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# Global hypomethylation of peripheral leukocyte DNA in male patients with schizophrenia: A potential link between epigenetics and schizophrenia

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**Abstract**

Genetic and epigenetic factors can potentially alter susceptibility to psychiatric disorders such as schizophrenia. In order to explore the effect of epigenetics on the pathogenesis of schizophrenia, we examined the global methylation level of leukocyte DNA from 210 patients with schizophrenia (124 males and 86 females) and 237 healthy subjects (108 males and 129 females). Methylated deoxycytidine (mC) content in peripheral leukocyte DNA was measured by high performance liquid chromatography (HPLC). We confirmed in the healthy subjects our previous finding that there are sex-dependent differences in mC content (males > females; beta = 0.319, $p < 0.001$), in addition to the effect of age (beta = −0.141, $p = 0.022$). We therefore used multiple regression to analyze the data from all subjects by sex, with age as a co-variant. In males, a tendency was observed toward lower mC content in patients than in controls (beta = −0.115, $p = 0.075$), with a significant effect of age (beta = −0.212, $p < 0.001$). This difference was more prominent in younger individuals. In females, no effect of age or disease status on mC content was observed. These results established that there is significant sex-dependent difference in the mC content of human peripheral leukocyte DNA, and raise the possibility that alterations in DNA methylation state are present in patients with schizophrenia.


*Keywords:* Schizophrenia; Epigenetics; DNA methylation; Gender effect; Peripheral leukocytes; HPLC

## 1. Introduction

Epigenetic regulation of cellular function is involved in a wide range of normal and pathological phenomena (Jones and Laird, 1999; Jaenisch and Bird, 2003). Immunodeficiency, centromeric region instability, and facial anomalies (ICF) syndrome and Rett syndrome are inherited disorders caused by mutations in the DNA methyltransferase 3B gene (*DNMT3B*), and the gene encoding methyl-CpG-binding protein-2 (*MECP2*), respectively. Both of these syndromes are also associated with mental dysfunction. In particular, patients with Rett syndrome show symptoms that partially resemble those observed in individuals with autism and schizophrenia (Robertson and Wolffe, 2000;

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Shahbazian and Zoghbi, 2002). X-linked mental retardation with alpha-thalassemia (ATR-X syndrome) is also caused by mutations of *ATRX*, which encodes a member of the SWI/SNF family of chromatin remodeling proteins (Gibbons et al., 2000). These findings suggest that the disruption of the normal epigenetic state plays a role in mental disorders, including schizophrenia (Petronis, 2004).

Epidemiological studies of twins and families demonstrated that genetic factors play a major role in the development of schizophrenia. The molecular etiology of the disease however remains enigmatic (Owen et al., 2000; Baron, 2001). The discordance rate between monozygotic co-twins is approximately 50%, indicating that environmental factors may cause differential patterns of gene expression in the brains of co-twins. Although the causes of the high discordance rate remain to be explored, DNA methylation has been proposed as a potential underlying mechanism (Tsujita et al., 1998; Kato et al., 2002).

DNA is demethylated in the early embryo, and re-methylated as embryogenesis proceeds. Once established, genomic methylation is generally very stable, but is infrequently subjected to unexpected, stochastic changes, or changes induced by environmental or intrinsic biological factors (Hsieh, 2000). Although the traditional view of DNA methylation is that it is not passed from parents to offspring, there is increasing evidence that some epigenetic signals may have partial meiotic stability and be transmitted from one generation to the next (Roemer et al., 1997; Morgan et al., 1999; Sutherland et al., 2000; Rakyan et al., 2002). Thus, DNA methylation and/or other epigenetic modifications of the genome could help to explain the ambiguity of inherited schizophrenia and the putative role of environmental factors in the etiology of the disease. To date, however, the role of DNA methylation in disorders such as schizophrenia has not been rigorously examined.

We have developed a model of schizophrenia (Nakamura et al., 2003), in which the genes involved in embryonic reconstruction and subsequent maintenance of DNA methylation are the primary genetic factors of schizophrenia. The presence of single nucleotide polymorphisms (SNP) in one or a combination of such genes could cause subtle changes in structure and function that would render the genome epigenetically altered. Suboptimal epigenetic modifications of the genome may increase vulnerability to stress, which in turn might alter susceptibility to schizophrenia. According to this model, methylation of genomic DNA would be altered not only in the brain, but also in other tissues and cells, including peripheral leukocytes. Thus, the model predicts that the methylation level of leukocyte DNA in subjects with schizophrenia may be different from that of unaffected subjects. The goal of the present study was to examine whether differences in DNA methylation exist between subjects with schizophrenia and healthy individuals. We obtained blood samples from subjects with and without schizophrenia and analyzed DNA methylation in peripheral leukocytes using HPLC (Fuke et al., 2004).

## 2. Methods and materials

### 2.1. Subjects and DNA extraction

The schizophrenic subjects consisted of 210 patients (124 males and 86 females) who satisfied DSM-IV criteria for schizophrenia. Control subjects consisted of 237 healthy volunteers (108 males and 129 females). The ethnicity of all the subjects was Japanese. Schizophrenic patients were recruited from inpatient or outpatient psychiatric wards of hospitals around Tokyo, Nagasaki, and Okinawa, and were under chronic pharmacological treatment with antipsychotics, although detailed data about their treatment were not always available. The clear history (past or present) of hallucinations, delusions, disorganized speech and negative symptoms were observed in 79 (53%), 78 (53%), 40 (27%) and 110 (74%) among 148 patients. Age of onset ranged from twelve to 61 years (mean ± SD: 27.3 ± 10.2 years) in those patients. Control subjects were recruited mostly from among the staff members of the hospitals. Their close relatives, their parents, siblings, and offspring, were interviewed by the psychiatrists (T.S., A.I., T.T., T.U., M.T., K.H., and Y.O.) among the co-authors of the current study and confirmed not to have psychiatric disorders. Written informed consent was obtained from all subjects. The study was approved by the ethics boards of the Universities of Tokyo, Nagasaki, and Ryukyu (Okinawa). Blood samples were obtained from the subjects and peripheral leukocyte DNA was extracted using the standard proteinase K/phenol method.

### 2.2. HPLC analysis

Global methylation levels of peripheral leukocyte DNA were evaluated by measuring mC content with HPLC. All reagents and conditions for HPLC analysis have been previously described (Fuke et al., 2004). All DNA samples were analyzed in duplicate, and we eliminated samples for which the difference in mC content between duplicate samples was greater than 3%. Care was taken to analyze samples from the patient group and from the control group during every experiment, to minimize the effect of experimental errors between groups. mC content is reported as the ratio of mC to the sum of 2′-deoxycytidine and mC (%).

### 2.3. Statistical analysis

Statistical analysis was performed using Stat View software (SAS Institute Inc., Cary, NC, USA). Effects of age, sex, and diagnosis (patient or control) on the mC content were evaluated using linear multiple regression. Correlation between age and mC content was evaluated using the Pearson correlation test. Results were considered statistically significant if the $p$ value was less than 0.05.

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## 3. Results

### 3.1. Effects of age and sex on mC content

Global DNA methylation in leukocytes decreases with age in rodents and humans (Drinkwater et al., 1989; Golbus et al., 1990; Wilson and Jones, 1983; Wilson et al., 1987). Sex-dependent differences in global methylation in peripheral leukocyte DNA also seem to exist (Fuke et al., 2004). In the current study, we first confirmed our previous observation of sex-dependent differences in global DNA methylation as well as the effect of age in 237 healthy subjects (108 males and 129 females) using linear multiple regression. As shown in Table 1 (a), the effects of sex and age were statistically significant in the healthy subjects: the effect of sex (males > females) was stronger (beta = 0.319, $p < 0.001$) than the effect of age (beta = −0.141, $p = 0.022$). The correlation between age and mC content was statistically significant in males (Pearson's correlation coefficient (CC) = −0.231, $p = 0.016$), whereas the correlation was not significant in females (Pearson's CC = −0.082, $p = 0.354$).

These statistical analyses were then repeated for 210 patients with schizophrenia (124 males and 86 females). As shown in Table 1 (b), the effects of sex and age were also statistically significant in this group, although the magnitudes of the sex and age effects were not as high in the patients as in the controls. Table 2 also shows that the sex difference was not as pronounced in the patients as in the controls. The correlation between age and mC content was statistically significant in male patients (Pearson's CC = −0.200, $p = 0.026$), but not in female patients (−0.045, $p = 0.680$), consistent with the results in the controls.

### 3.2. Comparison between patients and controls

Taking into account the significant effects of sex and age, we next conducted a linear multiple regression analysis by sex to test the effects of disease status (patient vs. control) on the mC content, controlling for the effect of age (Table 3). In males, a trend toward an effect of disease status was observed, although the effect did not reach the significance level of 5% (beta = −0.115, $p = 0.075$). The effect of age was statistically significant (beta = −0.212, $p = 0.001$). As shown in Fig. 1 and Table 2, the differences in global DNA methylation between male schizophrenic patients and healthy male subjects seemed to be more marked in individuals of young ages. No effect of age or disease status on mC content was observed in females (Table 3).

### 3.3. Comparison of mC content in male patients between DSM-IV-based subtype groups

Finally, we compared mC content in male patients classified into 4 groups according to DSM-IV criteria. Means and SD of mC content and ages were shown in Table 4.

Table 1
Effects of sex and age on 5-methylcytosine DNA (mC) content in 237 healthy controls (a) and 210 schizophrenia patients: (b) analysis using linear multiple regression

| | Standardized coefficients (beta) | $t$ | $p$ |
|---|---|---|---|
| (a) *Controls* | | | |
| Sex | 0.319 | 5.207 | <0.001 |
| Age | −0.141 | −2.308 | 0.022 |
| (b) *Patients* | | | |
| Sex | 0.142 | 2.076 | 0.039 |
| Age | −0.138 | −2.024 | 0.044 |

Table 3
Analysis by gender of the effects of age and disease status (patient vs. control) on mC content in 232 males (124 patients and 108 controls) and 215 females (86 patients and 129 controls) using linear multiple regression

| | Standardized coefficients (beta) | $t$ | $p$ |
|---|---|---|---|
| *Males* | | | |
| Age | −0.212 | −3.302 | 0.001 |
| Affected status | −0.115 | −1.788 | 0.075 |
| *Females* | | | |
| Age | −0.067 | −0.969 | 0.334 |
| Affected status | 0.035 | 0.506 | 0.614 |

Table 2
Comparison by age of mC content (%) between male and female patients with schizophrenia (SCZ) and healthy controls

| | | | <30 | <40 | <50 | <60 | 60≦ |
|---|---|---|---|---|---|---|---|
| Male | Control | mC (mean ± SD) | 4.082 ± 0.087 | 4.030 ± 0.071 | 4.026 ± 0.079 | 4.003 ± 0.042 | 4.038 ± 0.079 |
| | | Age (mean ± SD) | 26.04 ± 2.77 | 34.52 ± 2.81 | 43.46 ± 2.50 | 54.93 ± 2.52 | 64.63 ± 4.33 |
| | | $n$ | 24 | 27 | 26 | 15 | 16 |
| | SCZ | mC (mean ± SD) | 4.033 ± 0.069 | 4.020 ± 0.067 | 4.035 ± 0.074 | 4.003 ± 0.090 | 3.984 ± 0.106 |
| | | Age (mean ± SD) | 25.11 ± 3.14 | 34.62 ± 2.82 | 44.84 ± 3.18 | 53.75 ± 3.09 | 65.75 ± 4.59 |
| | | $n$ | 28 | 21 | 31 | 20 | 24 |
| Female | Control | mC (mean ± SD) | 3.981 ± 0.085 | 3.996 ± 0.052 | 3.986 ± 0.066 | 4.001 ± 0.080 | 3.969 ± 0.051 |
| | | Age (mean ± SD) | 23.84 ± 2.95 | 36.38 ± 2.42 | 45.13 ± 2.68 | 53.44 ± 2.62 | 67.58 ± 4.67 |
| | | $n$ | 32 | 21 | 30 | 27 | 19 |
| | SCZ | mC (mean ± SD) | 3.983 ± 0.087 | 4.002 ± 0.096 | 3.985 ± 0.075 | 4.009 ± 0.075 | 3.976 ± 0.077 |
| | | age (mean ± SD) | 25.73 ± 2.22 | 35.87 ± 2.97 | 44.65 ± 3.35 | 53.76 ± 3.11 | 65.44 ± 4.99 |
| | | $n$ | 15 | 15 | 17 | 21 | 18 |

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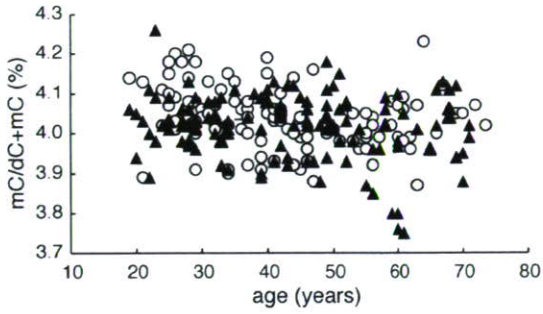


Fig. 1. Distributions of mC content (% mC) by age in males. The mC content in schizophrenic and healthy subjects was depicted by filled triangles and open circles, respectively.

Table 4
Mean ages and mC content (%) in male schizophrenia patients by DSM-IV-based subtypes

| Subtypes | *N* | Ages (years) | mC (%) |
|---|---|---|---|
| | | mean ± SD | mean ± SD |
| Disorganized | 48 | 40.1 ± 14.3 | 4.02 ± 0.08 |
| Paranoid | 39 | 45.1 ± 14.3 | 4.01 ± 0.09 |
| Residual | 11 | 57.1 ± 10.7 | 4.03 ± 0.10 |
| Undifferentiated | 26 | 44.8 ± 14.6 | 4.01 ± 0.07 |

We evaluated by ANOVA difference in mean mC content of the groups except residual type, because of higher mean age and lesser number of samples in that group than in the others. No statistically significant difference was found ($F = 0.21$, $p = 0.81$, $\lambda = 0.42$).

## 4. Discussion

The relationship between psychiatric disorders and methylation has long been debated, although the debate has not necessarily centered on DNA methylation (Reynolds et al., 1984). It is well recognized that methylation of genomic DNA is intimately involved in human diseases (Robertson and Wolffe, 2000). There are now several emerging lines of evidence implicating DNA methylation, and more broadly epigenetics, in psychiatric disorders (Impagnatiello et al., 1998; Tsujita et al., 1998; Tremolizzo et al., 2002). The results of the current study confirmed previous findings, that in healthy subjects, there is a higher mC content in males than in females (Fuke et al., 2004). Furthermore, we suggested that there is the presence of hypomethylation of leukocyte DNA in male patients with schizophrenia compared to healthy male subjects, and that the effect is more marked in younger patients. In contrast, we observed no evidence of hypomethylation in female patients with schizophrenia.

The observed hypomethylation in male schizophrenic patients in the current study supports a model in which a suboptimal or altered epigenetic state alters susceptibility to schizophrenia. Some of the major targets of DNA methylation are retrotransposons (Yoder et al., 1997). In the context of an altered epigenetic state, "stress"-induced demethylation of retrotransposons may restore their activities, which would result in altered regulation of nearby genes (Walsh et al., 1998; Morgan et al., 1999). In support of this model, previous studies in schizophrenic and depressive patients showed that these patients frequently have the genes responsible for the reduced methionine adenosyltransferase and/or methylenetetrahydrofolate reductase activity (Kelsoe et al., 1982; Morere et al., 1986; Smythies et al., 1986; Regland et al., 1994). These are enzymes of one-carbon metabolism involved in the synthesis of the methyl donor *S*-adenosylmethionine. However, it is also important to note that hypomethylation in male schizophrenics may be a secondary effect of antipsychotic medications. This may also explain the sex-disparity in the levels of DNA methylation between the patients and healthy controls. Male patients may have received multiple drugs and higher doses than female patients. Interestingly, our data (Fig. 1 and Table 2) suggested that there are higher correlations between global methylation and younger schizophrenia patients. This might reflect the high variability of global methylation in the older patients, which seems being in accordance with the observation by Fraga et al. (2005). It will likewise be important to examine whether the mC content of the brain DNA parallels that of leukocyte DNA in schizophrenic patients.

In a recent study by our lab, we found inter-individual differences in DNA methylation at some loci of the human endogenous retrovirus (HERV) K family of repetitive sequences in placentas and, to a lesser degree, in fetal livers. The inter-individual variation in methylation levels of HERV-K loci in the placentas was conserved in the livers (Shen et al., 2006). These results suggested that global methylation level might be a good indicator of the cell's capacity to maintain proper state of genomic methylation.

In contrast to males, we did not find a statistically significant difference in the mC content between female schizophrenic patients and healthy female controls. Sex hormones have been implicated in schizophrenia and brain development (Rao and Kolsch, 2003; Gogos and Van den Buuse, 2004; Huber et al., 2004, 2005), and female schizophrenia generally has a later onset and a better prognosis than male schizophrenia (Szymanski et al., 1995). The gender effect observed in the current study may be due to differences in the type and concentration of sex hormones between the two sexes, and raises the possibility that female sex hormones play a protective role in the pathology of altered or suboptimal epigenetic modifications.

To our knowledge, these results are the first indication that gender has an effect on global methylation of the mammalian leukocyte genome, although it has recently been shown that the inactive X-chromosome in females is hypomethylated in gene-poor regions (Wilson et al., 2006). Our results also suggested that there are differences in the methylation of leukocyte DNA in schizophrenic and healthy males. In the light of the fact that most methylated cytosines are derived from repetitive sequences such as satellite DNA and retrotransposons, it is clear that dif-

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ferences in methylation at the global level may be meaningful. However, the results of the current will need to be developed further.

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# Gender-Common and -Specific Neuroanatomical Basis of Human Anxiety-Related Personality Traits

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**Exploration of the relationships between regional brain volume and anxiety-related personality traits is important for understanding preexisting vulnerability to depressive and anxiety disorders. However, previous studies on this topic have employed relatively limited sample sizes and/or image processing methodology, and they have not clarified possible gender differences. In the present study, 183 (male/female: 117/66) right-handed healthy individuals in the third and fourth decades of life underwent structural magnetic resonance imaging scans and Temperament and Character Inventory. Neuroanatomical correlates of individual differences in the score of harm avoidance (HA) were examined throughout the entire brain using voxel-based morphometry. We found that higher scores on HA were associated with smaller regional gray matter volume in the right hippocampus, which was common to both genders. In contrast, female-specific correlation was found between higher anxiety-related personality traits and smaller regional brain volume in the left anterior prefrontal cortex. The present findings suggest that smaller right hippocampal volume underlies the basis for higher anxiety-related traits common to both genders, whereas anterior prefrontal volume contributes only in females. The results may have implications for why susceptibility to stress-related disorders such as anxiety disorders and depression shows gender and/or individual differences.**

**Keywords:** anxiety, gender, hippocampus, MRI, prefrontal cortex

## Introduction

Anxiety is a universal mechanism for generating adaptive behavior as it guides appropriate responses to environmental cues such as danger and threat; however, in humans, an excessive amount of anxiety predisposes individuals toward psychiatric conditions such as phobia, depression, and post-traumatic stress disorder (PTSD) (reviewed in Gross and Hen 2004). Recent structural magnetic resonance imaging (MRI) studies have revealed significant neural correlates of these anxiety and depressive disorders in hippocampus, amygdala, and prefrontal cortex (reviewed in Pitman et al. 2001; Hasler et al. 2004). However, whether or not brain functional/structural deviations represent a predispositional vulnerability to develop the disorders remains unclear.

A previous volumetric MRI twin study reported smaller-than-normal hippocampal volume as a preexisting vulnerability factor for PTSD after exposure to psychological trauma rather than a shrinkage resulting from strong and chronic stress (Gilbertson et al. 2002). Based on this notable finding, healthy individuals who are vulnerable to develop anxiety disorders should show smaller-than-normal hippocampal volume. Evaluating healthy individuals may have advantage in avoiding a number of potential confounds that could affect regional brain volume in studies of patients with anxiety and depression, such as alcohol abuse (Agartz et al. 1999), psychiatric comorbidity (Schuff et al. 2001), chronic illness (Sheline et al. 1999), and chronic medication (Vermetten et al. 2003). However, neuroanatomical correlates of human anxiety-related personality traits remain unclear.

Recent functional neuroimaging studies have revealed that individual differences in anxiety-related traits are associated with differences in neural response to emotional activation in the amygdala and prefrontal regions among healthy individuals (Grachev and Apkarian 2000; Hariri et al. 2002, 2005; Etkin et al. 2004; Milad et al. 2005; Pezawas et al. 2005; Most et al. 2006). In addition, a recent animal study reported a negative correlation between hippocampal volume and trait anxiety in rats with normal anxiety-related behavior (Kalisch et al. 2006). In contrast, a limited number of previous studies have examined relationships between brain morphological variability, a highly heritable trait marker (e.g., Lyons et al. 2001; Thompson et al. 2001), and anxiety-related traits in healthy human adults (Knutson et al. 2001; Pujol et al. 2002; Omura et al. 2005; Wright et al. 2006). Previous studies reported that high anxiety-related traits correlated with small whole brain volume ($n = 86$ [male/female = 38/48]; Knutson et al. 2001) and large anterior cingulate surface area ($n = 100$ [50/50]; Pujol et al. 2002). More recent studies employed computational morphological analysis to identify regional correlates of anxiety-related traits throughout the entire brain, although the study sample sizes were relatively small ($n = 41$ [19/22] in Omura et al. 2005; $n = 28$ [11/17] in Wright et al. 2006). Because the previous human studies employed relatively limited image processing methodology and/or small sample size as overviewed above, the relationship between regional brain volume and anxiety-related traits has not yet been examined in the whole brain in a study employing a large sample size.

Among healthy individuals, one of the major factors contributing to individual difference in anxiety-related traits is gender difference. Previous studies have reported sex dimorphism in both anxiety-related traits (e.g., Cloninger et al. 1993; Farmer et al. 2003) and brain anatomy (e.g., Good et al. 2001; Luders et al. 2004). Furthermore, a few studies have suggested gender differences in neural correlates of emotional modulation (Canli et al. 2002). The above findings suggest it is necessary to consider gender effects in attempting to uncover the neuroanatomical underpinnings of human anxiety-related personality traits.

The use of self-report questionnaires such as Temperament and Character Inventory (TCI) has been well established as a means to assess individual differences in behavioral traits (Cloninger 1987; Cloninger et al. 1993). Cloninger and

colleagues describe 4 dimensions of temperament in the TCI including harm avoidance (HA), a frequently measured anxiety-related personality trait, as a marker of genetic and biological origins (Cloninger et al. 1993). In accordance with the theory, previous studies have reported high heritability of HA (e.g., Farmer et al. 2003) and significant early environmental and genetic backgrounds, an example of the latter being serotonin transporter promoter polymorphism (5-HTTLPR) (e.g., Lesch et al. 1996). However, no specific genetic variants contributing to the traits have been conclusively identified (reviewed in Sen et al. 2004). Furthermore, previous studies have reported that individuals with panic disorder, generalized anxiety disorder (Starcevic et al. 1996), PTSD (Richman and Frueh 1997), and depression, as well as those with genetic vulnerability to depression (Farmer et al. 2003), scored high in HA. Therefore, the HA of TCI is suitable as a probe to index neuroanatomical correlates of individual differences in anxiety-related personality traits.

The present study was thus designed to use computational voxel-by-voxel morphometric analysis to explore the relationship between individual differences in HA scores and regional gray matter volumes in the hippocampus, amygdala, and prefrontal cortex as well as throughout the whole gray matter in 183 healthy young adults. Furthermore, the gender difference in the correlation between anxiety-related traits and regional gray matter volume was also examined. The possible confounding effects of aging, handedness, and psychiatric illness were controlled in order to identify neuroanatomical correlates of personality traits as directly as possible.

## Materials and Methods

### *Subjects and Clinical Evaluation*

One hundred and eighty three right-handed Japanese subjects (117 males/66 females), mainly college students, hospital staff, and their acquaintances, participated in the present study. Because the present study was concerned with trait aspects of brain morphology and personality, the age of the subjects was restricted to the third and fourth decades of life to minimize the effects of aging and the menopause on brain morphology. The socioeconomic status (SES) and parental SES were assessed using the Hollingshead scale (Hollingshead 1965). Handedness was assessed based on the Edinburgh Inventory (Oldfield 1971). The participants were interviewed by a trained psychiatrist (H.Y. or M.S.) to be screened for the presence or absence of neuropsychiatric disorders through the structured clinical interview for DSM-IV axis I disorder, non-patient edition (First et al. 1997). The exclusion criteria were current or past DSM-IV Axis I or II psychiatric disorder including alcohol/substance-related disorders in themselves, neurological illness, and traumatic brain injury with any known cognitive consequences or loss of consciousness for more than 5 min. All participants had to have IQ greater than 75. These interviews were performed on the same day as MR scanning. The ethical committee of the University of Tokyo Hospital approved this study. After a complete explanation of the study to the subjects, written informed consent was obtained.

### *Personality Assessment*

A valid Japanese translation (Kijima et al. 2000) of TCI (Cloninger 1987; Cloninger et al. 1993) was used for measuring the personality trait of each subject. Each subject completed a 240-item TCI questionnaire within 3 months before or after MR scan. In this study, we focused on the HA subscale of the TCI.

### *MRI Acquisition*

The method of 1.5-mm-slice high-spatial resolution MRI acquisition was the same as that of our previous study (Yamasue et al. 2003). Briefly, the MRI data were obtained using a 1.5-T scanner (General Electric Signa Horizon Lx version 8.2, GE Medical Systems, Milwaukee, WI). Three-dimensional Fourier transform spoiled gradient recalled acquisition with steady state was used because it affords excellent contrast between the gray matter and white matter in the evaluation of brain structures. The repetition time was 35 ms, the echo time 7 ms with one repetition, the nutation angle 30 degrees, the field of view 24 cm, and the matrix 256 × 256 (192) × 124. A trained neuroradiologist (H.Y. or O.A.) evaluated the MRI scans and found no gross abnormalities in any of the subjects.

### *Image Processing for Voxel-Based Morphometry*

Image processing for voxel-based morphometry (VBM) (Ashburner and Friston 2000; Good et al. 2001), a fully automatic technique for computational analysis of differences in local brain tissue volume throughout the entire brain, was conducted using SPM 2 (Institute of Neurology, London, UK). This method involves the following steps: 1) spatial normalization of all images to a standardized anatomical space by removing differences in overall size, position, and global shape; 2) extraction of gray and white matter from the normalized images; and 3) analysis of differences in local gray and white matter volume across the whole brain (Ashburner and Friston 2000). Spatial normalization to the standard anatomical space was performed in a 2-stage process. In the first step, each image was registered to the International Consortium for Brain Mapping template (Montreal Neurological Institute, Montreal, Canada), which approximates Talairach space. This step applied a 12 parameter affine transformation to correct for image size and position. Regional volumes were preserved while corrections for global differences in whole brain volume were made. The normalized images of all participants were averaged and smoothed with a Gaussian kernel of 8 mm full-width at half-maximum (FWHM) and then used as a new template with reduced scanner- and population-specific bias. In the second normalization step, we locally deformed each image of our entire group to the new study-specific template using a nonlinear spatial transformation. This accounts for the remaining shape differences between the images and the template and improves the overlap of corresponding anatomical structures. Finally, using a modified mixture model cluster analysis, normalized images were corrected for nonuniformities in signal intensity and partitioned using study-specific customized prior probability map into gray and white matter, cerebrospinal fluid, and background. To remove unconnected nonbrain voxels (e.g., rims between brain surface and meninges), a series of morphological erosions and dilations to the segmented images were applied (Good et al. 2001). In an intensity modulation step, voxel values of the segmented images were multiplied by the measure of warped and unwarped structures derived from the nonlinear step of the spatial normalization (Jacobian determinant). This step converts relative regional gray matter density to absolute gray matter density expressed as the amount of gray matter per unit volume of brain tissue prior to spatial normalization. The resulting modulated gray matter images were smoothed with a Gaussian kernel of 12 mm FWHM.

### *Statistical Analysis of VBM*

Statistical analyses were performed using an analysis of covariance model (Friston et al. 1990). To account for global anatomical variations, the intracranial volume calculated from VBM procedure was treated as a confounding covariate. To detect the neuroanatomical correlates of individual differences in HA, statistical analysis treated intracranial volume as confounding covariate and the score of HA in TCI as the covariate of interest. To test hypotheses with respect to regionally specific association with HA, the estimates were compared using 2 linear contrasts. The resulting set of voxel values for each contrast constituted a statistical parametric map of the *t*-statistic (SPM[*t*]). The SPM(*t*)s were displayed at an uncorrected threshold of $P < 0.001$ for graphical reporting. We only discuss results in the text and in tables that survive a correction at 0.05 for the search volumes. The statistics in the tables are transformed to a *Z*-score to make them more intuitive. The significance of each region was corrected for multiple comparisons using false discovery rate (FDR) because previous literature suggests that multiple hypothesis testing (Bonferroni type) family-wise error (FWE) correction tends to wipe out both false and true positives when

applied to the entire data in neuroimaging (Genovese et al. 2002). The innovation of FDR is that they control the expected proportion of the rejected hypotheses that are falsely rejected. Thus, the statistical significance level was set at FDR-corrected $P < 0.05$. Whereas significant effects were explored throughout the entire gray matter regions, small volume correction was employed in predicted regions based on previous literature: hippocampus (Gilbertson et al. 2002), amygdala (Hariri et al. 2002; Pezawas et al. 2005), and prefrontal cortex (Grachev and Apkarian 2000; Canli et al. 2002; Yamasue et al. 2003; Milad et al. 2005). In contrast to the whole gray matter exploration, FWE-corrected $P$ was conservatively employed to detect findings within the searched volumes (SVs) (hippocampus: 3.5 ml; amygdala: 2 ml; Prefrontal cortex: 60 ml, bilaterally).

Furthermore, the gender difference in the correlation between HA and regional gray matter volume was tested using the condition by covariates interaction analysis. This interaction analysis treated gender as a condition, the score of HA as the covariate of interest, and intracranial volume as confounding covariate. The threshold for statistical significance was the same as that in the correlational analysis between the score of HA and regional gray matter volume. Once a significant interaction was found, post hoc correlational analysis between the score of HA and regional gray matter volume was then conducted in each gender separately.

## Results

Although the group mean score of HA was higher in the female group than in the male group, a Mann-Whitney test revealed that the group difference did not reach statistical significance ($P = 0.58$). Whereas the score of HA showed no significant correlations with age, self-SES, parental-SES, and handedness ($0.032 <$ Spearman's rho $< -0.124$, $0.095 < P < 0.668$), Mann-Whiteny test showed significant gender differences in age ($P = 0.01$) and self-SES ($P = 0.002$). (Table 1) To control the gender differences in age and self-SES, the VBM interaction analysis between gender and HA employing these variables as confounding as covariates was added.

The VBM revealed that the score of HA showed a significant negative correlation with regional gray matter volume in the right hippocampus (peak coordinate = [34, -36, -6], $z = 3.57$, FWE-corrected $P = 0.005$ with 3.5 ml SV, cluster size = 1096 $mm^3$) (Fig. 1, Table 2). The regional gray matter volume in the other brain regions showed no significant correlation with the score of HA in the male and female combined group.

**Table 1**
Subject characteristics

| Variable | Male ($n$ = 117) | | Female ($n$ = 66) | | Mann-Whitney | |
|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | $Z$ value | $P$ |
| Demographic variables | | | | | | |
| Age (range) | 29.2 (21-40) | 4.1 | 27.8 (22-40) | 4.2 | −2.60 | 0.009 |
| Handedness (range)[a] | 95.7 (25-100) | 10.9 | 96.2 (50-100) | 9.8 | −1.34 | 0.18 |
| SES[b] | 1.44 | 0.5 | 1.77 | 0.7 | −3.07 | 0.002 |
| Parental SES[b] | 2.09 | 0.6 | 2.14 | 0.6 | −0.47 | 0.64 |
| TCI | | | | | | |
| HA | 16.4 | 7.2 | 16.7 | 6.6 | −0.56 | 0.58 |
| Novelty seeking | 22.3 | 5.9 | 22.3 | 5.3 | −0.17 | 0.87 |
| Reward dependence | 15.1 | 3.4 | 16.9 | 3.2 | −3.25 | 0.001 |
| Persistence | 4.7 | 1.8 | 4.6 | 1.6 | 0.00 | 1 |
| Self direcctedness | 29.6 | 6.5 | 31.0 | 6.4 | −1.19 | 0.24 |
| Cooperativeness | 28.9 | 5.2 | 30.4 | 5.3 | −1.87 | 0.061 |
| Self transcendence | 9.0 | 4.9 | 11.4 | 6.2 | −2.40 | 0.017 |

Note: SD, standard deviation.
[a]Determined using Edinburgh Inventory (Oldfield 1971): Scores greater than 0 indicate right handedness. A score of 100 indicates strong right handedness.
[b]Assessed using the Hollingshead scale (Hollingshead 1965). Higher scores indicate lower educational and/or occupational status.

A significant gender difference in the correlation with HA was found in regional gray matter volume of the left anterior prefrontal cortex ([-20, 56, -2], $z = 4.11$, FWE-corrected $P = 0.008$ with 60 ml SV, cluster size = 1016 $mm^3$). Consequently, post hoc correlational analyses showed a significant negative correlation between the score of HA and the regional gray matter volume in left anterior prefrontal cortex only in the female ([-18, 56, 2], $z = 3.58$, FWE-corrected $P = 0.046$ with 60 ml SV, cluster size = 632 $mm^3$) but not in the male subjects ([-18, 56, 2], $z = 1.83$, FWE-corrected $P = 0.82$ with 60 ml SV) (Fig. 2, Table 2). The interaction remained significant after the effect of aging, and self-SES was eliminated. The regional gray matter volume in the other brain regions, including right hippocampus ([34, -36, -6], $z = 0.88$, FWE-corrected $P = 0.55$ with 3.5 ml SV), shows no significant gender difference in correlation between HA and regional brain volume.

Neither significant correlation nor significant gender difference in the correlation was observed between HA and regional gray matter volume in amygdala of 12-mm FWMH smoothed images, although a trend level negative correlation was found in the left amygdala of 4-mm FWHM smoothed images of female subjects ([-16, -12, -16], $z = 2.73$, FWE-corrected $P = 0.08$ with SV 2 ml).

To compare the current results with previous findings, the correlation with voxel density was additionally examined. Then, HA showed no significant correlation or gender difference in the correlation with the regional white or gray matter density, which should mainly reflect probability of tissue existence rather than regional brain volume.

## Discussion

The present study demonstrated evidence that smaller right hippocampus is a gender common neuroanatomical correlate of higher anxiety-related traits in a relatively large sample of young healthy individuals. Of note, a personality trait, a behavioral index thought to have multiple, complex determinants, showed a statistically significant association with a localized brain region, the right hippocampus, which was common to both genders. In contrast, the current analysis also revealed that regional brain volume in the left anterior prefrontal cortex showed a negative correlation with HA that was present only in the female group.

The negative association between right hippocampal volume and anxiety-related traits revealed by the current study is in line with previous reports of smaller hippocampal volume in patients with PTSD (reviewed in Pitman et al. 2001) and depression (reviewed in Hasler et al. 2004). In particular, patients with long-lasting PTSD symptoms consistently demonstrated smaller-than-normal hippocampus volume, although several previous studies examining acute and shortly recovered patients with PTSD reported no significant volume decrease in patients with PTSD compared with healthy individuals (Bonne et al. 2001; Yamasue et al. 2003). In addition, a previous study reported that high HA predicts increased PTSD symptom severity (Richman and Frueh 1997). The current study reveals that small right hippocampal volume predicts high HA in healthy young individuals and further supports the suggestion by a twin study that smaller-than-normal right hippocampus is a preexisting vulnerable factor to develop long lasting and severe PTSD after exposure to psychological trauma (Gilbertson et al. 2002).

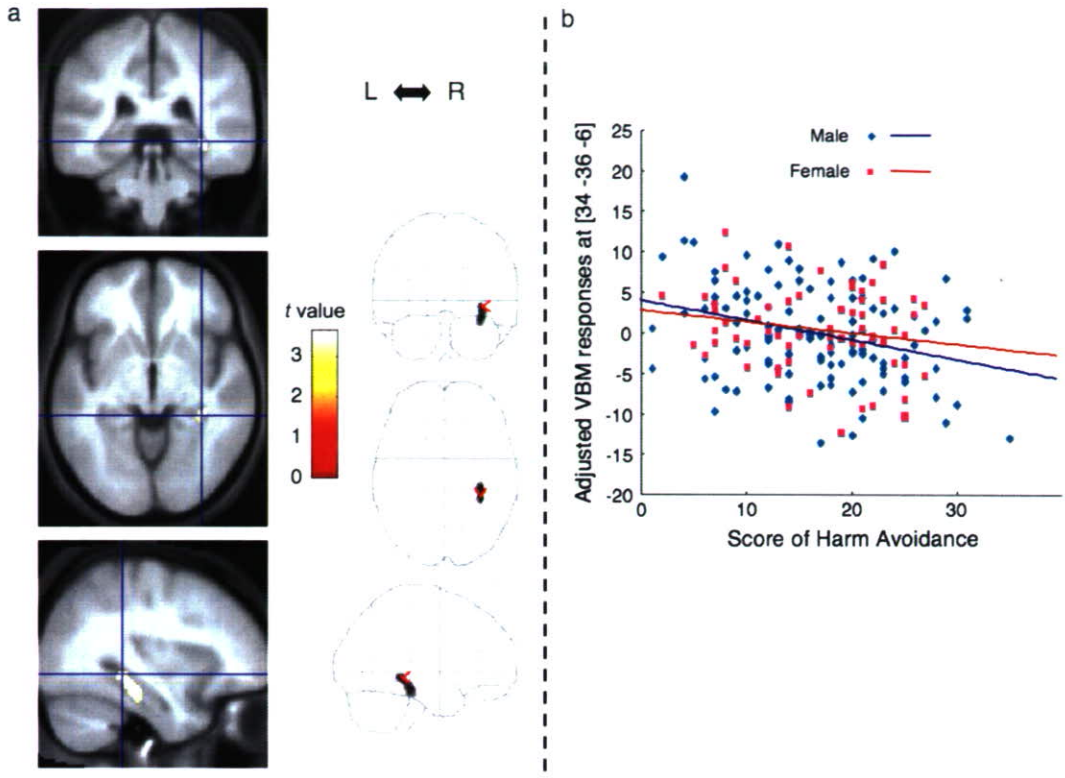


**Figure 1.** Gender-common negative correlation between HA and regional gray matter volume in the right hippocampus. (*a*) Gray matter regions showing significant correlations with the individual variability of HA were rendered in the Montreal Neurological Institute space. (Right) Statistical parametric map in the 3 orthogonal projections shows voxels where negative correlations with HA emerged. (Left) The gray matter voxels showing negative correlations with the individual variability of HA were rendered onto the averaged images of the whole sample ($N = 183$) (voxel threshold: uncorrected $P < 0.001$). L, left; R, right. (*b*) Scatter plots depicting correlations between regional gray matter volume at the peak voxel [34, −36, −6] and individual variability in HA in females ($N = 66$) and males ($N = 117$).

**Table 2**
Neuroanatomical correlates of HA

| Anatomical location | Peak coordinate | | | *Z* score | Correlation coefficient | Corrected *P* | Cluster size ($mm^3$) (voxel threshold: uncorrected $P < 0.001$) |
|---|---|---|---|---|---|---|---|
| | *x* | *y* | *z* | | | | |
| Negative correlation ($n = 183$) (Fig. 1) | | | | | | | |
| Right hippocampus | 34 | −36 | −6 | 3.57 | −0.26 | 0.005* | 1096 |
| Interaction with gender on the correlation between HA and regional brain volume** ($n = 183$) | | | | | | | |
| Left anterior prefrontal (Fig. 2)[a] | −20 | 56 | −2 | 4.11 | — | 0.008** | 1016 |
| Post hoc analyses: Negative correlation in female ($n = 66$) | | | | | | | |
| Left anterior prefrontal (Fig. 2)[b] | −18 | 56 | 2 | 3.58 | −0.43 | 0.046** | 632 |

[a]In contrast to the anterior prefrontal, the interaction with gender on the correlation between HA and right hippocampal volume did not reach statistically significant level ([34, −36, −6], $z = 0.88$, corrected $P = 0.55$ with 3.5 ml SV).
[b]In contrast to the correlation in female, the correlation between HA and left anterior prefrontal volume did not reach statistically significant level in male ($n = 117$, [−18, 56, 2], $z = 1.83$, corrected $P = 0.82$ with 60 ml SV).
*FWE-corrected *P* with 3.5 ml SV.
**FWE-corrected *P* with 60 ml SV.

The current study is also consistent with previously reported associations between anxiety-related traits and hippocampal function and chemical condition in healthy human subjects (Gallinat et al. 2005) and experimental animals (Kalisch et al. 2006). Gallinat et al. (2005) reported a significant correlation between lower *N*-acetylaspartate, a putative neural integrity marker, and higher trait anxiety, using MR spectroscopy and state and trait anxiety inventory, in 38 healthy subjects. Kalisch et al. (2006) recently reported a negative correlation between hippocampal volume and trait anxiety in normal anxiety-related behavior rats, although they found a positive correlation in extreme anxiety-related behavior rats. Animal studies (e.g., Nakao et al. 2004) and recent functional MRI studies (e.g., Dolcos et al. 2004; Strange and Dolan 2004) further reported a modulating role for hippocampus in processing of emotional memory interacting with amygdala. The current human in vivo finding is consistent with these suggestions. The present study identified possible involvement of the hippocampus in trait anxiety even at the brain structural level, a static trait marker, in healthy young human individuals.

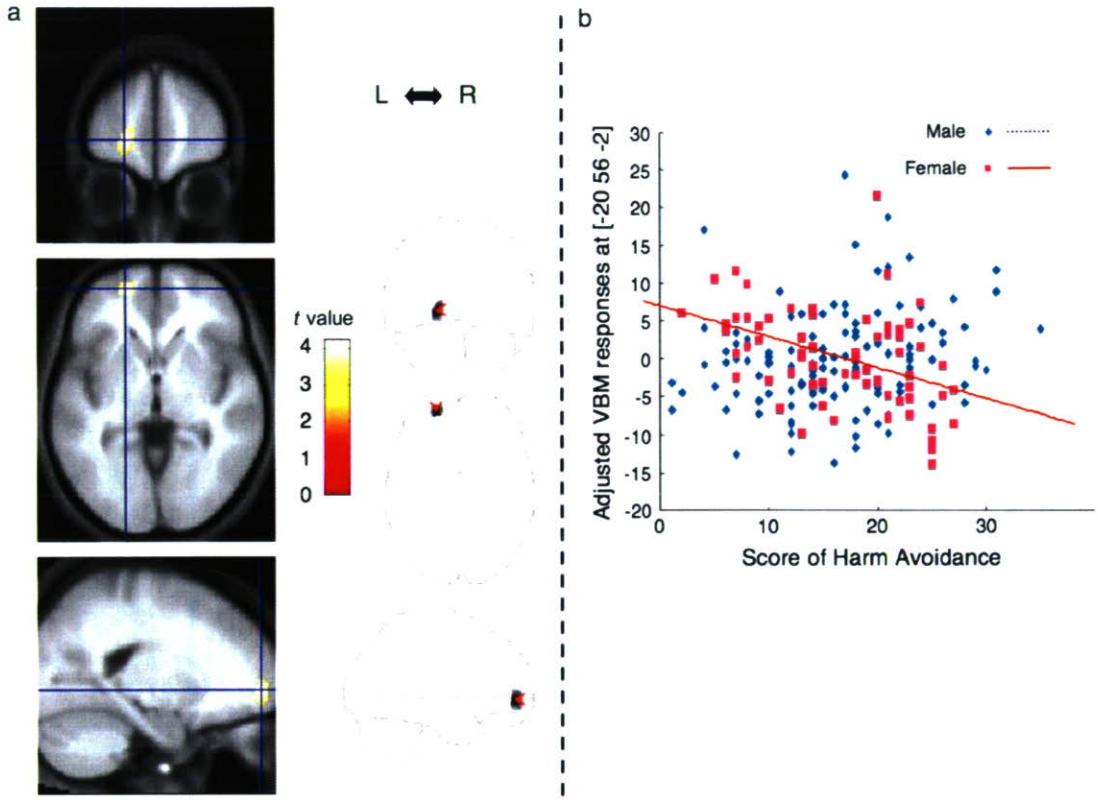


**Figure 2.** Female-specific negative correlation between HA and regional gray matter volume in the left anterior prefrontal cortex. (*a*) The gray matter regions where interaction between gender and HA was found are rendered in the Montreal Neurological Institute space. (Right) Statistical parametric map in the 3 orthogonal projections shows voxels where interaction between gender and HA emerged. (Left) The gray matter voxels showing interactions between gender and the individual variability of HA were rendered onto the averaged images of the whole sample ($N = 183$) (voxel threshold: uncorrected $P < 0.001$). (*b*) Scatter plots depicting correlations between regional gray matter volume at the peak voxel [−20, 56, −2] and individual variability in HA in females ($N = 66$) and males ($N = 117$).

Moreover, the present study revealed a significant female-specific association between HA and regional volume in the left anterior prefrontal cortex. Previous postmortem and brain activation studies have reported a similar location to that in the current study, anterior prefrontal as well as frontopolar cortex, as a neural substrate of emotional modulation, anxiety, and depression. A previous postmortem brain study reported a significant decrease in growth-associated protein levels and related mRNA expression in anterior prefrontal cortex of suicide brains compared with controls (Hrdina et al. 1998). In addition, Merali et al. (2004) reported that corticotropin-releasing hormone (CRH) levels were elevated in frontopolar and dorsomedial prefrontal cortex of suicide victims relative to the comparison group. Conversely, using quantitative polymerase chain reaction analyses, it was observed that mRNA for CRH1 receptors was reduced in frontopolar cortex of suicide brains. The same research group (Merali et al. 2006) further reported that immunoreactivity levels of CRH among brains of suicides were elevated in several brain regions including frontopolar cortex. Using single-photon emission-computed tomography, Segawa et al. (2006) reported that an improvement of depressive symptom severity due to electroconvulsive therapy showed a correlation with the change in regional cerebral blood flow of left frontopolar cortex. Papousek and Schulter (2002) reported that a frontopolar activation revealed by electroencephalography is related to emotional modulation. Urry et al. (2006) also revealed an activation of anterior prefrontal cortex (Brodmann's area 10) using functional MRI, which was associated with regulation of negative affect. Of note, Wright et al. (2006) recently found an inverse correlation between neuroticism scores and cortical thickness of the anterior portion of the left orbitofrontal cortex in a location close to that of the current finding. Moreover, related to sex dimorphism, a recent lesion study reported a gender difference in the role of the anterior portion of ventromedial prefrontal cortex in emotional and social dysfunction (Tranel et al. 2005). In addition, it was further reported that both ongoing self evaluation of emotional experience and subsequent memory performance for the highly emotionally arousing pictures showed correlations with activations in more extensive brain regions including anterior cingulate cortex of women than in those of men (Canli et al. 2002). They suggested that greater overlap in brain regions sensitive to current emotion and contributing to subsequent memory may be a neural mechanism for emotions to enhance memory more powerfully in women than in men. The present study supports this notion and further extends these gender differences in emotional processing at the brain structural level. Individual differences in emotional modulation in females are more likely to reflect individual differences at the level of brain structure. The present findings at least partially explain individual and gender differences in the susceptibility to develop anxiety and depressive disorders. Female individuals with smaller anterior prefrontal cortex as well as higher anxiety-related

traits might be more susceptible to depression or anxiety disorders.

Regional brain volume in amygdala showed no significant correlation with the score of HA in the current study sample, which is consistent with previous studies reviewed below. For example, Hariri et al. (2002) reported no significant association between amygdala responsivity/morphology and individual differences in HA, although they reported genetic contribution (5-HTTLPR) to amygdala responsivity to fearful stimulus (Hariri et al. 2002; Pezawas et al. 2005). Similarly, Omura et al. (2005) found no significant correlation between amygdala volume and neuroticism using VBM in 41 healthy individuals, although they reported a significant association between gray matter concentration and neuroticism without intensity modulation. In addition, Wright et al. (2006) found no correlation between amygdala volume and neuroticism in 28 healthy subjects. Previous studies, most of which reported smaller-than-normal hippocampus, have consistently revealed no volumetric abnormality involving the amygdala in patients with PTSD (reviewed in Rauch et al. 2006), although one study reported small amygdala volume in cancer survivors with intrusive recollections compared with those without such symptom (Matsuoka et al. 2003). However, the reasons for a consistent lack of association with HA in the amygdala in these studies including ours, and of structural MRI reports of reduction in amygdala volume in PTSD, remain unclear. In consistent with the current results, recent lesion studies suggested that the human amygdala may be recruited during phenomenal affective states in the intact brain but is not necessary for the production of these states (Anderson and Phelps 2002). Thus, the failure of amygdala size to relate to anxiety trait scores in our study may fit well with the hypothesis that human amygdala may not be critical for emotion per se. Another possibility may be that the functional heterogeneity of the amygdala, which is divided into functionally distinctive subnuclei, might obscure the association. This speculation may be supported by our finding of a subthreshold association between HA and the left amygdala of 4-mm FWHM smoothed images of female subjects. To clarify this issue, therefore, studies with larger sample sizes for both genders are necessary.

Here we address the methodological considerations and limitations of the current study. First, cross-sectional study design cannot access the etiology of the neuroanatomical correlates of anxiety-related traits, although the current study design minimized aging and pathological effects on regional brain volume. Second, the number of male subjects was disproportionally larger than that of females. Thus, the ability to identify female-specific correlation might be weaker than that to find male-specific correlation, although in fact the present analysis revealed a female-specific correlation. Third, gender differences in age and SES were observed in the current study sample, although statistical analyses controlling these effects preserved a significant interaction with gender on the association between HA and regional volume in anterior prefrontal cortex. Fourth, the specificity of current hippocampus findings for HA was limited in the current study because a significant positive correlation between the regional gray matter volume in right hippocampus and the score of reward dependence of TCI in the combined subjects ([34, -30, -8], FWE-corrected $P = 0.002$ with 3.5 ml SV, $z = 3.79$) was found with no significant gender difference in the correlation.

In conclusion, the present study provides evidence that smaller right hippocampal volume contributes to higher anxiety-related traits in human individuals. These results are consistent with a previous study reporting small right hippocampal volume as predisposing factor to develop stress-related disorders. Together with the female-specific relationship between left anterior prefrontal cortical volume and HA, the present findings may at least partly explain individual and gender differences in the susceptibility to develop anxiety and depressive disorders.

## Notes

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特集

# 気分障害研究の最前線

# 気分障害のエピジェネティクス

加藤忠史*

**KEY WORDS**

・双極性障害
・大うつ病
・エピジェネティクス
・DNA メチル化
・ヒストン修飾

**SUMMARY**

うつ病はストレス脆弱性とストレスの相互作用で発症すると考えられる．ラットにおいて，養育の影響によるストレス脆弱性がグルココルチコイド受容体遺伝子の DNA メチル化変化を介しているとの説が提唱されている．抗うつ薬や電気けいれん療法は，BDNF（脳由来神経栄養因子）遺伝子のプロモーターにおけるヒストン修飾の変化を介していると報告されている．双極性障害では，HDAC（ヒストン脱アセチル化阻害酵素）阻害を介して DNA メチル化を低下させる作用をもつバルプロ酸が躁状態に有効である一方，DNA メチル化を促進する S-アデノシルメチオニンが双極性うつ病に有効であるとの報告があることなどから，DNA メチル化の関与が示唆されている．双極性障害患者死後脳における膜結合型 COMT のプロモーターの DNA メチル化変化の報告もあるが，方法論的に疑問が残る．双極性障害に関して不一致な一卵性双生児の研究から，罹患双生児で PPIEL の低メチル化が見出され，症例対照研究では，双極Ⅱ型障害で PPIEL の低メチル化がみられた．気分障害におけるエピジェネティクス研究は始まったばかりであり，更なる研究が必要である．

## 1．エピジェネティクスとは

エピジェネティクスとは，DNA 塩基配列以外で，細胞から細胞に受け継がれ，遺伝子発現に長期的に影響する要因，およびそれを研究する学問のことをいう．こうしたメカニズムの代表が DNA メチル化とヒストン修飾である．この両者は密接に関連しており，一方が他方を変化させるという相互関係がある．

### 1）DNA メチル化

ゲノム DNA は，シトシンとグアニンが連なっている配列（CpG 配列という）の部分で，シトシンがメチル化修飾を受ける．シトシンはメチル化されてもグアニンと対合することには変わりないため，DNA 複製には影響せず，アミノ酸配列にも影響しない．

ゲノムには CpG アイランドと呼ばれる，C-G 配列が連なって存在している領域があり，こうした領域はしばしばプロモーターとして機能している．その細胞でよく発現している遺伝子のプロモーターの CpG アイランドでは，CpG 配列のシトシンがメチル化されていないことが多い．逆に DNA メチル化を受けている CpG アイランドは，メチル化 DNA 結合蛋白との相互作用などのメカニズムを介して，転写因子との相互作用が阻害され，一般に遺伝子発現が抑制される[1]．

細胞分裂時，ゲノムの DNA メチル化状態は DNA メチルトランスフェラーゼ（DNMT）により娘細胞へと

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コピーされる．DNAの二本鎖が二つに分かれ，相補鎖が形成された際には，Cm（メチル化シトシン）-Gと対合するのはC-Gである．しかし，一方の鎖のCpGのシトシンがメチル化されていた場合に反対鎖のシトシンをメチル化する酵素であるDNMTが存在することにより，ゲノムのメチル化状態を完全にコピーすることができる．配偶子形成の際，インプリンティング領域以外はDNAメチル化がほぼ消去され，発生の間に再び組織特異的なメチル化（*de novo*メチル化）を受け，リプログラミングされる．ある程度発生が進んでからは，DNAメチル化は安定と考えられる．

### 2）脳内DNAメチル化の年齢依存性

最近，ヒト大脳皮質における50の遺伝子のDNAメチル化状態を，妊娠17週から104歳に至る幅広い年齢層の125名で解析した論文が報告され，初めてヒト脳におけるDNAメチル化の年齢依存性の一端が明らかにされた．その結果，多くの遺伝子で年齢依存的なDNAメチル化状態の変化がみられ，年齢依存性変化には以下の4パターンがみられることがわかった[2]．

1）加齢とともに次第にメチル化されていく遺伝子（*HOXA1*など8遺伝子）

2）10歳頃まで（ほとんどは新生児期）にDNAメチル化が急速に増加し，その後は安定である遺伝子（*PAX8*など18遺伝子）

3）50歳以降，一部の者で急速にDNAメチル化が増加する遺伝子（*HGMT*の1遺伝子のみ）

4）10歳頃までにDNAメチル化が低下し，その後は安定している遺伝子（くり返し配列Aluのみ）

5）明確な年齢依存性のない遺伝子（その他）

このように，新生児期から10歳頃までに脳のDNAメチル化は大きく変動するようである．これが脳の細胞構成や解剖学的変化によるのか，各神経細胞における変化なのかなど，まだ不明な点は多いが，0～10歳の間にこのように脳のDNAメチル化状態が大きく変化するとすれば，その間の環境が，生涯にわたり脳内のDNAメチル化状態に永続的な影響を与える可能性も考えられる．

### 3）ヒストン修飾

ヒストンは，DNAをコンパクトに折り畳む際に必要とされる蛋白で，ヒストンの修飾状態はDNAのほぐれ方に影響することで，転写因子とDNAの相互作用を変化させ，遺伝子発現を制御する．ヒストンは，最もよく研究されているアセチル化の他，メチル化，スモイル化，ユビキチン化など，種々の修飾を受ける[3]．ヒストンH3の9番目および14番目のリジン残基がアセチル化されると，クロマチンが開放された状態となり，転写が活性化される，といった所見が代表的なものである[4]．

ヒストンの修飾状態が細胞分裂に際して保存されるメカニズムは明らかにされていない．有力な説の一つは，細胞分裂時にDNAの2本鎖がそれぞれに分かれる際，H3が2つとH4が2つという4量体であるヒストンが，H3-H4という2量体ずつに分かれ，DNAの相補鎖を合成する際に，ヒストンの修飾状態もコピーされるとの考え方である．しかし，4量体ヒストンにおいて，H3-H3の相互作用が強固であることから，このように対称な形でヒストンが分離することは化学的にはあり得ないと考えられていた．最近，ヒストンシャペロンであるCIAがH3，H4と相互作用することによって，ヒストンの修飾状態が複製される可能性が示された[5]．しかしながら，ヒストンの修飾状態を複製するメカニズム自体は解明されておらず，ヒストン修飾状態が，細胞分裂時に伝達されて，エピジェネティックシグナルとして働いているかどうかは，いまだ証明されているとは言えない．

## 2　うつ病とエピジェネティクス

### 1）遺伝環境相互作用とエピジェネティクス

うつ病は，遺伝子と養育環境などの相互作用によりストレス脆弱性が形成された者がストレスに曝された時に発症する疾患と考えられる．こうした遺伝・環境要因は，疫学的な大規模研究により明らかにされ，特にセロトニントランスポーターのプロモーター多型（HTTLPR）とストレス，あるいは虐待との遺伝環境相互作用の報告が有名である[6]．しかし，その後の4,175名という大サンプルの研究では確認されていない[7]．

エピジェネティクス研究は，こうした遺伝環境相互作

用の結果として細胞・組織レベルに刻印された持続的な変化を検出することができると期待される[8].

### 2）養育によるストレス脆弱性のエピジェネティック記憶

Meaney らのグループ[9]は，母子分離などの人工的な操作以外で養育が仔に与える影響を研究するため，高養育（high licking and glooming：High LG）ラットから生まれた仔ラットと，低養育（Low LG）ラットから生まれた仔ラットを比較するというパラダイムで研究を進めてきた．その結果，High LG と Low LG の母親の仔では，成熟後の母性行動やストレスに対する脆弱性が異なり，これが非遺伝的に伝達されることを明らかにした．

彼らはその分子メカニズムに関して研究を進めた結果，以下の可能性を示した．出生直後に十分な母性行動を受けた仔ラットではセロトニンが放出され，セロトニン 7 受容体を介して転写因子 NGFI-A が誘導される．これが海馬グルココルチコイド受容体（GR）上流配列に作用すると，母性行動を受けた仔ラットは，GR 上流配列に NGFI-A が結合しているため，DNA メチル化を免れ，GR の発現量が保たれる．一方，十分な母性行動を受けなかったラットでは，生涯にわたり海馬 GR 上流配列が DNA メチル化され，GR 発現量が低下し，これが仔ラットの行動に長期的な影響を与える．histon deacetylase（HDAC）阻害薬で，ヒストンアセチル化の増加を介して DNA メチル化を低下させる薬剤，トリコスタチン A を脳室内投与すると，Low LG ラットの仔におけるグルココルチコイド受容体遺伝子の DNA メチル化が低下し，それに伴ってストレス脆弱性が改善する[10].

この研究は，それまで仮想的なものであったエピジェネティック記憶を現実に証明した初めての研究といえる．しかしながら，その後他のグループからこの所見が追試されたとの報告はなされておらず，更なる検討が必要と思われる．

もしこの所見が確認されれば，ヒトでも同様の現象が存在し，うつ病の発症脆弱性と関係しているのかどうかを解明していくことが必要であろう．

### 3）抗うつ治療とヒストン修飾

Nestler らのグループ[11]は，抗うつ薬および電気けいれん療法のメカニズムにヒストン修飾の変化が関与すると考えて研究を進めている．慢性社会的敗北ストレスを受けたマウスでは，brain-derived neurotrophic factor (BDNF)-Ⅲおよび-Ⅳという転写物が低下し，これらのプロモーターにおけるヒストンメチル化（発現に対し抑制的に作用する）が増加していた．イミプラミンの慢性投与は，こうした BDNF 低下に拮抗し，これらのプロモーターのヒストンアセチル化（発現に対し促進的に作用する）を増加させた．イミプラミンのヒストンアセチル化増加作用は，HDAC5 の低下を介しており，ウイルスベクターにより HDAC5 を海馬に発現させると，イミプラミンの行動学的作用（社会的敗北ストレスによる社会的相互作用低下の改善）が消失した．

この研究で観察されたヒストン修飾状態の変化は，単に BDNF の遺伝子発現状態を反映したものにも思え，ヒストンの修飾状態を調べることにどれだけの意味があるのかという疑問も残る．彼らは，イミプラミンによるヒストンの変化が，コントロールマウスへの投与ではみられず，社会的敗北ストレスを受けたマウスのみでみられたという点において，BDNF の変化とは異なっていると考えているようである．

一方，電気けいれん療法（electroconvulsive therapy：ECT）による研究では，c-Fos，CREB，BDNF のヒストン H4 アセチル化は，これらの mRNA のレベルとよく相関し，単回の ECT でも変化する一方，H3 のヒストンアセチル化はより選択的であり，慢性 ECT 施行による BDNF の増加はプロモーターⅢおよびⅣの H3 アセチル化により制御されていると報告された[12]．このように，ECT の作用が長期に持続するメカニズムとして，ヒストン修飾の変化の関与が示唆されている．

### 4）双極性障害とエピジェネティクス

#### ①遺伝学的所見

遺伝子が片親性に発現する現象，すなわちゲノムインプリンティングは，DNA メチル化を介している．インプリンティングが関与する遺伝病では，その遺伝子が母親から遺伝したか，父親から遺伝したかによって，表現

型が大きく異なる．双極性障害でも，どちらの親から遺伝したかによって，発症するか否か，あるいは症状の重症度が異なる現象，すなわち parent-of-origin effect (POE) が報告されている[13)~15)]．双極性障害は，母方から遺伝する場合のほうが多い[15)]が，父方から遺伝した場合には重症で[16)]，発症年齢が早く[17)]，母方からの遺伝にくらべて同胞における罹患率が高い[18)]．

18番染色体との連鎖は，父方からの遺伝に関してのみみられる[19)~21)]．この所見にもとづいて，18番染色体の連鎖部位のインプリンティングを受ける遺伝子が探索されている[22)]．他にも，いくつかの領域，すなわち 13q12，1q41[21)]，2p24-21，2q31-q32，14q32，16q21-q23[23)]で POE を伴った連鎖が報告されているが，実際にインプリンティングが証明された例はない．

トリオサンプルにおける伝達不平衡テスト（transmission disequilibrium test：TDT）でも，母方からの遺伝と父方からの遺伝を分けて検討されるようになっており，こうした解析で関連を認めた報告もある[24)~26)]．しかしながら，父方からの遺伝のみに関して有意な関連が認められた HSPA5 について検討した結果，脳内で両アレルとも発現しており，インプリンティングを受けているとは考えられなかった[26)]．

**② 薬理学的所見**

バルプロ酸は躁状態に有効であるが，その作用メカニズムは諸説ある．バルプロ酸とリチウムが共通に神経保護作用や神経新生への作用を有していることが注目されているが，バルプロ酸は HDAC 阻害作用をもっており[27)]，これが神経保護作用[28)29)]や神経幹細胞の分化を促進する作用[30)]に関係していると考えられた．

S-アデノシルメチオニン（SAM）は，DNA メチル化反応においてメチル基を供与する分子であり，DNA メチル化を促進する作用がある．SAM がうつ病に有効であるとする報告は多く[31)]，特に双極性うつ病患者 11 名中 9 名に躁転を引き起こしたとの報告は注目される[32)]．ラットにおいて，SAM の前駆体である L-メチオニンの投与は，GR プロモーターのメチル化を促進し，High LG の行動学的影響に拮抗する[33)]．

**③ 死後脳の DNA メチル化解析**

最近 Abdolmaleky ら[34)]は，膜結合型 COMT（catechol-O-methyltransferase）の DNA メチル化状態を双極性障害患者死後脳で調べた．COMT には 2 つの mRNA アイソフォーム，膜結合型（membrane-bound COMT：MB-COMT）と可溶性（soluble COMT：S-COMT）があり，それぞれに異なったプロモーターがある．彼らは，双極性障害患者死後脳の前頭前野（ブロードマン 46 野）で MB-COMT のメチル化状態を，メチル化特異的 PCR（MS-PCR）により調べた．その結果，この領域はほとんどメチル化されていないが，メチル化された DNA 由来の PCR 産物がみられる割合が対照群（35 名中 60%）にくらべ，双極性障害患者（35 名中 29%）では有意に低かったと報告している．

一方，Dempster ら[35)]は，スタンレー脳バンクの 60 名の患者群および対照群において，パイロシーケンス法により S-COMT の DNA メチル化を調べ，患者群と対照群に差を認めなかったと報告している．

Abdolmaleky ら[34)]の用いた MS-PCR 法は元々インプリンティング病の診断のために用いられていたものであり[36)]，定量性がないため，PCR がかかった回数を比較しても意味がないであろう．

Costa らのグループ[37)]は Reelin 遺伝子（*RELN*）のメチル化が統合失調症に関与しているという説を唱え，統合失調症患者の死後脳で RELN の DNA メチル化が亢進していると報告した．しかしながら，このデータは，CpG でないサイトが強くメチル化されているというやや理解しがたい結果であった．この所見を前述の MS-PCR 法で確認したとの報告[38)]もあるが，メチル化感受性制限酵素処理と定量的ゲノム PCR 法を用いた検討では，患者群と対照群の差はみられなかった[39)]．Tochigi ら[40)]は，パイロシーケンス法を用いた定量法を確立し，段階希釈した標準サンプルによる検量線を測定して測定精度を検証したうえで患者サンプルで検討した結果，統合失調症患者の脳内で *RELN* はほとんどメチル化されていないことを見出した．Costa らのグループ[37)]の報告は，sodium bisulfite 処理が不完全であるなど，種々の技術的問題点によるアーチファクトである可能性が高いと考えられた．

### ④一卵性双生児双極性障害不一致例におけるDNAメチル化差異の探索

われわれは以前，双極性障害に関して不一致な一卵性双生児間で遺伝子発現差異のある遺伝子として*XBP1*を同定した[41]．ゲノム配列がほぼ同じである双生児の間で，一人だけが精神疾患を発症する理由としては，周産期の脳障害や，感染症など，さまざまな環境因が関与する場合がある．しかしながら，これらの双生児では，こうした原因が特定できない．このように，DNA配列そのものには違いがないはずの一卵性双生児間で大きな表現型の違いが存在する理由として，DNAメチル化の異常，すなわちepimutation（エピ変異）が考えられる[42]．しかし，これらの双生児では，*XBP1*の塩基配列，コピー数，およびDNAメチル化には違いがなかった．

そこで，双生児間の不一致の原因は他の遺伝子のDNAメチル化差異によるものと考え，双生児間でDNAメチル化が異なる遺伝子を特定するため，これまでがんの研究に用いられてきた研究手法であるmethylation-sensitive representational difference analysis（MS-RDA）法を応用した．この方法は，正常な細胞とがん細胞の間で，DNAメチル化が異なる部分をスクリーニングする方法として開発された[43]．メチル化感受性制限酵素を用いていることから，ゲノム塩基配列の差異の影響も受けてしまうため，これまでは主として同一人物の細胞での比較のために応用されてきた．しかしながら，一卵性双生児であれば，この方法を応用することで，双生児の間でDNAメチル化差異を特定できると考えられた．

MS-RDA法による検索で得られた多数のクローンの中から，リピート配列などを除き，CpGアイランドあるいはエクソン1付近の遺伝子として10クローンが選択された．さらにbisulfite法で確認されたものとして4つに絞られ，症例対照研究で確認されたものとして，2つの遺伝子に絞られた．うち一つであるSMS（spermine synthase）は，X染色体上の遺伝子であるため男女に分けて解析した結果，女性患者のみで関連していた．しかし，変化の方向が双生児と逆であること，メチル化状態と発現量が相関しないことなどから，その意義は不明であった．一方，もう一つの機能不明の遺伝子断片では，健常双生児ではほぼ完全にメチル化されており，罹患双生児では半分程度メチル化がはずれているという，より明確なDNAメチル化差異が見出された[44]．この遺伝子は，*PPIE*という既知遺伝子に配列が類似していたため，*PPIEL*（PPIE-like）と命名した．*PPIEL*のDNAメチル化は，多数例の被験者由来の培養リンパ芽球において，その遺伝子発現量とよく相関していた．

一卵性双生児間のDNAメチル化差異は，年齢とともに次第に増加することが報告されている[45]．したがって，一卵性双生児で差異がみられたからといって，それが病気と関係していると断定はできない．

そこで症例対照研究を行ったところ，双極Ⅱ型障害患者で，PPIELの低メチル化がみられた．この所見は，独立の双極Ⅱ型障害患者群でも確認された．また，培養リンパ芽球のメチル化状態は，末梢血液のDNAにおけるメチル化状態と相関していた．

PPIELは，齧歯類と霊長類が分かれた後に遺伝子重複により生じたと考えられる，霊長類特異的な遺伝子である．ヒト脳におけるPPIEL発現部位を調べたところ，下垂体および黒質に多く発現しており，ドパミン神経，あるいは神経内分泌への関与の可能性が考えられた．

今回の結果では，PPIELのDNAメチル化変化が，病気の原因なのか，結果なのかは不明であるが，PPIELが何らかの形で双極Ⅱ型障害の病態に関与している可能性が考えられた．

## おわりに

このように，気分障害におけるエピジェネティクス研究は，ようやくスタート地点に立ったところである．

前述のとおり，統合失調症研究においては，方法論的問題により不一致な結果が報告される事例があったことから，更なる方法論の洗練が必要と考えられる．これまで報告された定量的DNAメチル化解析法の中では，sodium bisulfite処理DNAのパイロシーケンス法による解析[35][40][44]，あるいはメチル化感受性制限酵素処理後に定量的リアルタイムPCR法でゲノムDNA定量を行う方法[39]が，比較的簡便かつ定量性の得られる方法と考えられる．

また，ゲノム解析と異なり，エピジェネティクス解析

では，どのような組織を用いるかが重要となる．脳を用いたとしても，脳内のニューロン/グリア比の影響を受ける可能性も考えられる．エピジェネティクス研究を目指すうえでは，患者死後脳を対象とする必要があるだけでなく，形態学をも視野に入れた解析が必要となっていくであろう．こうした面でも今後，更なる技術革新が必要と考えられる．

❖❖❖

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# XBP1 induces WFS1 through an endoplasmic reticulum stress response element-like motif in SH-SY5Y cells

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**Abstract**

XBP1 is a key transcription factor in the endoplasmic reticulum (ER) stress response pathway. In a previous study, we suggested a possible link between XBP1 and bipolar disorder, but its role in neuronal cells has not yet been clarified. Here we examined the target genes of XBP1, using DNA microarray analysis in SH-SY5Y cells transfected with an XBP1-expressing vector. Among the genes up-regulated by XBP1, the most significant *p*-value was observed for *WFS1*, which is an ER stress response-related gene. Examining the promoter region of *WFS1*, we found a conserved sequence (CGAGGCGCACCGTGATTGG) that is highly similar to the ER stress response element (ERSE). A promoter assay showed that this ERSE-like motif is critical for the regulation of *WFS1* by XBP1. An electrophoretic mobility shift assay suggested that XBP1 does not directly bind to this sequence. Our results demonstrate that *WFS1* is one of the target genes of XBP1 in SH-SY5Y cells.

**Keywords:** endoplasmic reticulum stress response, endoplasmic reticulum stress response element, WFS1, XBP1.

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The endoplasmic reticulum (ER) is responsible for protein folding within each cell. When unfolded proteins accumulate in the ER, the ER stress response begins. The ER stress response consists of four signaling cascades: (i) induction of ER chaperones such as *HSPA5* (*GRP78/BiP*), which promotes the folding of unfolded proteins (unfolded protein response, UPR); (ii) inhibition of protein synthesis; (iii) induction of the ER-associated degradation pathway; and (iv) induction of apoptosis (Yoshida 2004; Schroder and Kaufman 2005). The UPR begins when HSPA5 proteins are used to fold unfolded proteins. Dissociation of HSPA5 from ATF6 protein on the ER membrane causes cleavage of ATF6, and cleaved ATF6 protein induces the expression of ER chaperones and *XBP1*. In parallel, dissociation of HSPA5 from IRE1 protein on the ER membrane causes dimerization of IRE1, which splices *XBP1* mRNA. The spliced *XBP1* mRNA encodes an active form of XBP1 that strongly induces the expression of target genes such as ER chaperones (Yoshida 2004).

We previously showed, by DNA microarray analysis, that *XBP1* and *HSPA5* are down-regulated in the lymphoblastoid cells of monozygotic twins with bipolar disorder, compared with healthy co-twins (Kakiuchi *et al.* 2003). However, the role of the ER stress response pathway in the brain has not yet been clarified, except for the possible role of the UPR in the modulation of glutamate receptor trafficking (Shim *et al.* 2004; Vandenberghe *et al.* 2005).

To clarify the role of XBP1 in neuronal cells, we investigated the target genes of XBP1 in SH-SY5Y cells. First, we performed DNA microarray analysis in SH-SY5Y cells transfected with an XBP1-expressing vector, and we identified *WFS1* as the most up-regulated gene. Next, we found an ER stress response element (ERSE)-like sequence in the promoter region of *WFS1*. We further searched for ERSE-like sequences in the genes altered by XBP1 overexpression, and found that the gene for glycine cleavage system H protein (*GCSH*) is another candidate gene with an ERSE-like sequence in its upstream region. We confirmed by a

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*Abbreviations used*: DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; ER, endoplasmic reticulum; ERSE, endoplasmic reticulum stress response element; FAM, 6-carboxyfluorescein; FBS, fetal bovine serum; MEF, mouse embryonic fibroblast; MGB, minor groove binder; NSRE, nutrient-sensing response element; RMA, robust multiarray average; s*XBP1*, spliced-form *XBP1*; UPR, unfolded protein response.

 