matter (WM) integrity, provides information about the diffusion of water in biological tissues. In the WM, water diffusion is highly anisotropic, with greater diffusion in the direction parallel to axonal tracts. Thus, diminished anisotropy of water diffusion has been proposed to reflect compromised WM integrity (Lim et al., 1999). Fractional anisotropy (FA) (Basser, 1995) is a quantitative measure of diffusion anisotropy acquired from DTI.

The normally aging brain exhibits an assortment of micro- and macroscopic changes in the WM as well as the cerebral cortex. Histological studies demonstrate a decrease in myelin density and in the number of myelinated fibers (Meier-Ruge et al., 1992). Postmortem brain (Meier-Ruge et al., 1992) and volumetric neuroimaging studies (Christiansen et al., 1994; Salat et al., 1999) have suggested that WM changes are more prominent than cortical changes with aging, at least during certain segments of the age span and in certain regions of the brain. For example, volume loss in prefrontal WM is disproportionately greater than that in prefrontal cortex with late aging {comparison of elderly adults aged 60–75 with those aged >85 years (Salat et al., 1999)}. Several DTI studies have demonstrated age-related reductions of WM anisotropy in the genu of the corpus callosum (Pfefferbaum et al., 2000b), anterior WM (Pfefferbaum et al., 2000a; O'Sullivan et al., 2001), periventricular WM (Nusbaum et al., 2001), and the prefrontal WM (Nusbaum et al., 2001; Pfefferbaum et al., 2005; Salat et al., 2005).

Regarding schizophrenia, impairments of the neural connectivity between certain cortical areas, such as frontal and temporal areas, have been implicated in the pathophysiology of the disease (Frith and Dolan, 1996; Andreasen et al., 1997; Bullmore et al., 1997). Indeed, volumetric magnetic resonance (MR) studies and pathological studies demonstrated abnormalities of the WM in schizophrenia (Miyakawa et al., 1972; Cannon et al., 1998; Davis et al., 2003; Ho et al., 2003; Uranova et al., 2004). Changes in WM integrity in schizophrenia has relevance to the neural disconnection model of the disorder and may provide a basis for focal abnormalities as well. Several previous DTI studies in chronic schizophrenia showed decrease of FA in schizophrenics mainly in the front-temporal white matter and corpus callosum (Buchsbaum et al., 1998; Lim et al., 1999; Agartz et al., 2001; Burns et al., 2003). Furthermore, FA decrease in patients with first

episode schizophrenia might be less pronounced compared to chronic patients (Price et al., 2005; Szeszko et al., 2005), suggesting that the decreased FA in schizophrenics might be attributed, at least in part, to progressive and exaggerated age-dependent changes in schizophrenics rather than neurodevelopmental abnormalities in the WM. To date, there is only one cross-sectional study with a small sample size investigating age-related FA changes in schizophrenia that demonstrated an age-related FA increase in schizophrenics (Jones et al., 2006).

The present study was aimed to examine whether patients with chronic schizophrenia do have reduced FA values compared to controls and whether such changes in FA progress in an age-dependent manner.

## 2. Methods

### 2.1. Subjects

Table 1 shows the characteristics of participants of this study. Forty-two patients with chronic schizophrenia were recruited at the National Center of Neurology and Psychiatry, Tokyo, Japan. Consensus diagnosis was made for each patient by at least two trained psychiatrists according to the DSM-IV criteria (American Psychiatric Association, 1994), based on all available information, including clinical interviews, medical records and other research assessments. All patients were stable and/or partially remitted and had been taking antipsychotic medication at the time of MR measurement and neuropsychological tests. Forty-two healthy volunteers who had no current or past contact to any psychiatric services served as controls. All the subjects were biologically unrelated Japanese. After description of the study, written informed consent was obtained from every subject. The study protocol was approved by the ethics committee of the National Center of Neurology and Psychiatry, Tokyo, Japan. Exclusion criteria for all the participants included asymptomatic or symptomatic cerebral infarctions detected by T2 weighted MRI, serious neurological or endocrine disorder, any medical condition that could potentially affect the central nervous system, or mental retardation according to DSM-IV criteria.

### 2.2. Image acquisition

MR studies were performed on a 1.5 tesla Magnetom Vision Plus system (Siemens, Erlangen, Germany). Axial DTI scans aligned to the plane containing anterior and posterior commissures were acquired with a pulsed-

Table 1  
Characteristics of participants

|  | Controls     | Schizophrenics | Two-sample     |                             |
|--|--------------|----------------|----------------|-----------------------------|
|  |              |                | <i>t</i> -test | (Two-tailed; <i>df</i> =82) |
|  |              |                | <i>t</i>       | <i>P</i>                    |
| Number of subjects   | 42           | 42             |                |                             |
| Gender (male/female)   | 26/16        | 26/16          |                |                             |
| Handedness (right/left)  | 41/1         | 41/1           |                |                             |
| Age (years)  | 39.2 (9.0)   | 40.0 (9.3)     | -0.42          | 0.68                        |
| Range of age (years)   | 22–59        | 22–59          |                |                             |
| Education (years)  | 17.1 (3.5)   | 13.0 (2.9)     | 8.1            | <0.001                      |
| Full-scale IQ (WAIS-R)   | 114.3 (11.6) | 86.0 (21.3)    | 6.0            | <0.001                      |
| Age of onset   |              | 23.3 (7.0)     |                |                             |
| Duration of illness (years)  |              | 16.8 (9.0)     |                |                             |
| Duration of hospitalization (months)                                     |              | 31.2 (61.3)    |                |                             |
| Dose of total antipsychotic drugs (mg/day, chlorpromazine equivalent)    |              | 1005.1 (735.3) |                |                             |
| Dose of typical antipsychotic drugs (mg/day, chlorpromazine equivalent)  |              | 694.8 (748.3)  |                |                             |
| Dose of atypical antipsychotic drugs (mg/day, chlorpromazine equivalent) |              | 310.3 (464.2)  |                |                             |

Mean (S.D.).

WAIS-R: Wechsler Adult Intelligence Scale-Revised.

gradient, spin-echo, single-shot echo planar imaging (EPI) sequence (TR/TE=4000/100 ms; acquisition matrix, 256 × 256; NEX=4, FOV 240 mm;  $b=1000$  s/mm<sup>2</sup>; 20 slices, slice thickness 5 mm, gap 1.5 mm). Diffusion was measured along six non-collinear directions. For each of six gradient directions, four acquisitions were averaged. Four acquisitions without diffusion weighting ( $b=0$ ) were also averaged. Additionally, a three dimensional volumetric acquisition of a T1-weighted gradient echo sequence with a gapless series of thin sagittal sections using an MPRage sequence (TR/

TE=11.4/4.4 ms; flip angle, 15 degree; acquisition matrix, 256 × 256; NEX=1, FOV 315 mm; slice thickness 1.23 mm) was acquired for evaluating the volume of grey matter (GM), WM and cerebrospinal fluid (CSF) space.

### 2.3. Image processing

FA images for each subject were computed from seven diffusion images acquired as above by an in-house script on Matlab 6.5 software (Mathworks, Inc., MA, USA). Then, the FA images were spatially-normalized using high-dimensional-warping algorithm (Ashburner et al., 1999) and were matched to the FA template image. To make the FA template image, we warped FA images of 4 normal subjects (other than 42 control subjects) to the single-subject T1 template (skull stripped image) using spatial normalization function of SPM2 and averaged the 4 warped FA images. The transformed FA images were smoothed with a Gaussian kernel. The filter size (full-width at half-maximum: FWHM) was varied from zero to 16 mm in steps of 2 mm to validate the consistency of results of SPM analyses, because a previous study (Jones et al., 2005) reported that the statistical results of SPM analyses were differed depending on filter size. For measuring the volume of GM, WM and CSF space, an additional function of an optimized VBM script (<http://dbm.neuro.uni-jena.de/vbm>) was used (Good et al., 2001).

### 2.4. Statistical analysis

#### 2.4.1. Voxel-by-voxel analysis

The resultant FA images were analyzed using statistical parametric mapping with the framework of the General Linear Model in SPM2 (Wellcome Department of Cognitive Neurology, London, UK) (Friston et al., 1995). We constituted following three

Table 2

The relationship between smoothing kernel sizes (FWHM) and number of resels in our sample

| FWHM (mm)    | Number of resels |
|--------------|------------------|
| None         | 12460.4          |
| 2 × 2 × 2    | 5131.1           |
| 4 × 4 × 4    | 1720.2           |
| 6 × 6 × 6    | 706.0            |
| 8 × 8 × 8    | 289.4            |
| 10 × 10 × 10 | 119.7            |
| 12 × 12 × 12 | 52.1             |
| 14 × 14 × 14 | 24.4             |
| 16 × 16 × 16 | 12.4             |

statistical analyses: 1) a two-sample *t*-test for estimating group differences (controls versus schizophrenics), 2) a correlational analysis between age and FA values in both

controls and the schizophrenics and 3) a correlational analysis of FA values with duration of illness, age of onset, duration of hospitalization, and daily dose of

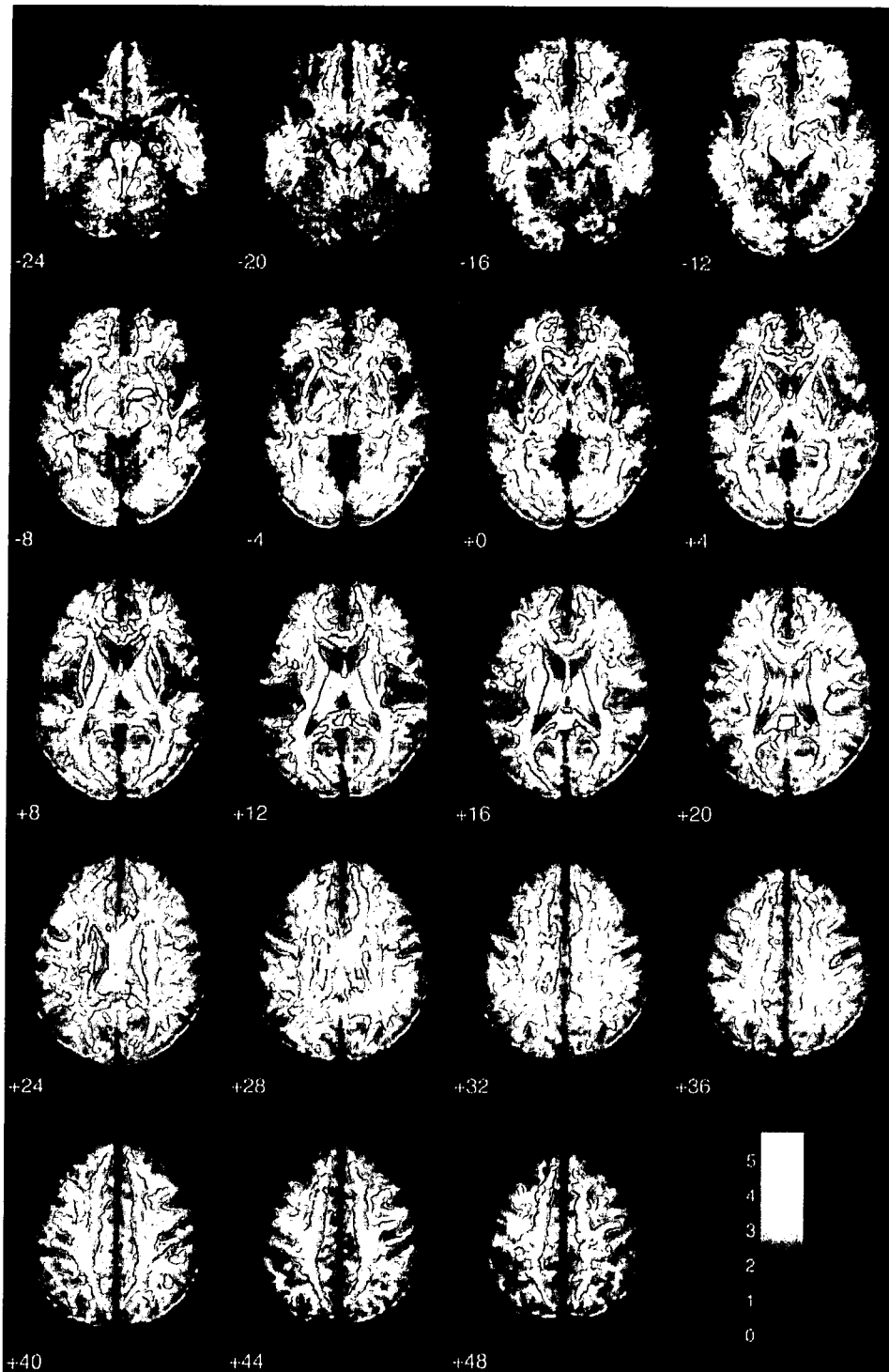


Fig. 1. Comparison in FA values between patients with schizophrenia and controls. The SPM  $\{t\}$  is displayed onto axial FA template images. The WM areas in which significantly lower FA values in patients compared with controls were observed, including the bilateral frontal and temporal WM, uncinate fasciculi, cingulum bundles, and genu and splenium of the corpus callosum ( $P < 0.001$ , uncorrected).