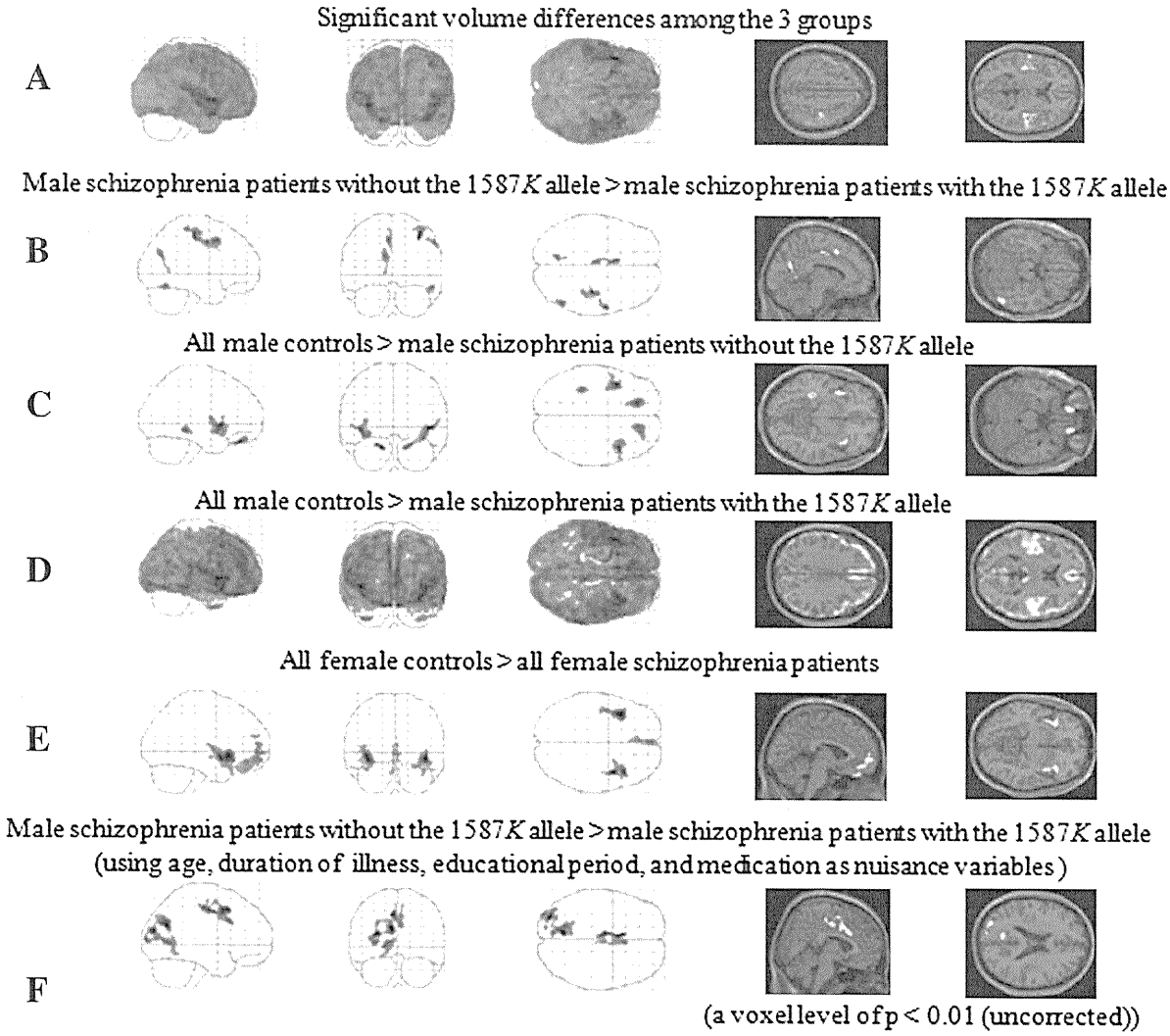

Fig. 1. Group effect was assessed using analysis of covariance (ANCOVA) (SPM2). Age was used as a nuisance variable. (A); There were statistically significant volume differences among the 3 groups of men, i.e., the male schizophrenia patients with and without the 1587*K* allele and the entire male control group. (B); Male schizophrenia patients carrying the 1587*K* allele showed gray matter volume reduction in the bilateral occipital regions and posterior cingulate cortices compared with those who did not carry this allele. (C); There were volume decreases in the bilateral insulae and orbitofrontal regions, and the left parahippocampal region in male patients with schizophrenia without the 1587*K* allele compared with all male controls. (D); Male patients with schizophrenia carrying the 1587*K* allele showed volume reduction in almost all the gray matter areas, compared with all male controls. (E); When all female schizophrenia patients were analyzed collectively, they showed gray matter volume reduction in the bilateral insulae, anterior cingulate cortex, and orbitofrontal cortex, compared with all female controls. (F); We also evaluated the difference in gray matter volume between the schizophrenic groups with and without the 1587*K* allele for each sex using ANCOVA, controlling for age, duration of illness, educational period, and medication. Male patients with schizophrenia carrying the 1587*K* allele showed small gray matter volume in the left occipital region and bilateral posterior cingulate cortices, compared with those who did not carry the 1587*K* allele controlling for age, duration of illness, educational period, and medication, at nominal trend level ($P<0.01$ uncorrected).

2007). Two broad theories have been proposed to describe the pattern of cerebral changes: the global and macro-circuit theories (Buchsbaum et al., 2006). According to the global theory, white matter reductions occur uniformly throughout the brain, possibly as a result of genetic abnormalities in the protein pathways controlling myelination (Konrad and Winterer, 2008). The alternative macro-circuit theory proposes that specific white matter tracts are disrupted in schizophrenia either as a cause or a consequence of a disorder in the gray matter regions they connect (Konrad and Winterer, 2008). The present results may accord with the global theory by showing smaller volume in almost the entire gray matter in male schizophrenia patients carrying the 1587*K* allele of *ABCA1*, because ABCA1 was regarded as the key regulator of brain cholesterol homeostasis and associated with structure and function in neurons such as myelination (Karasinska et al., 2009). Both male and female schizophrenia patients who did not carry the 1587*K* allele showed smaller volume in the medial temporal region, insulae, and anterior cingulate cortex, which have been referred to as predominantly impaired brain regions in schizophrenia, than control subjects (Glahn et al., 2008; Ellison-Wright et al., 2008). On the other hand, the male patients with schizophrenia carrying the 1587*K* allele showed the smaller volume in the occipital regions and posterior cingulate cortices, where it is known to remain unchanged from illness, than male patients not carrying 1587*K* allele. Intricate analysis controlling for age, duration of illness, educational period, and medication, male patients carrying 1587*K* allele showed the smaller volume in occipital and posterior cingulate cortices compared with male patients not carrying 1587*K* allele, only at the trend level, but these tendencies could not be detected in females even at the trend level. From these points, we suggest a male-specific association of the 1587*K* allele of *ABCA1* with susceptibility to schizophrenia and smaller gray matter volume in schizophrenia. In this study, we evaluated only a gray matter volume change, and no consideration was paid to the white matter. Further work with the diffusion tensor imaging data will be necessary to confirm our results.

Schizophrenia is a multifactorial disorder caused by a complex interaction of genetic and environmental factors (Bassett et al., 2001).

In this study, we found no significant difference in gray matter volume related to the *R1587K* polymorphism in healthy subjects. This may be accounted for by the possibility that *ABCA1* polymorphism interacts with other risk factors for schizophrenia and that these collectively influence brain vulnerability.

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Effects of Metabotropic Glutamate Receptor 3 Genotype on Phonetic Mismatch Negativity

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Abstract

Background: The genetic and molecular basis of glutamatergic dysfunction is one key to understand schizophrenia, with the identification of an intermediate phenotype being an essential step. Mismatch negativity (MMN) or its magnetic counterpart, magnetic mismatch field (MMF) is an index of preattentive change detection processes in the auditory cortex and is generated through glutamatergic neurotransmission. We have previously shown that MMN/MMF in response to phoneme change is markedly reduced in schizophrenia. Variations in metabotropic glutamate receptor (GRM3) may be associated with schizophrenia, and has been shown to affect cortical function. Here we investigated the effect of *GRM3* genotypes on phonetic MMF in healthy men.

Methods: MMF in response to phoneme change was recorded using magnetoencephalography in 41 right-handed healthy Japanese men. Based on previous genetic association studies in schizophrenia, 4 candidate SNPs (rs6465084, rs2299225, rs1468412, rs274622) were genotyped.

Results: *GRM3* rs274622 genotype variations significantly predicted MMF strengths ($p = 0.009$), with C carriers exhibiting significantly larger MMF strengths in both hemispheres compared to the TT subjects.

Conclusions: These results suggest that variations in *GRM3* genotype modulate the auditory cortical response to phoneme change in humans. MMN/MMF, particularly those in response to speech sounds, may be a promising and sensitive intermediate phenotype for clarifying glutamatergic dysfunction in schizophrenia.

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Introduction

Synaptic pathology through glutamatergic dysfunction is a key to understand the pathophysiology of schizophrenia [1]. Functions of susceptibility genes for schizophrenia have converged to synaptic plasticity and glutamatergic neurotransmission [2]. Postmortem studies have shown reduced spine density in prefrontal and auditory cortices in schizophrenia [3,4].

Consistently observed reduction of mismatch negativity (MMN) event-related potentials or its magnetic counterpart (magnetic mismatch field; MMF), an index of auditory sensory memory with a major generator in the auditory cortex, has supported the hypothesis [5], since the generation of MMN is regulated through the N-methyl-D-aspartate (NMDA) receptor agonistic effect [6]. Thus, MMN/MMF may be a useful intermediate phenotype to assess glutamatergic function in schizophrenia.

MMN/MMF is also elicited by change in speech sounds [7], which has been recognized as an index of language-specific speech-sound traces and learning-induced short-term plasticity. We have shown that phonetic MMN, rather than tonal MMN, exhibited more marked reduction in schizophrenia [8]. We replicated the findings using MMF [9]. Moreover, the reduced MMF strengths were significantly associated with reduced left planum temporale gray matter volume reduction in patients with schizophrenia [10]. These results suggest that MMN/MMF in response to phoneme change may be a promising candidate as an intermediate phenotype of glutamate-related genes.

Metabotropic glutamate receptors, by augmenting the function of, or co-acting with, NMDA receptors, are important in synaptic plasticity [1]. Metabotropic glutamate receptor 3 (*GRM3*) may be associated with schizophrenia phenotype [11–14], although controversial [15,16]. Among studies reporting positive findings, the polymorphisms showing association were totally different.

Egan *et al.* [12] observed that the single nucleotide polymorphism (SNP) rs6465084 (hCV11245618) and a related haplotype are associated with the disease in a U.S. population. The same SNP was studied by Norton *et al.* [15], and no significant association was observed. In Asians, Chen *et al.* [13] observed a significant association of the SNP rs2299225 and a related haplotype with schizophrenia in Chinese subjects. Fujii *et al.* [11] found an association between rs1468412 and schizophrenia in their Japanese study. Bishop *et al.* [14] has shown that *GRM3* rs274622 modulated the effect of olanzapine on negative symptoms in Caucasian patients with schizophrenia.

Moreover, *GRM3* genotype modulates prefrontal BOLD signals and N-Acetylaspartate (NAA) levels [12,17] and mGlu3 protein levels were reduced in the prefrontal cortex in postmortem brains of schizophrenia [18].

Accordingly, we predicted that phonetic MMF would be modulated by variations in *GRM3* in healthy subjects. Our hypothesis stems from the facts: 1) we previously found more marked reduction of phonetic MMN rather than tonal MMN in patients with schizophrenia [8,9]; 2) phonetic MMN has been recognized as an index of language-specific speech-sound traces and learning-induced short-term plasticity [7]; 3) MMN is thought to be a promising intermediate phenotype for glutamatergic system which is involved in synaptic plasticity [5]. We addressed all of the SNPs that showed significant results in previous studies [11–14].

Methods

Subjects

Participants were 41 healthy Japanese men (mean age: 28.8+/−5.5 years). All participants were right-handed according to the Edinburgh Inventory and were native Japanese speakers. The average years of education of the subjects were 17.0 [SD = 1.2]; the average IQ was 112.3 [SD = 7.6]. For screening of healthy subjects, SCID non-patient edition (SCID-NP) was used. Exclusion criteria were neurological illness, hearing dysfunction, traumatic brain injury with any known cognitive consequences or loss of consciousness for more than 5 minutes, a history of substance abuse or addiction. The ethical committee of the Faculty of Medicine, University of Tokyo approved this study (No. 784-2 for MEG experiment; No. 639-9 for imaging-genetics project). All subjects gave written informed consent after a complete explanation of the study.

MEG recording and analysis

MEG recording and analysis was described in detail elsewhere [19]. Briefly, the subjects were presented with sequences of auditory stimuli to both ears, consisting of standard (Japanese vowel /a/ with a 250-msec duration, 80 dB SPL and a rise/fall time of 10 ms; probability = 90%) and deviant (Japanese vowel /o/ with a 250-msec duration, p = 10%) stimuli. The stimulus onset asynchrony was 445±15 msec. Measurements were performed in the early afternoon (from 2 p.m. through 3 p.m.). The subjects were instructed to perform a visual detection task, in order to keep attention away from the auditory stimuli. MEG signals were recorded using VectorView (Elekta Neuromag, Helsinki, Finland), which has 204 first-order planar gradiometers at 102 measuring sites on a helmet-shaped surface that covers the entire scalp. The recorded data were filtered online with a band-pass filter of 0.03–100 Hz, digitalized at a sampling rate of 512 Hz, and averaged online separately for standard and deviant stimuli. The duration of the averaging period was 400 ms, including an 80-msec prestimulus baseline. Trials with EOG movement exceeding 150 μV or MEG exceeding 3,000 fT/cm were excluded the analysis. The number of accepted responses for deviant stimuli was above 100 for all subjects. The averaged data were further filtered offline with a band-pass filter of 1–20 Hz.

The MMF is defined as the difference between the evoked magnetic fields of the standard stimuli and those of the deviant tone. The strength of MMF was indexed by the magnetic counterpart of the global field power (mGFP), which was calculated as the root mean squares of the differences over the 54 channels positioned over the temporal region, separately for each hemisphere [9,20].

Genotyping

Genotyping procedures were also described in detail elsewhere [16]. Briefly, genomic DNA was extracted from leukocytes by using the standard phenol-chloroform method. Based on previous literature, we genotyped 4 candidate SNPs (rs6465084, rs2299225, rs1468412, rs274622) of *GRM3*.

Statistical analysis

The mixed model repeated measures analysis of variance (ANOVA) was performed on the mGFPs of MMF, adopting the genotype as a between-subject factor and the hemisphere as a within-subject factor. For rs6465084, AA subjects (N = 33) and AG subjects (N = 8) were compared; for rs2299225, TT subjects (N = 33) and TG subjects (N = 8) were compared; for rs1468412, AA subjects (N = 25) and AT/TT subjects (N = 16 [AT = 15/TT = 1]) were compared; for rs274622, CC/CT subjects (N = 19 [CC = 2/CT = 17]) and TT subjects (N = 22) were compared. The threshold for statistical significance was set at $p<0.0125$ based on the Bonferroni correction.

Results

Figure 1 shows grand mean mGFP waveforms of MMF averaged for each GRM3 rs274622 genotype group for each hemisphere. The repeated measures ANOVA showed a significant main effect of *GRM3* rs274622 genotype ($F[1,39] = 7.59$, $p = 0.009$), with no genotype X hemisphere interaction ($F[1,39] = 0.036$, $p = 0.85$). The *GRM3* rs274622 C carriers (CC+TC) exhibited significantly larger mGFP values in both hemispheres compared to the TT subjects (post-hoc t-tests: left hemisphere: $t[39] = 2.20$, $p = 0.034$; right hemisphere: $t[39] = 2.41$, $p = 0.021$). Demographic (age, education, IQ, handedness), behavioral (sleepiness, response time and hit rate for visual task), and MEG (the number of accepted sweeps for deviant stimuli, MMF peak latency) variables did not significantly differ between the genotypes ($p's>0.32$).

Variations in the other 3 SNPs did not significantly influence individual differences in MMF (main effect of genotype: $p's>0.17$).

Discussion

Variations in *GRM3* rs274622 genotype are associated with individual differences in phonetic MMF power in the bilateral auditory cortices. To our knowledge, this is the first report that demonstrates the association between variations in a glutamate-related gene and individual differences in MMN/MMF in humans. These results confirm that MMF generation is regulated through glutamatergic neurotransmission and suggest that MMN/MMF may be a useful intermediate phenotype for genes coding the glutamatergic system in humans and possibly in patients with schizophrenia. In patients with schizophrenia, progressive decrease of gray matter volume of the superior temporal gyrus and MMN could concurrently occur [21,22]. Therefore, the association

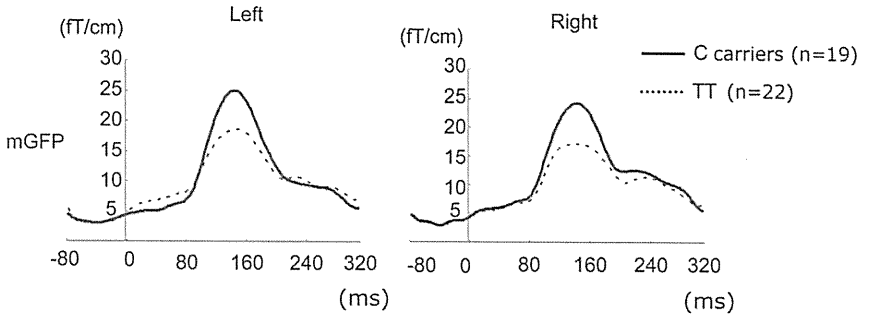

Figure 1. Grand mean mGFP waveforms of MMF. These waveforms were averaged for each GRM3 rs274622 genotype group for each hemisphere (C carriers [N = 19], solid line; TT individuals [N = 22], dashed line).
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between intermediate phenotypes and genes could be obscured to some extent in patients with schizophrenia. In this sense, MMF could be considered as both trait and state markers in schizophrenia. In contrast, young healthy subjects do not exhibit markedly progressive decline in these indices. Thus, MMF could be a relatively robust trait marker and the significant association between gene and intermediate phenotype could be predicted more clearly.

Bishop et al. [14] found that improvement in negative symptoms when treated with olanzapine was significantly greater in patients with schizophrenia who carry C allele of GRM3 rs274622 than in TT subjects. Taken together with the present findings, this SNP may be functionally relevant for cortical synaptic plasticity.

Our research group has previously demonstrated that effect size of the difference between healthy subjects and schizophrenia is larger for phonetic MMN/MMF than for tonal MMN/MMF [8,9]. Thus, phonetic MMN/MMF may be a particularly sensitive intermediate phenotype for clarifying the molecular pathway of the glutamate system in the pathophysiology of schizophrenia.

Limitations of this study include small sample size and limited number of genes and SNPs investigated, and data on patients with schizophrenia being unavailable. Our next step should be a multivariate analytic approach to understand the complex relationship between various types of MMN/MMF and genes related to the glutamatergic system and synaptic plasticity identified through a genome-wide search in the general population and its alteration in patients with schizophrenia.

Author Contributions

Conceived and designed the experiments: TS Y. Kano KK. Performed the experiments: Y. Kawakubo MS MT MY. Analyzed the data: Y. Kawakubo MS MT TS KK. Contributed reagents/materials/analysis tools: MY KI. Wrote the paper: Y. Kawakubo KK.

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Serum Levels of Mature Brain-Derived Neurotrophic Factor (BDNF) and Its Precursor proBDNF in Healthy Subjects

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Abstract: BACKGROUND: Accumulating evidence points to the brain-derived neurotrophic factor (BDNF) as a biomarker for neuropsychiatric diseases, such as major depression. Mature BDNF is synthesized from its precursor form, proBDNF. Although BDNF levels in human blood can be measured using commercially available human BDNF ELISA kits, due to limited specificity of the BDNF antibody, these kits are unable to distinguish between proBDNF and mature BDNF. In this study, we measured serum levels of proBDNF and mature BDNF in healthy subjects, using human proBDNF and BDNF ELISA kits, respectively.

METHODS: Serum levels of proBDNF and mature BDNF in healthy subjects (n = 40) were measured using the sandwich human proBDNF and BDNF ELISA kits.

RESULTS: In healthy subjects, serum levels of mature BDNF were 23.71 ± 5.61 ng/mL (mean ± S.D., n=40). Serum levels of proBDNF in healthy subjects were 7.58 ± 7.68 ng/mL (mean ± S.D., n=25). However in 15 subjects, serum levels of proBDNF were less than the minimum detectable concentration (0.5 ng/mL) of the kit.

CONCLUSIONS: This study shows that serum levels of proBDNF and mature BDNF are measurable using either the commercially available human proBDNF or BDNF ELISA kits, although the sensitivity of proBDNF kit was unacceptably low. These ELISA kits may be useful for measuring proBDNF and mature BDNF in the body fluids of patients with neuropsychiatric, cardiovascular and other diseases.

Keywords: Biomarker, brain-derived neurotrophic factor (BDNF), mature BDNF, proBDNF, ELISA, blood.

1. INTRODUCTION

At present, there are no clinical laboratory tests that can be used by doctors to assist with the diagnosis of patients with neuropsychiatric diseases. Identification of biomarkers in human body fluids such as blood, urine, and cerebral spinal fluid (CSF) would aid both in the diagnosis of neuropsychiatric diseases, and development of effective therapies [1].

Mature brain-derived neurotrophic factor (BDNF) is a 13 kDa polypeptide, known to play an important role in the survival, differentiation, and outgrowth of select peripheral and central neurons during development and adulthood [2,3]. Accumulating evidence suggests a pivotal role for BDNF in the pathophysiology of major depression, as well as in the therapeutic mechanisms of antidepressants [4-9]. Mature BDNF is initially synthesized as a precursor protein, prepro BDNF, in the endoplasmic reticulum. Following cleavage of the signal peptide, proBDNF (~32 kDa) is converted to mature BDNF (13 kDa) by extracellular proteases (Fig **1**) [9-12]. It was initially thought that only secreted, mature BDNF was biologically active, and that proBDNF, localized intracellularly, serving as an inactive precursor.However, recent studies show that proBDNF and mature BDNF elicit opposing effects *via* the $p75^{NTR}$ and TrkB receptors, respectively, and that both proBDNF and mature BDNF play important roles in several physiological functions (Fig. **1**) [9-12].

BDNF is present in human blood, although it is highly concentrated in brain tissue. Previously, we reported that serum BDNF levels were significantly lower in patients with neuropsychiatric diseases, such as major depression [13], eating disorders [14,15], pediatric depression [16], and high-functioning autism [17]. Subsequent meta-analyses confirmed our findings on major depression [18-21]. Therefore, it is likely that accurate measurement of blood BDNF levels could serve as a potential biomarker for major depression [9].

Considering the important role that both proBDNF and mature BDNF play in the physiological functioning of the brain, it would be valuable to measure individual levels of precursor and mature BDNF in the body fluids of human subjects [9]. In this study, we measured serum levels of proBDNF and mature BDNF in healthy subjects using the human either the proBDNF or BDNF ELISA kits (Adipo Bioscience) (Table **1**). Furthermore, we also measured serum levels of total BDNF, including proBDNF and mature BDNF, using other commercially available human BDNF ELISA kits from Millipore, R&D Systems and Promega

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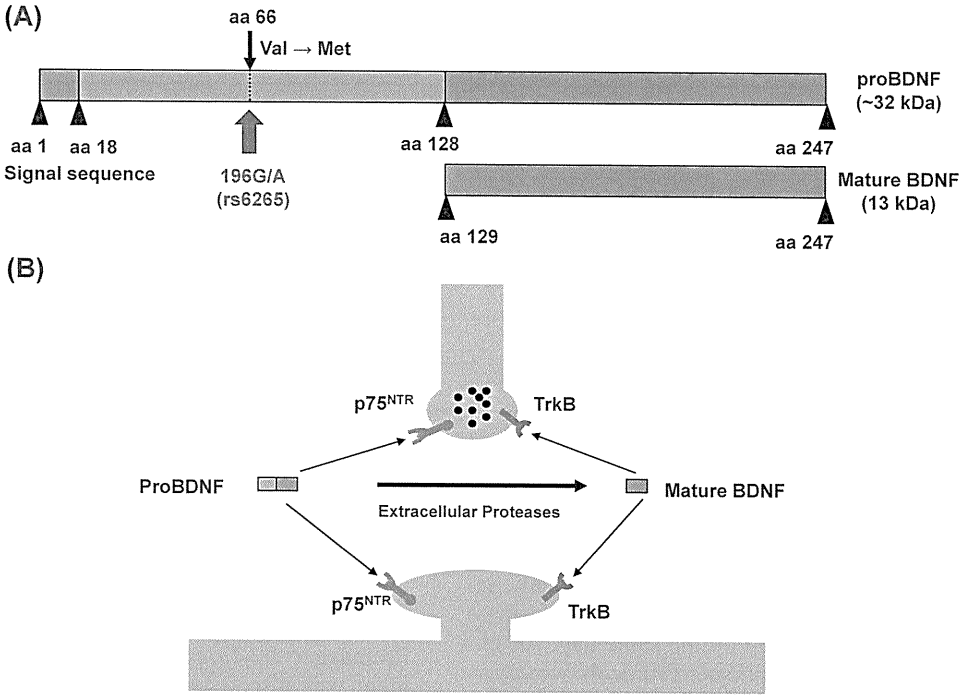

Fig. (1). (**A**) Structure of proBDNF and mature BDNF. Arrowheads indicate known protease cleavage sites involved in the processing of mature BDNF. The position of the single nucleotide polymorphism (rs6265, Val66Met) in the human *BDNF* gene is indicated by an arrow. (**B**) Extrasynaptic cleavage of proBDNF to mature BDNF. ProBDNF preferentially binds $p75^{NTR}$. ProBDNF is cleaved by extracellular proteases at the synapses and converted to mature BDNF. Mature BDNF preferentially binds the TrkB receptor. This figure is a modified version of previously published figures [9-11].

Table 1. Properties of Human proBDNF and BDNF ELISA Kits

	Human proBDNF ELISA Kit	Human BDNF ELISA Kit	Chem Kine BDNF Kit	Quantikine BDNF ELISA Kit	BDNF Emax ImmunoAssay System
Company	Adipo Bioscience	Adipo Bioscience	EMD Millipore Corporation	R&D Systems	Promega Corporation
Catalog No.	SK00752-06	SK00752-01	CYT306	DBD00	G7611
Sensitivity	0.5 ng/ml	5 - 8 pg/ml	7.8 pg/ml	20 pg/ml	15.6 pg/ml
Cross-reactivity	proBDNF	Mature BDNF	proBDNF and mature BDNF	proBDNF and mature BDNF	proBDNF and mature BDNF

(Table **1**), since these kits are routinely used worldwide. It is known that the BDNF Emax ImmunoAssay System (Promega) is based on an antibody to the carboxy terminal region of BDNF, and recognizes both the precursor and mature forms of BDNF [22].

2. METHODS AND MATERIALS

2.1. Subjects

Forty healthy subjects (age: 30.7 ± 6.87 years old, range: 21-40 years old) participated in this study as normal controls (Table **2**). The ethics committee of Chiba University Graduate School of Medicine approved the study protocol, and all subjects provided written, informed consent for participation in the study. Healthy subjects were recruited from the local Chiba area, by advertisement. Subjects were screened using the Structured Clinical Interview for DSM-IV Axis I Disorders, Non-Patient Edition, to exclude Axis I disorders, according to DSM-IV criteria.

2.2. Procedures

Serum samples from normal control subjects were collected between 9:00 to15:00, and stored at -80°C until use. Serum levels of proBDNF and mature BDNF were measured by using the human proBDNF ELISA Kit (Cat #: SK00752-06, Adipo Bioscience, Santa Clara, CA, USA) and the human BDNF ELISA Kit (Cat #: SK00752-01, Adipo Bioscience, Santa Clara, CA, USA), respectively (Table **1**). Serum levels of total BDNF, including proBDNF and mature BDNF, were also measured using the BDNF Emax® ImmunoAssay System (Cat #: G7611, Promega Corporation, Madison, WI, USA), Quantikine® human BDNF Immunoassay (Cat #: DBD00, R&D Systems, Minneapolis, MN, USA), and *ChemKine*™ BDNF Sandwich ELISA (Cat#:

Table 2. Serum Levels of proBDNF and BDNF in Healthy Subjects

Age (years old)	30.7 ± 6.87 (21-40)(n=40)
Gender (Male/Female)	19/21
proBDNF (Adipo Bioscience)	7.58 ± 7.68 ng/mL (0.656 – 31.85)(n=25) <0.5 ng/mL (n=15)
BDNF mature (Adipo Bioscience)	23.71 ± 5.61 ng/mL (12.56 – 36.64)(n=40)
BDNF (Millipore)	23.75 ± 16.82 ng/mL (5.80 – 79.19)(n=40)
BDNF (R&D Systems)	24.81 ± 5.87 ng/mL (11.34 – 36.62)(n=40)
BDNF (Promega)	16.50 ± 3.88 ng/mL (7.23 – 24.76)(n=40)

The values are the mean ± S.D.
The values in the parenthesis are the range.

CYT306, EMD Millipore Corporation, Billerica, MA, USA) (Table **1**). To minimize assay variance, serum levels of proBDNF and mature BDNF from each subject were measured using the Adiopo Bioscience kits, on the same day. Serum levels of total BDNF in each subject, using the three remaining kits were also measured on the same day. All experiments were performed in duplicate. Protocols were performed according to the manufacturer's instructions. The optical density of each well was measured using an automated microplate reader (Emax; Molecular Devices, Sunnyvale, CA, USA).

2.3. Statistical Analysis

The data were presented as the mean ± standard deviation (S.D.). Analysis of BDNF serum levels from four groups was performed using one-way analysis of variance (ANOVA), and the *post hoc* Dunnett test. The relationship between the two variables was ascertained using Pearson's correlation coefficients. Values of $p<0$ .05 were considered statistically significant.

3. RESULTS

Using the human proBDNF and BDNF ELISA kits (Adipo Bioscience), we measured serum levels of proBDNF and mature BDNF respectively, in healthy subjects. Serum levels of mature BDNF were 23.71 ± 5.61 ng/mL (n=40) (Table **2**). Where proBDNF was measurable, serum levels were 7.58 ± 7.68 ng/mL (n=25). In 15 subjects, serum levels of proBDNF were below the minimum detectable concentration (0.5 ng/mL) of the proBDNF ELISA kit (Table **2**). In 38 subjects, levels of proBDNF were lower than those of mature BDNF although, in two male subjects, this pattern was reversed.

Next, we measured total BDNF in the serum of healthy subjects using the three other ELISA kits. Total BDNF levels were 23.75 ± 16.82 ng/mL (Millipore, n=40), 24.81 ± 5.87 ng/mL (R&D Systems, n=40), and 16.50 ± 3.88 ng/mL (Promega, n=40) (Table **2**). One-way ANOVA revealed significant differences [$F(3, 156) = 6.439$, $p<0.001$] within the four groups, Adipo Bioscience, Millipore, R&D Systems and Promega, and *post hoc* analysis indicated that serum levels of total BDNF as measured by Promega were significantly ($p=0.003$) lower than those of mature BDNF as measured by Adipo Bioscience. In contrast, there were no differences among the three kits from Adipo Bioscience, Millipore and R&D Systems.

As shown in Fig. (**2**), there were significantly positive correlations between mature BDNF (Adipo Bioscience), and total BDNF serum levels (R&D Systems, $r=0.701$, $p<0.001$, Promega, $r=0.673$, $p<0.001$) (Figs. **2A** and **2B**). Furthermore, there was also a weak but significant correlation between mature BDNF (Adipo Bioscience) and total BDNF serum levels (Millipore, $r=0.377$, $p=0.016$) (Fig. **2C**).

4. DISCUSSION

In this study, we measured serum levels of proBDNF and mature BDNF in human subjects, using either the human proBDNF or human BDNF ELISA kits. To the best of our knowledge, this is the first report demonstrating the measurement of proBDNF as well as mature BDNF from the serum of human subjects. According to the manufacturer's information, the human proBDNF ELISA kit (Adipo Bioscience: Cat No. SK00752-06) recognizes proBDNF, but not the mature form, while the human BDNF ELISA kit (Adipo Bioscience: Cat No. SK00752-01) recognizes mature BDNF, but not the precursor form (Table **1**). In tests, we were unable to measure serum levels of proBDNF in some subjects, since the values fell below the minimum detectable threshold of the proBDNF ELISA kit. The manufacturer's instructions state that the minimum detectable concentrations of the proBDNF and BDNF ELISA kits are 0.5 ng/mL and 5-8 pg/mL, respectively (Table **1**), indicating that the sensitivity of the proBDNF kit is markedly lower than that of the mature BDNF ELISA kit. Therefore, accurate measurements of low levels of proBDNF in human body fluids require the development of an ELISA kit of higher sensitivity than is currently available. Nonetheless, we were able to measure proBDNF and mature BDNF in the serum of human subjects using these ELISA kits.

In this study, we also measured serum levels of total BDNF, including proBDNF and mature BDNF, using the human BDNF ELISA kits from Millipore, R&D Systems and Promega, since these kits are commonly used worldwide. The manufacturer's instructions state that the Millipore *ChemiKine*™ BDNF ELISA kit is based on mouse monoclonal antibodies generated against human mature BDNF. The Quantikine® BDNF ELISA kit (R&D Systems) is based on monoclonal antibodies generated against human recombi-

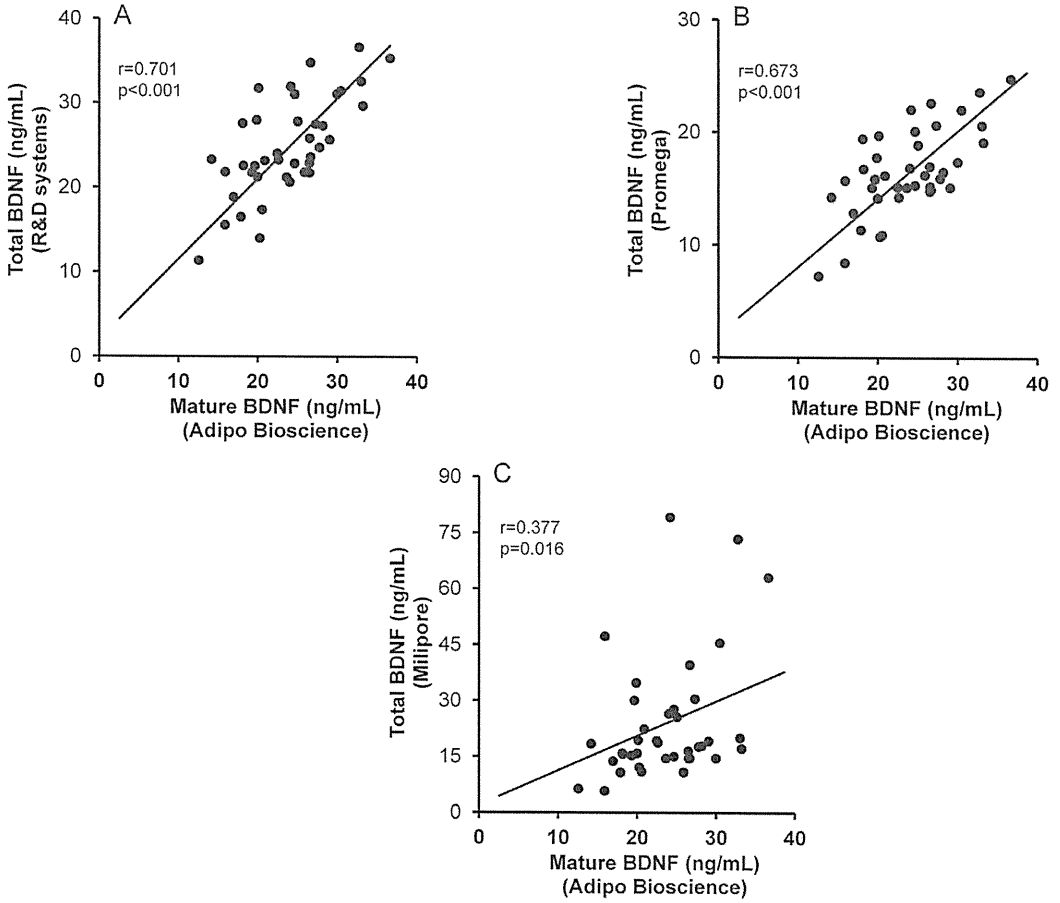

Fig. (2). Correlations between mature BDNF (Adipo Bioscience) and total BDNF (R&D System, Promega, Millipore) in human serum. (**A**) There was a significant correlation (r=0.701, p<0.001) between mature BDNF serum levels (Adipo Bioscience), and total BDNF serum levels (R&D Systems). (**B**) There was a significant correlation (r=0.673, p<0.001) between mature BDNF serum levels (Adipo Bioscience), and total BDNF serum levels (Promega). (**C**) There was also a significant correlation (r=0.377, p=0.016) between mature BDNF serum levels (Adipo Bioscience) and total BDNF serum levels (Millipore).

nant mature BDNF, and shows approximately 10% cross reaction against recombinant human proBDNF. The BDNF Emax® ImmunoAssay System (Promega Corporation) is based on an antibody to the carboxy terminal of mature BDNF [22], and also recognizes proBDNF. Each of these three kits recognizes proBDNF as well as mature BDNF, making them unsuitable for quantification of mature BDNF. In this study, we found positive correlations between the serum levels of mature BDNF (Adipo Bioscience) and the serum levels of total BDNF (R&D System and Promega) (Fig. **2A** and **2B**). It would therefore seem that serum levels of total BDNF using the R&D System and Promega ELISA kits correlate with serum levels of mature BDNF (Adipo Bioscience), although these two kits recognize both proBDNF and mature BDNF.

Alterations in the levels of total BDNF, including proBDNF, have been reported in the body fluids (e.g., blood) of patients with major depression [13, 18-21], schizophrenia [23], anorexia nervosa [14, 15, 24-26], bipolar disorders [27], and cardiovascular disease [28-30]. Furthermore, the presence of proBDNF and mature BDNF in human saliva has been reported by Western blotting analysis, but not ELISA method [31]. Given the opposing physiological roles of proBDNF and mature BDNF in the brain and peripheral organs, it would be of great interest to determine the exact concentrations of precursor and mature BDNF in the body fluids (e.g., blood, CSF, saliva) of patients with these diseases, and healthy control subjects [9].

In this study, we found significant levels of proBDNF present in the serum of human subjects, although levels of proBDNF were lower than those of mature BDNF, with the exception of two subjects. ProBDNF is converted to mature BDNF by extracellular proteases (Fig. **1**) [9-11]. Again, given the opposing biological effects of proBDNF and mature BDNF, it would be informative to study the precise mechanisms controlling the cleavage of proBDNF to mature BDNF [9]. To address these issues, it will first be necessary to develop highly sensitive ELISA systems that can differentiate between proBDNF and mature BDNF [9].

The Val66Met gene variant (196G/A: rs6265) of the human BDNF gene is thought to affect intracellular trafficking and mature BDNF secretion, as well as being associated with hippocampal volume and episodic memory in humans (Fig. **1**) [8, 9, 32]. The frequency of this genotype is highest in Asian populations, including the Japanese [33]. It is predicted that both proBDNF-66Met and proBDNF-66Val could well exist in body fluids of subjects with the BDNF 196G/A genotype [9]. This raises the possibility that conversion of the two proBDNFs variants to mature BDNFs may differ between subjects who carry the BDNF 196G/A geno-

type, suggesting that in subjects with the BDNF 196G/A genotype, levels of proBDNF-66Met and proBDNF-66Val may vary [9,34]. If a highly sensitive ELISA system could be developed to distinguish between proBDNF-66Met and proBDNF-66Val, it would then be possible to quantify levels of the wild type and variant forms of proBDNF as well as the mature BDNF levels in body fluids of healthy subjects and patients with neuropsychiatric or other diseases [9,34].

CONCLUSION

This study shows that serum levels of proBDNF and mature BDNF in human subjects can be measured individually, using the human proBDNF and BDNF ELISA kits, although the sensitivity of the current proBDNF ELISA kit is currently unsatisfactory. Since it would be highly informative to measure serum levels of proBDNF and mature BDNF in patients with neuropsychiatric and other diseases, the development of a highly sensitive proBDNF ELISA system is a priority.

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CONFLICT OF INTEREST

None declared.

ABBREVIATIONS

BDNF	=	Brain-derived neurotrophic factor
CSF	=	Cerebral spinal fluid
ELISA	=	Enzyme-Linked ImmunoSorbent Assay
proBDNF	=	Precursor of brain-derived neurotrophic factor
SD	=	Standard deviation

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Longitudinal volume changes of the pituitary gland in patients with schizotypal disorder and first-episode schizophrenia

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ABSTRACT

An enlarged volume of the pituitary gland has been reported in the schizophrenia spectrum, possibly reflecting the hypothalamic–pituitary–adrenal (HPA) hyperactivity. However, it remains largely unknown whether the pituitary size longitudinally changes in the course of the spectrum disorders. In the present study, longitudinal magnetic resonance imaging (MRI) data were obtained from 18 patients with first-episode schizophrenia, 13 patients with schizotypal disorder, and 20 healthy controls. The pituitary volume was measured at baseline and follow-up (mean, 2.7 years) scans and was compared across groups. The pituitary volume was larger in the schizophrenia patients than controls at baseline, and both patient groups had significantly larger pituitary volume than controls at follow-up. In a longitudinal comparison, both schizophrenia (3.6%/year) and schizotypal (2.7%/year) patients showed significant pituitary enlargement compared with controls (−1.8%/year). In the schizophrenia patients, greater pituitary enlargement over time was associated with less improvement of delusions and higher scores for thought disorders at the follow-up. These findings suggest that the pituitary gland exhibits ongoing volume changes during the early course of the schizophrenia spectrum as a possible marker of state-related impairments.

1. Introduction

Hypothalamic–pituitary–adrenal (HPA) axis hyperactivity is thought to reflect stress-related hormonal dysregulation and has been described in schizophrenia and related disorders (Phillips et al., 2006; Walker et al., 2008). Neuroendocrine studies in schizophrenia and schizotypal (personality) disorder (SPD), a prototypic disorder within the schizophrenia spectrum (Siever and Davis, 2004), have demonstrated that these disorders might share similar HPA axis dysfunctions, such as higher salivary cortisol level (Mittal et al., 2007; Walker et al., 2001) or blunted cortisol response to acute metabolic stress (Mitropoulou et al., 2004), as a potential indicator of common stress vulnerability. Furthermore, the association of HPA axis dysfunction with symptom severity (Goyal et al., 2004; Walder et al., 2000; Walker et al., 2001), medication (Cohrs et al., 2006; Scheepers et al., 2001), and illness stages (Phillips et al., 2006; Walker et al., 2008) in these disorders suggests that HPA activity reflects state-related impairments in the course of the schizophrenia spectrum.

The pituitary gland, an integral part of the HPA axis, may be one of the brain regions most affected by the hormonal stress response (Phillips et al., 2006). Recent magnetic resonance imaging (MRI) findings of increased pituitary volume in first-episode psychosis (Pariante et al., 2004, 2005), recent onset schizophrenia or schizotypal disorder (Takahashi et al., 2009), and individuals at high risk of developing psychosis (Garner et al., 2005) have been attributed to HPA hyperactivity in the early stages of these disorders, whereas normal (Tournikioti et al., 2007) or even decreased (Pariante et al., 2004) pituitary volume in chronically medicated schizophrenia patients could be explained by the notion that pituitary size is reduced over time as a result of prolonged HPA activation (Pariante et al., 2004; Sassi et al., 2001). However, the few longitudinal MRI studies of pituitary volume in psychotic disorders have yielded inconsistent findings from non-significant decrease (<2% over a 3-month period) (Nicolo et al., 2010) to 12% increase over 12 months (MacMaster et al., 2007b) during the first episode of illness. The effect of medication is also an important consideration for the pituitary

Abbreviations: ANCOVA, analysis of covariance; ANOVA, analysis of variance; CASH, Comprehensive Assessment of Symptoms and History; HPA axis, hypothalamic–pituitary–adrenal axis; ICV, intracranial volume; MMPI, Minnesota Multiphasic Personality Inventory; MRI, magnetic resonance imaging; SANS, Scale for the Assessment of Negative Symptoms; SAPS, Scale for the Assessment of Positive Symptoms; SPD, schizotypal personality disorder.

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findings (MacMaster et al., 2007b; Nicolo et al., 2010; Phillips et al., 2006), but a cross-sectional finding of decreased pituitary volume in antipsychotic-naïve schizophrenia patients with relatively recent onset (Upadhyaya et al., 2007) suggests that factors other than illness stages or medication, such as early treatment response (Garner et al., 2009), might also affect the pituitary volume. However, the precise effect of these clinical factors in schizophrenia remains unclear, especially for pituitary volume changes over time. In addition, no longitudinal MRI studies have examined the pituitary volume in schizotypal subjects, who have no overt and sustained psychosis but partly share stress vulnerability with full-blown schizophrenia (Siever and Davis, 2004).

This longitudinal MRI study investigated the pituitary volume changes over time in patients with first-episode schizophrenia and schizotypal disorder compared with those in healthy equivalents. On the basis of the potential role of the pituitary volume as an indicator of HPA dysfunction in the schizophrenia spectrum (Takahashi et al., 2009), which could reflect state influences of the disorders (Garner et al., 2009; Phillips et al., 2006; Walker et al., 2008), we predicted that both schizophrenia and schizotypal patients would show progressive pituitary enlargement. We also explored the relationship between the pituitary volume changes over time and several clinical factors (e.g., antipsychotic medication and early treatment response) in these disorders.

2. Methods

2.1. Participants

Eighteen first-episode schizophrenia patients who fulfilled the ICD-10 research criteria (World Health Organization, 1993) were recruited from inpatient and outpatient clinics of the Department of Neuropsychiatry of Toyama University Hospital. In accordance with the literature (Hirayasu et al., 2000; Kasai et al., 2003; Schooler et al., 2005; Takahashi et al., 2009; Yap et al., 2001), first-episode patients were defined as patients experiencing their first episode of schizophrenia whose illness onset was within 1 year of baseline scanning ($N=14$) or those undergoing their first psychiatric hospitalization ($N=4$). The diagnosis of schizophrenia was confirmed at the follow-up scan for all cases.

Schizotypal disorder patients ($N=13$) who met the ICD-10 research criteria (World Health Organization, 1993) were recruited from among patients who visited the clinics of the Department of Neuropsychiatry of Toyama University Hospital. This patient group had exhibited at least four of the schizotypal features (inappropriate affect, odd behavior, social withdrawal, magical thinking, suspiciousness, ruminations without inner resistance, unusual perceptual experiences, stereotyped thinking, and occasional transient quasi-psychotic episodes) over a period of at least 2 years, accompanied by distress or associated problems in their lives and required clinical care including low-dose antipsychotics. Their characteristics have been described previously (Kawasaki et al., 2004; Suzuki et al., 2005; Takahashi et al., 2006). All available clinical information and data obtained from a detailed review of the patients' clinical records and structured interviews for Comprehensive Assessment of Symptoms and History (CASH) including the chapter on premorbid or intermorbid personality (Andreasen et al., 1992) were stored in a database. The subjects were diagnosed by a consensus reached by at least two psychiatrists using these data. Although all of the schizotypal subjects in this study also fulfilled the DSM-IV criteria for SPD on Axis II, two subjects had previously experienced transient quasi-psychotic episodes fulfilling a DSM Axis I diagnosis of brief psychotic disorder (American Psychiatric Association, 1994). The mental condition of each subject was regularly assessed by experienced psychiatrists to check for the emergence of full-blown psychotic symptoms, and none of the 13 patients has developed overt schizophrenia to date (mean clinical follow-up period after baseline scanning = 5.1 years, SD = 2.1).

The control subjects consisted of 20 healthy volunteers recruited from members of the community, hospital staff, and university students. They were given a questionnaire consisting of 15 items concerning their personal (13 items; e.g., a history of obstetric complications, substantial head injury, seizures, neurological or psychiatric diseases, impaired thyroid function, hypertension, diabetes, and substance use) and family (2 items) histories of illness. They did not have any personal or family history of psychiatric illness among their first-degree relatives. All controls were interviewed and administered the Minnesota Multiphasic Personality Inventory (MMPI) by experienced psychologists to obtain a relatively homogeneous control group without eccentric profiles on the MMPI and were excluded if they had an abnormal profile, namely, any T-score on the validity or clinical scales exceeding 70.

The clinical symptoms of the schizophrenia and schizotypal patients were rated at the time of scanning (baseline and follow-up) using the Scale for the Assessment of Negative Symptoms (SANS; Andreasen, 1984) and the Scale for the Assessment of Positive Symptoms (SAPS; Andreasen, 1984). At the baseline, 12 schizophrenia and 6 schizotypal patients were treated with atypical antipsychotics, and 6 schizophrenia and 7 schizotypal patients were receiving typical antipsychotics. The patients were also receiving benzodiazepines (15 schizophrenia and 8 schizotypal patients), anticholinergics (14 schizophrenia and 9 schizotypal patients), antidepressants (1 schizophrenia and 6 schizotypal patients), and/or mood stabilizers [lithium carbonate (1 schizotypal patient), sodium valproate (1 schizophrenia patient), or carbamazepine (2 schizotypal patients)]. At the follow-up scan, 11 schizophrenia and 10 schizotypal patients were on atypical antipsychotics, and 7 schizophrenia and 3 schizotypal patients were on typical antipsychotics. Some patients were also receiving benzodiazepines (13 schizophrenia and 10 schizotypal patients), anticholinergics (15 schizophrenia and 9 schizotypal patients), antidepressants (1 schizophrenia and 4 schizotypal patients), and/or mood stabilizers [sodium valproate (1 schizophrenia and 1 schizotypal patients), carbamazepine (1 schizophrenia and 2 schizotypal patients), or a combination of lithium and carbamazepine (1 schizophrenia and 1 schizotypal patients)]. During the follow-up period between scans, 9 patients (4 schizophrenia and 5 schizotypal patients) were predominantly treated with typical antipsychotics, 18 patients (11 schizophrenia and 7 schizotypal patients) were treated mostly with atypical antipsychotics (although 2 patients received typical antipsychotics for <1 month), and 4 (3 schizophrenia and 1 schizotypal patients) received substantial amounts of both typical and atypical antipsychotics.

All subjects were right-handed and physically healthy, and none of the participants were pregnant or taking exogenous estrogens at the time of the study. None had a history of serious head trauma, neurological illness, substance abuse disorder, or serious medical disease (e.g., primary hypothyroidism). All participants were also screened for gross brain abnormalities (e.g., pituitary or hypothalamic tumor) by neuroradiologists. However, hormonal levels as well as menstrual cycle in females were not assessed at scanning. Of the 51 participants in this study, 48 subjects (17 schizophrenia, 12 schizotypal, and 19 control subjects) were included in our previous cross-sectional study of pituitary volume (Takahashi et al., 2009). This study was approved by the Committee on Medical Ethics of Toyama Medical and Pharmaceutical University. After a complete description of the study was provided, written informed consent was obtained from all subjects.

2.2. Magnetic resonance imaging procedures

The subjects were scanned twice on a 1.5-T Magnetom Vision (Siemens Medical System, Inc., Erlangen, Germany) with a three-

dimensional gradient-echo sequence FLASH (fast low-angle shots) yielding 160–180 contiguous T1-weighted slices of 1.0-mm thickness in the sagittal plane. The imaging parameters were: repetition time = 24 ms; echo time = 5 ms; flip angle = 40°; field of view = 256 mm; and matrix size = 256 × 256 pixels. The voxel size was 1.0 × 1.0 × 1.0 mm. The scanner was calibrated weekly with the same phantom to ensure measurement stability.

Image processing for volumetric analysis has been described in detail elsewhere (Takahashi et al., 2002). Briefly, on a Unix workstation (Silicon Graphics, Inc., Mountain View, CA, USA), the image data were processed using the software package Dr View 5.3 (AJS, Tokyo, Japan). Brain images were realigned in three dimensions to standardize for differences in head tilt during image acquisition and were then reconstructed into entire contiguous coronal images, with a 1-mm thickness, perpendicular to the anterior commissure–posterior commissure line. The signal-intensity histogram distributions from the T1-weighted images across the whole cerebrum were then used to semi-automatically segment the voxels into brain tissue components and cerebrospinal fluid. The intracranial volume (ICV) was measured to correct for differences in head size as described previously (Zhou et al., 2003).

2.3. Pituitary measurements

The volume of the pituitary gland was manually traced on consecutive 1-mm coronal slices on the basis of a method used by Garner et al. (2005). Briefly, we traced around the usually well-defined borders of anterior and posterior pituitary: the diaphragma sellae, superiorly; the sphenoid sinus, inferiorly; and the cavernous sinuses, bilaterally. As presented in Fig. 1, the pituitary stalk was excluded from the tracings, but we included a posterior bright spot, corresponding to the posterior pituitary (the intensity of which is thought to reflect vasopressin concentration). All measurements were carried out by a trained rater (TT) without knowledge of the subjects' identities and the times of their scans. Inter- (TT and VL) and intra-rater intraclass correlation coefficients in a subset of 10 randomly selected brains were over 0.93.

2.4. Statistical analysis

Clinical and demographic differences between groups were examined with one-way analysis of variance (ANOVA) or chi-square test. The absolute volume of the pituitary gland was analyzed using a repeated measures analysis of covariance (ANCOVA) with age at first scan, ICV, inter-scan interval, and cumulative dose of antipsychotics during scans as covariates, diagnosis as a between-subject factor, and time of scan (baseline and follow-up) as a within-subject variable. We also investigated the pituitary volume change over time using ANCOVA with the percentage volume change [100 × (absolute volume at follow-up scan − absolute volume at baseline) / absolute volume at baseline] as the dependent variable. Post hoc Tukey honestly significant difference test was used. While gender was not used as a between-subject factor owing to the small sample size, especially for females, none of the ANCOVA results reported herein changed when we included gender as a covariate.

While our previous cross-sectional study in a larger sample (Takahashi et al., 2009) found no significant correlation between pituitary volume and clinical variables (including onset age, illness duration, daily dosage or duration of antipsychotic medication, and the severity of both positive and negative symptoms), longitudinal changes in HPA activity in psychosis have been implicated in the manifestation of clinical symptoms (Phillips et al., 2006; Walker et al., 2010). We therefore examined the correlation between the percentage pituitary volume change per year and SANS/SAPS subscale scores (absolute score change between scans and score at follow-up) using Spearman's rho. The association between the annual pituitary volume change and cumulative dose of antipsychotics as well as illness (schizophrenia) or medication (schizophrenia and schizotypal disorder) duration at baseline was also analyzed. Statistical significance was defined as $p<0.05$.

3. Results

3.1. Demographic and clinical data

The groups were matched for age, gender, height, parental education, and inter-scan interval, but the controls had attained a higher level of education than the patients with either disorder (Table 1). While the baseline SAPS score for the schizophrenia patients was higher than that for the schizotypal patients, no significant group difference was found at follow-up, indicating relatively good response of positive symptoms to medication in our first-episode schizophrenia group (Table 1). There were significant differences in medication dosage at both time points; the schizotypal patients took significantly smaller amounts of antipsychotics than the schizophrenia patients. ANCOVA with age as a covariate showed that the schizotypal patients had a larger ICV compared with the schizophrenia patients ($p=0.015$) and controls ($p=0.017$) [$F(2, 47)=3.65$, $p=0.034$].

3.2. Comparison of absolute pituitary volume

ANCOVA showed a significant effect of time [$F(1, 48)=11.87$, $p=0.001$] and a time-by-diagnosis interaction [$F(2, 48)=14.01$, $p<0.001$]. The pituitary volume was larger in the schizophrenia patients than controls at baseline ($p=0.021$), and both patient groups

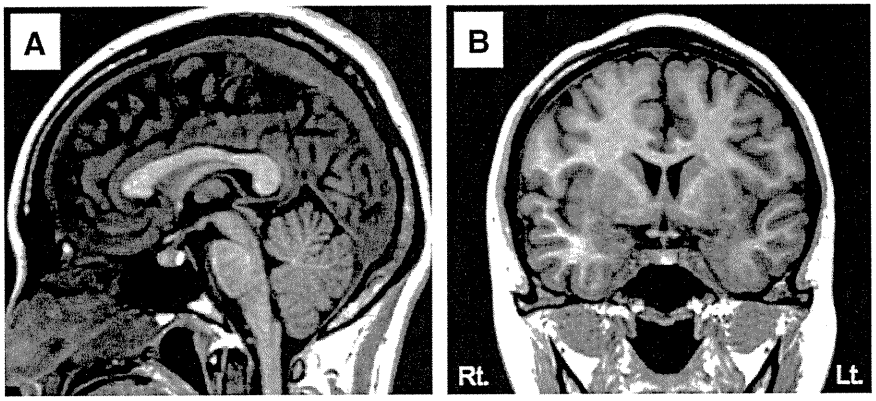

Fig. 1. Sagittal (A) and coronal (B) views of the pituitary gland manually traced in this study (blue). The pituitary stalk was excluded from the tracings, but we included a posterior bright spot.

had significantly larger pituitary volume than controls at follow-up ($p<0.001$) (Table 2). The schizophrenia patients had a larger pituitary volume compared with schizotypal patients (baseline, $p=0.004$; second scan, $p=0.002$).

While no significant difference was found between the pituitary volume at baseline and follow-up for the controls ($p=0.250$), the pituitary gland exhibited a significant volume enlargement (follow-up>baseline) in both patient groups (schizophrenia, $p=0.002$; schizotypal, $p=0.020$).

3.3. Comparison of pituitary percentage volume change

ANCOVA of the longitudinal volume change showed a significant group difference [$F(2, 44)=11.92$, $p<0.001$], with both schizophrenia ($p<0.001$) and schizotypal ($p<0.001$) patients having a greater pituitary enlargement over time than the controls (Table 2, Fig. 2). However, there was no difference between the schizophrenia and schizotypal patients ($p=0.984$).

The pituitary volume changes over time did not differ between the patients who were predominantly treated with typical ($N=9$) and atypical ($N=18$) antipsychotics during the follow-up period [$F(1, 21)=2.23$, $p=0.150$].

3.4. Correlational analysis

In the schizophrenia patients, greater pituitary enlargement over time was correlated with less improvement of delusions ($N=14$, rho$=0.59$, $p=0.026$) and higher scores for thought disorders at the follow-up ($N=17$, rho$=0.73$, $p<0.001$) (Fig. 3), although the former result did not reach statistical significance after Bonferroni correction for multiple comparisons. There was an outlier with a high SAPS thought disorder score at the follow-up (Fig. 3, *right*), but the result did not change even when we excluded this patient ($N=16$, rho$=0.70$, $p=0.002$). No such correlations were found for the schizotypal patients (all $p>0.427$). The SANS subscale scores did not correlate with pituitary changes over time in both patient groups (all $p>0.100$).

A higher cumulative dose of antipsychotics during follow-up was significantly correlated with greater improvement of hallucinations (rho$=-0.57$, $p=0.035$) in schizophrenia, but there was no significant correlation between the cumulative dose and pituitary volume changes over time in the schizophrenia patients (rho$=-0.33$, $p=0.179$), schizotypal patients (rho$=-0.31$, $p=0.306$), or in the patient group as a whole (rho$=-0.25$, $p=0.175$). When we examined only the patients treated with atypical antipsychotics during follow-up ($n=18$), a higher cumulative dose of antipsychotics tended to correlate with less severe pituitary enlargement (rho$=-0.43$, $p=0.075$). The longitudinal pituitary changes did not correlate with illness (schizophrenia, rho$=0.25$, $p=0.312$) or medication (schizophrenia, rho$=0.21$, $p=0.406$; schizotypal, rho$=0.00$, $p=1.000$) duration.

Table 2
Intracranial and pituitary volumes in the study participants.

Brain region	Controls (11 males, 9 females)		Schizotypal patients (9 males, 4 females)		Schizophrenia patients (12 males, 6 females)	
	Mean	SD	Mean	SD	Mean	SD
ICV (cm^3)	1494	142	1593[a]	104	1477	137
Pituitary gland (mm^3)						
Baseline	719	159	703	141	768[b]	125
Second scan	688	164	762[c]	162	830[b]	134
% change/year	−1.8	2.0	2.7[c]	1.7	3.6[c]	5.0

Values indicate absolute volumes except % change/year values.
% change/year was calculated as follows: [100×(absolute volume at follow-up − absolute volume at baseline)/absolute volume at baseline]/inter-scan interval. Negative value indicates a decrease in volume.
The statistical analyses for longitudinal changes reported herein were based on % changes covarying with inter-scan interval.
For the results of analyses of covariance and post hoc tests, see text.
[a] Significantly larger than controls and schizophrenia patients.
[b] Significantly larger than controls and schizotypal patients.
[c] Significantly larger than controls.

4. Discussion

In the present longitudinal MRI study, we found a significant pituitary enlargement over time in both first-episode schizophrenia and schizotypal disorder patients compared with age- and gender-matched

Table 1
Demographic and clinical data of healthy controls, schizotypal disorder patients, and first-episode schizophrenia patients.

	Control subjects ($N=20$)	Schizotypal patients ($N=13$)	Schizophrenia patients ($N=18$)	Group comparisons
Male/female	11/9	9/4	12/6	Chi-square$=0.87$, $p=0.649$
Height at first scan (cm)	165.6 (7.2)	166.6 (9.5)	166.1 (6.7)	ANOVA: $F(2,48)=0.08$, $p=0.925$
Education (years)	15.1 (2.4)	12.6 (2.5)	13.0 (1.6)	ANOVA: $F(2,48)=6.58$, $p=0.003$
Parental education (years)	12.9 (2.8)	12.2 (1.7)	12.4 (2.1)	ANOVA: $F(2,48)=0.40$, $p=0.670$
Age at baseline scan (years)	23.2 (5.7) [18.0–38.0]	22.8 (5.0) [16.3–34.4]	23.1 (4.7) [17.9–31.9]	ANOVA: $F(2,48)=0.32$, $p=0.727$
Inter-scan interval (years)	2.6 (0.4) [2.0–3.2]	2.9 (0.8) [1.8–4.4]	2.7 (0.6) [1.3–3.9]	ANOVA: $F(2,48)=0.84$, $p=0.437$
Age of onset (years)	–	–	21.9 (4.7) [16.0–30.0]	–
Illness duration at baseline (months)	–	–	10.8 (9.7) [1–41] (median$=6.6$)	–
Duration of medication at baseline (months)	–	38.7 (61.0) [1.2–204] (median$=10.8$)	9.1 (10.4) [1–36] (median$=3.6$)	ANOVA: $F(1,29)=4.12$, $p=0.052$
Drug dose (haloperidol equivalent[a])				
At baseline (mg/day)	–	4.6 (3.8)	15.7 (11.9)	ANOVA: $F(1,29)=10.36$, $p=0.003$
At follow-up (mg/day)	–	5.7 (5.0)	13.2 (10.4)	ANOVA: $F(1,29)=5.86$, $p=0.022$
Mean dose during follow-up (mg/day)	–	5.4 (4.2)	9.9 (6.8)	ANOVA: $F(1,29)=4.52$, $p=0.042$
Cumulative dose during follow-up (mg)	–	5970 (6307)	10,213 (8974)	ANOVA: $F(1,29)=2.13$, $p=0.155$
Total SAPS score[b]				
Baseline	–	17.0 (9.7)	34.3 (25.2)	ANOVA: $F(1,25)=5.00$, $p=0.035$
Follow-up	–	12.1 (10.2)	20.8 (17.7)	ANOVA: $F(1,28)=2.49$, $p=0.126$
Total SANS score[b]				
Baseline	–	52.1 (21.6)	58.8 (24.2)	ANOVA: $F(1,25)=0.56$, $p=0.462$
Follow-up	–	41.8 (17.2)	38.9 (24.4)	ANOVA: $F(1,28)=0.13$, $p=0.718$

Data are presented as mean (SD) [range]. SANS, Scale for the Assessment of Negative Symptoms; SAPS, Scale for the Assessment of Positive Symptoms.
[a] The different typical and atypical antipsychotic dosages are converted into haloperidol equivalents using the guideline by Toru (2001).
[b] Data missing for 4 patients (1 schizotypal and 3 schizophrenia patients) at the baseline and for 1 schizophrenia patient at the follow-up.

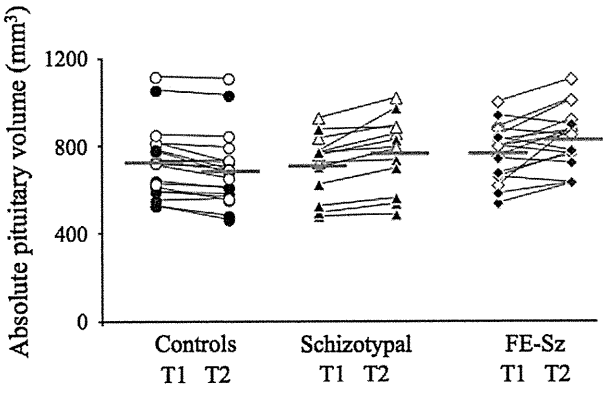

Fig. 2. Progressive volume changes of the pituitary gland in healthy controls, patients with schizotypal disorder, and first-episode patients with schizophrenia (FE-Sz). Values of baseline (T1) and follow-up (T2) scans in each subject are connected with a straight line. Horizontal bars indicate the means of each group. Male and female participants in each group were colored in black and white, respectively.

healthy controls. Our baseline findings replicated the findings by Pariante et al. (2004) in showing that first-episode schizophrenia patients had significantly larger pituitary volume compared with controls. Furthermore, in the schizophrenia group, greater pituitary enlargement over time was associated with less improvement of delusions and higher scores for thought disorders at the follow-up. These findings provide evidence that the pituitary gland exhibits ongoing volume changes during the early course of the schizophrenia spectrum, which might be a marker of state-related impairments.

To date, longitudinal volume changes of the pituitary gland have not been well documented, even for healthy subjects. Consistent with previous cross-sectional observations suggesting normal age-related pituitary atrophy after puberty (Lurie et al., 1990; MacMaster et al., 2007a; Takano et al., 1999), which are considered to reflect endocrinological change and ischemic degeneration of the anterior lobe (Kato et al., 2002), we directly demonstrated pituitary volume reduction over time in medically and psychiatrically healthy controls (mean = 23.2 years, 18 to 38 years of age) during a follow-up period of approximately 2.6 years. The rate of reduction in our sample (−1.8%/year) was less than but comparable to that in an earlier study by MacMaster et al. (2007b), who demonstrated a 3% decrease of pituitary volume in healthy controls (mean = 23.8 years, 12 to 32 years of age) over a 12-month follow-up.

In sharp contrast to the findings in healthy controls, both schizophrenia (3.6%/year) and schizotypal (2.7%/year) patients exhibited a substantial degree of ongoing pituitary enlargement over an approximately 3-year period. Pituitary volume changes in psychotic disorders might reflect HPA axis hyperactivity and a subsequent increase in the size and number of corticotrophs [cells producing adrenocorticotropic hormone (ACTH)], which could be explained by an activation of the hormonal stress response during psychotic experience (Pariante et al., 2004, 2005). The most common causes of pituitary enlargement [i.e., administration of estrogens, hypothalamic tumor, pregnancy, and primary hypothyroidism (Elster, 1993; Miki et al., 2005)] were excluded in our subjects. Age and gender have been reported to affect pituitary volume (Kato et al., 2002; MacMaster et al., 2007a), but the participants in this study were matched for these variables. The present findings might be consistent with the model of ongoing active pathological process related to HPA axis dysregulation in the course of schizophrenia spectrum disorders (Walker et al., 2008, 2010).

To our knowledge there have been only two MRI studies of longitudinal pituitary volume changes in psychotic disorders, which have yielded inconsistent findings such as a non-significant decrease (<2% over a 3-month period) in one study (Nicolo et al., 2010) and a 12% increase over 12 months in another study (MacMaster et al., 2007b) during the first episode of illness. Our finding of 3.6%/year pituitary enlargement in first-episode schizophrenia is similar to that of MacMaster et al. (2007b), although considerably less pronounced. These discrepancies could be the result of methodological and sample differences across studies [e.g., imaging techniques, gender ratios, and schizophrenia (MacMaster et al., 2007b) versus psychosis in general (Nicolo et al., 2010), medication, and other clinical factors]. As for the effect of medication, Nicolo et al. (2010) demonstrated a cumulative 3-month dose of atypical antipsychotics to be *negatively* correlated with pituitary volume changes in first-episode psychosis. Although not statistically significant, our results of correlational analyses are in line with their finding, suggesting that atypical antipsychotics may reduce pituitary volume in a dose-dependent manner in psychotic disorders (Nicolo et al., 2010), possibly due to suppression of HPA axis activity (Phillips et al., 2006; Scheepers et al., 2001; Walker et al., 2008). In contrast, MacMaster et al. (2007b) explained their finding of pituitary enlargement in drug-naïve patients following antipsychotic medication (especially prolactin-elevating drugs) as a consequence of an activation of prolactin-secreting cells. Owing to the lack of hormonal measures, however, it remains unclear whether pituitary expansion in their sample predominantly reflects the pathological

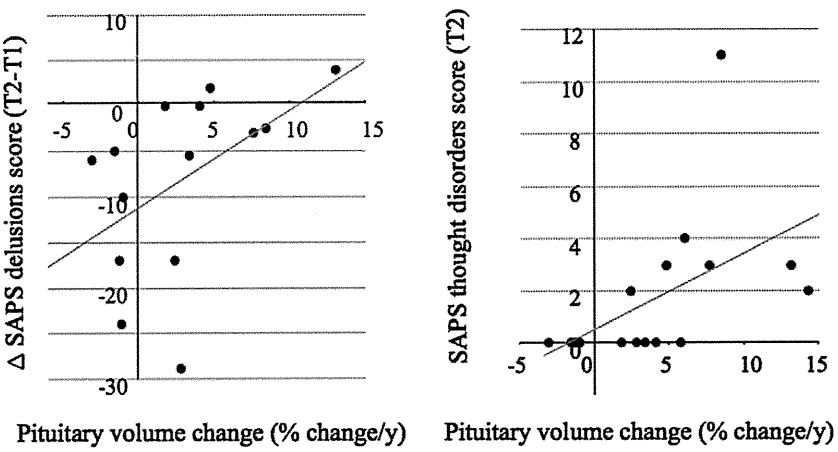

Fig. 3. Correlations between annual pituitary volume changes and absolute score changes of delusions between the baseline (T1) and follow-up (T2) scans on the Scale for the Assessment of Positive Symptoms (SAPS) (rho = 0.59, $p = 0.026$) (*left*) and SAPS thought disorder score at T2 (rho = 0.73, $p < 0.001$) (*right*) in first-episode schizophrenia patients. Annual pituitary volume change was calculated as follows: [100 × (absolute volume at T2 − absolute volume at T1)/absolute volume at T1]/inter-scan interval (in years). Positive values indicate increases in volume.

process of the disease itself or medication effects. Furthermore, a different breakdown of antipsychotics within the atypical group (Nicolo et al., 2010) as well as other psychotropics such as lithium (Bschor et al., 2002; Peiffer et al., 1991) or carbamazepine (Watson et al., 2004; Zobel et al., 2001) could differentially affect HPA function. On the other hand, our findings demonstrated that treatment response and severity of positive psychotic symptoms are also related to pituitary volume changes during the first episode of schizophrenia, consistent with recent cross-sectional MRI findings (Garner et al., 2009). Although the assessment of stress, anxiety, and cognitive or social impairments was not comprehensively undertaken in this sample, a possible effect of medication, positive psychotic symptomatology, and these other mediating factors on pituitary volume seem worthy of further examination. Furthermore, cross-sectional studies in chronic patients (illness duration>15 years) demonstrated normal (Tournikioti et al., 2007) or even decreased (Pariante et al., 2004) pituitary volume, suggesting its atrophy in the later course of the illness. Thus, it would be also worthwhile studying the pituitary changes over time in the chronic phase of psychosis.

In this study, schizotypal disorder patients exhibited similar pituitary enlargement over time to that of established schizophrenia patients, partly consistent with previous neuroendocrine investigations showing HPA hyperactivity in SPD (Mitropoulou et al., 2004; Mittal et al., 2007) and cross-sectional findings of pituitary enlargement in a larger schizotypal cohort (Takahashi et al., 2009). In combination with an association between elevated cortisol levels in SPD subjects and the severity of their clinical schizotypal signs (Walker et al., 2001), these findings suggest that the social distress related to schizotypal features (Dickey et al., 2005) could activate the stress response even without florid psychosis. On the other hand, a recent study of cortisol levels demonstrated longitudinal HPA changes in "at-risk" adolescents who subsequently developed psychosis (Walker et al., 2010). As schizotypal subjects have a higher incidence of developing psychosis (e.g., brief psychotic disorder) than the general population (Nordentoft et al., 2006), further study of the association of longitudinal pituitary volume changes with clinical course in a larger sample would allow us to test the hypothesis that HPA activity could trigger the expression of psychotic symptoms in vulnerable individuals (Walker et al., 2010).

A few possible confounding factors in this study should be taken into account. First, although our findings of ongoing pituitary volume changes might reflect state-related HPA axis dysregulation, we did not directly assess pituitary function. While our findings support the notion that atypical antipsychotics may reduce the pituitary volume, the pituitary gland is also considered to be sensitive especially to prolactin-elevating antipsychotics (MacMaster et al., 2007b; Mondelli et al., 2008). Thus, additional assessment of both pituitary volume and hormonal levels (e.g., cortisol, ACTH, and prolactin) is required. Second, the tracing protocol we used did not enable us to distinguish the anterior from the posterior lobe of the pituitary gland. However, the posterior pituitary that secretes oxytocin and vasopressin (Elster, 1993) constitutes less than 20% of the total pituitary gland and only tumors are associated with its enlargement (Krishnan et al., 1991; Mondelli et al., 2008). Third, despite sexual dimorphism of pituitary volume (males<females) reported in both healthy subjects and psychotic patients (MacMaster et al., 2007a; Takahashi et al., 2009), we could not reliably assess the effect of gender owing to the small sample size, especially for females. We found no overall effect of gender on longitudinal pituitary changes [males ($N=32$), mean $=-1.0$%/year; females ($N=19$), mean $=-1.7$%/year; ANCOVA, $F(1, 45)=2.27$, $p=0.139$], and the ANCOVA results for the longitudinal comparison reported herein did not alter even when we examined males and females separately [diagnosis effect for males, $F(2, 25)=5.04$, $p=0.015$; diagnosis effect for females, $F(2, 12)=5.03$, $p=0.026$]. However, further studies should examine the diagnosis-by-gender interaction on pituitary volume in a larger longitudinal sample. In addition, the current pituitary findings in schizotypal patients (no significant enlargement at baseline, smaller as compared with schizophrenia patients) are partly inconsistent with those in our previous cross-sectional study (Takahashi et al., 2009), which could be partly related to the small sample size. No correlations between the clinical symptoms and pituitary changes in schizotypal patients might be attributed to small sample size, mild and attenuated psychotic symptoms in this group, or substantial exposure to antipsychotics prior to the baseline scanning (median = 10.8 months). Finally, given that HPA axis functioning appears to be affected in various psychiatric populations, such as major depressive disorders (Axelson et al., 1992; Krishnan et al., 1991; MacMaster and Kusumakar, 2004), further investigation of the disease specificity of the pituitary findings is warranted, ideally in a longitudinal design across various stages.

5. Conclusion

In contrast to age-related pituitary volume reduction in healthy controls, we demonstrated ongoing volume expansion of the pituitary gland in both first-episode schizophrenia and schizotypal disorder patients. Furthermore, greater pituitary enlargement appears to be related to less improvement of positive psychotic symptoms during the early phases of schizophrenia. Although the effects of medication as well as hormonal levels should be further examined, the present longitudinal study complements and extends previous neuroendocrine and cross-sectional MRI findings in suggesting that the pituitary gland exhibits ongoing volume changes early in the course of the schizophrenia spectrum as a possible state-related marker.

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