Abstract

Chromosome 15q11-q13 has been a focus of genetic studies of autism susceptibility, because cytogenetic abnormalities are frequently observed in this region in autistic patients. An imprinted, maternally expressed gene within the region may have a role in autistic symptomatology. In the present study, we investigated the association between autism and the maternal expression domain (MED) in the region, containing the *UBE3A* and *ATP10C* genes, and the upstream imprinting center (IC), which mediates coordinate control of imprinted expression throughout the region. We analyzed 41 single nucleotide polymorphisms (SNPs) in 166 patients with autism and 416 controls from a Japanese population. As a result, a statistically significant difference after correction for multiple testing was observed between the patients and controls in the genotypic distribution of SNP rs7164989 (SNP8 in this study) located in *SNRPN*, whose promoter corresponds to the IC ($p = 0.018$, corrected for multiple testing). In the analysis of a four-marker haplotype located in *ATP10C*, a statistically significant difference after correction for multiple testing was observed in the frequency of one haplotype between male patients and controls (permutation $p = 0.033$, corrected for multiple testing). Thus, the present study may suggest the association between autism and the MED or the upstream IC in chromosome 15q11-q13 in the Japanese population.

Key words: autism, chromosome 15, Angelman syndrome, SNURF, genomic imprinting

Introduction

Autism is a developmental disorder characterized by three areas of abnormality: impairment in social interaction, impairment in communication, and restricted and stereotyped pattern of interest or behavior. Impairment in all three areas is observed before age three years and disrupted growth of the brain, with unknown mechanism, is implicated in the etiology of autism. Twin and family studies have indicated a robust role of genetic factors in the development of autism, while no susceptibility gene has been elucidated (Freitag, 2007).

Chromosome 15q11-q13 has been a focus of genetic studies of autism susceptibility because of the presence of cytogenetic abnormalities of this region in autistic patients. Deletions of the region lead to Prader-Willi syndrome and Angelman syndrome (AS) depending on the deleted chromosome's parent of origin – paternal and maternal, respectively (Knoll et al., 1989). Maternally, but not paternally, derived defects, such as duplications, within the AS critical region result in autistic symptomatology (Cook Jr et al., 1997). Supernumerary marker chromosomes (SMCs), called "idic" or "inverted duplication", of the region of maternal origin give rise to a more severe phenotype, arguably stemming from a dosage effect (Borgatti et al., 2001). Thus, a role of an imprinted, maternally expressed gene within 15q11-q13 was implicated in autistic symptomatology. However, it has been unclear which gene in the region contributes to the susceptibility.

The 15q11-q13 region consists of a large proximal domain (~2Mb) of paternally-expressed genes, a smaller maternal expression domain (MED: ~500kb), and a large distal region (~2Mb) of apparently biallelic expression (Nurmi et al., 2003) (Figure 1). Coordinate control of imprinted expression throughout the region is mediated by an imprinting center (IC) at the *SNRPN* promoter (Buiting et al., 2001; Chamberlain and Brannan, 2001). The MED contains two known imprinted, maternally expressed genes, *UBE3A* and *ATP10C*. *UBE3A* is implicated in the development of AS and encodes the ubiquitin-protein ligase E3A (Fang et al., 1999). The *ATP10C* gene product is thought to function as an amphipathic phospholipid transporter that may be involved in signaling in the central nervous system (Herzing et al., 2001). A study observed a significant association between autism and a microsatellite marker located at the 5' end of *UBE3A* (Nurmi et al., 2001). This association, however, was not replicated in a larger sample (Nurmi et al., 2003) and an earlier study did not observe a significant

associatin, either (Cook Jr et al., 1998). Nurmi et al. (2003) also investigated the association between *ATP10C* and autism. Two single nucleotide polymorphisms (SNPs) within the gene demonstrated preferential allelic transmission to the affected offspring. In addition, a haplotype within the gene displayed suggestive evidence for preferential transmission. However, earlier two studies (Nurmi et al., 2001; Kim et al., 2002) did not observe a significant association between *ATP10C* and autism.

Thus, the findings on genetic variants in these genes are inconclusive to date. Further accumulation of molecular genetic studies may be needed to elucidate the role of the MED in the pathophysiology of autism. Here we investigated 41 SNPs in the MED and the upstream IC in autism patients from a Japanese population.

Subjects and Methods

In this study, Japanese patients and control subjects around Tokyo, Japan, were recruited: 166 unrelated patients with autistic disorder diagnosed by the DSM-IV criteria (147 males and 19 females; age, 19.9 ± 9.8 years, mean ± SD) and 416 unrelated healthy volunteers (139 males and 277 females; age: 35.9 ± 11.5 years). Diagnosis of the patients was confirmed by two experienced child-psychiatrists independently through semi-structured behavior-observation of them and interview of their parents. At the interview, the Child Behavior Questionnaire Revised (CBQ-R; Supplementary material 1) was used to assist the evaluation of the autism-specific behaviors and symptoms. The CBQ-R is a parent-rating scale distinguishing pervasive developmental disorders from other child psychiatric conditions such as mental retardation. The validity and reliability of the CBQ-R have been confirmed (Izutsu et al., 2001). After the initial observation and interview, the patients were followed up for six months to confirm the diagnosis. In order to exclude other genetic syndromes or neurological diseases, studies were given to the patients including full exploration of medical and family history, physical and neurological examinations such as brain imaging, EEG, urinalysis, standard karyotyping and fragile X testing for the trinucleotide repeat expansion in the *FMR-1* gene (Chong et al., 1994). IQ levels were > 70 in 12 patients, 50 – 70 in 33 patients, 35 – 50 in 30 patients, and < 35 in 37 patients. The levels were evaluated mainly using a Japanese version of the Binet test. Thirty-nine patients were unable to take the IQ test due to their communication disorders or disability to

understand the questions. Data was not available in other 15 patients. All controls received a short interview by one of the authors to confirm that they had no history of psychiatric illnesses including autism spectrum disorders. The objective of the present study was clearly explained, and written informed consent was obtained from all parents. The consent was also obtained from the patients when they were able to follow the explanation. The study was approved by the Ethical Committee of the Faculty of Medicine, the University of Tokyo.

Genomic DNA was extracted from leukocytes by using the standard phenol-chloroform method. We selected 41 SNPs from the region including the MED and the IC from the list of the Assay-on-Demand™ Products for ABI PRISM 7900HT (Applied Biosystems, CA). All SNPs were analyzed by using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Detailed information of 31 SNPs were shown in Table I. Other 10 SNPs (rs11634286, rs904310, rs1377561, rs4906938, rs17115294, rs1564829, rs11858437, rs4906757, rs4906784, and rs4906788) were excluded in the later statistical analyses because they showed no polymorphism in the present subjects. Among the 31 SNPs, SNPs 1-17 are located in the upstream IC. The chi-square test was used to compare the SNP frequencies between the patients and controls. The pairwise linkage disequilibrium (LD) was measured and visualized by Lewontin's D' (Lewontin, 1964). Haplotype block analysis was conducted in the Gabriel method as well as the Four Gamete method (Gabriel et al., 2002; Wang et al., 2002). Haplotypes of the SNPs and their frequencies were estimated by the maximum-likelihood method with an expectation-maximization algorithm (Excoffier and Slatkin, 1995). Permutation p values were calculated in comparison of haplotype frequencies between the patients and controls (Fallin et al., 2001). The SNPAlyze 5.1 Standard software (DYNACOM, Japan) was used to conduct the LD, haplotype block, and haplotype analyses.

Results

Table I shows allelic frequencies of the 31 SNPs compared between the patients and controls. The distributions of all 31 SNPs follow the Hardy-Weinberg equilibrium in the patients. In the controls, the distributions of SNPs 6 and 8 nominal-significantly deviated from the Hardy-Weinberg equilibrium ($p = 0.024$ and 0.040, respectively), while the distributions of the other 29 polymorphisms were within the values expected

from Hardy-Weinberg equilibrium.

We observed a nominally significant difference in the allelic frequency of SNP 8 between the patients and controls (0.38 vs. 0.46, respectively, $\chi^2 = 5.96$, df = 1, p = 0.015). No significant difference was observed in the allelic frequencies of other 30 SNPs between the patients and controls. In the genotypic distributions, there were nominally significant differences between the patients and controls in SNP1 (major homo/hetero/minor homo = 0.23/0.47/0.30 vs. 0.24/0.54/0.22, respectively, $\chi^2 = 4.18$, df = 1, p = 0.041 in dominant model for major allele), SNP6 (0.44/0.39/0.17 vs. 0.43/0.49/0.08, respectively, $\chi^2 = 10.4$, df = 1, p = 0.0013 in dominant model for major allele and $\chi^2 = 11.6$, df = 2, p = 0.0030 in co-dominant model), and SNP8 (0.41/0.42/0.17 vs. 0.26/0.55/0.19, respectively, $\chi^2 = 11.8$, df = 1, p = 0.00058 in recessive model for major allele and $\chi^2 = 12.5$, df = 2, p = 0.0020 in co-dominant model). No significant difference was observed in the genotypic distributions of other 28 SNPs between the patients and controls.

Analysis after confining the subjects to males showed no significant difference in the allelic frequencies of the 31 SNPs between the patients and controls. In the genotypic distributions, there were nominally significant differences between the patients and controls in SNP1 (0.22/0.49/0.29 vs. 0.26/0.56/0.18, respectively, $\chi^2 = 4.10$, df = 1, p = 0.043 in dominant model for major allele), SNP6 (0.42/0.41/0.17 vs. 0.46/0.48/0.06, respectively, $\chi^2 = 8.85$, df = 1, p = 0.0029 in dominant model for major allele and $\chi^2 = 8.95$, df = 2, p = 0.011 in co-dominant model), SNP8 (0.42/0.40/0.18 vs. 0.26/0.56/0.18, respectively, $\chi^2 = 7.30$, df = 1, p = 0.0069 in recessive model for major allele and $\chi^2 = 8.32$, df = 2, p = 0.016 in co-dominant model), and SNP12 (0.55/0.40/0.05 vs. 0.48/0.40/0.12, respectively, $\chi^2 = 4.63$, df = 1, p = 0.031 in dominant model for major allele). No significant difference was observed in the genotypic distributions of other 27 SNPs between the patients and controls.

The strength of LD denoted as D' between pairs of SNPs is shown in Figure 2. Two haplotype blocks, SNPs 15-16 and 18-19 were suggested by the Gabriel method of haplotype block analysis (Gabriel et al., 2002); six haplotype blocks, SNPs 1-4, 6-10, 14-17, 18-19, 20-23, and 26-27 were suggested by the Four Gamete method (Wang et al., 2002). In the analysis of haplotype block consisting of SNPs 20-23, five haplotypes were observed with estimated frequencies >1% (Table II). The significantly associated haplotype was 'ACCT' (permutaion p = 0.011), while the global permutation p value for

these five haplotypes was 0.074. No significant difference was observed between the patients and controls in the distributions of any estimated haplotypes in the haplotype blocks consisting of other SNPs.

Analysis after confining the subjects to males showed almost the same LD maps with those in all subjects (data not shown). Haplotype block analyses suggested seven haplotypes blocks, including SNPs 14-16 and 18-19 by the Gabriel method and SNPs 1-4, 6-10, 14-17, 20-23, and 26-27 by the Four Gamate method. In the analysis of the haplotype block consisting of SNPs 20-23, five haplotypes were observed with estimated frequencies >1% (Table II). The global permutation p value for these five haplotypes was 0.048, of which the significantly associated haplotype was 'ACCT' (permutaion p = 0.0065). When studying other haplotype blocks, no significant difference was observed between the patients and controls in the distributions of any estimated haplotypes.

Discussion

In the present study, we investigated the association between autism and the MED or the upstream IC in chromosome 15q11-q13. Significant differences were observed in the allelic frequency of SNP 8 and genotypic distributions of SNPs 1, 6, and 8 between the patients and controls. The analysis in males showed similar results; no significant difference was observed in the allelic frequencies of any SNPs, while significant differences were observed in the genotypic distributions of SNPs 1, 6, 8, and 12. When the results were corrected for multiple testing for 31 SNPs, these associations became insignificant except for that of SNP8 in recessive model genotypic distribution in all subjects (corrected p = 0.018). This may suggest that having the minor-homo genotype of SNP8 may increase the risk for autism. In the analysis of haplotype block consisting of SNPs 20-23, a significant difference was observed in the distribution of estimated haplotypes between the patients and controls in male subjects. In this haplotype, the statistical level of the association of haplotype 'ACCT' was significant after correction for multiple testing for observed five haplotypes (corrected permutaion p = 0.033). Thus, the present study may suggest the association between autism and the MED or the upstream IC in chromosome 15q11-q13 in the Japanese population.

SNPs 1, 6, 8, and 12 are located in *SNRPN*, a bicistronic imprinted gene that encodes 2

polypeptides, the small nuclear ribonucleoprotein polypeptide N, and the SNRPN upstream reading frame (*SNURF*) polypeptide. *SNRPN* also encodes a long alternatively spliced transcript containing several small nucleolar RNAs (snoRNAs) and extends downstream to partially overlap the *UBE3A* gene in the antisense orientation (Runte et al., 2001). The *SNRPN* gene is transcribed exclusively from the paternally inherited chromosome and it was observed that maternally-only expression of *UBE3A* was regulated indirectly through the paternally expressed antisense transcript (Runte et al., 2004). In the present study, no association was observed between autism and SNPs in *UBE3A*. However, the observed association of SNPs in *SNRPN* may suggest the indirect involvement of *UBE3A* in the development of autism through regulation of the MED. In addition, a snoRNA, HBII-52, located in the locus was observed to regulate alternative splicing of serotonin 2C receptor, which has been implicated in autism (Kishore and Stamm, 2006). Further molecular genetic study of the locus including imprinting status or investigation of the transcript levels may contribute to elucidate the association.

SNPs 20-23 are located in *ATP10C*, which is an interesting candidate for autism susceptibility in chromosome 15q. The previous study (Nurmi et al., 2003) observed preferential transmission to affected offspring of alleles in two SNPs (rs1047700 and rs1345098) and a three-marker haplotype (rs2066703-rs1047700-rs901005). The three-marker haplotype is overlapping the haplotype consisting of SNPs 20-23 in the present study. In contrast, Nurmi et al. (2001) and Kim et al. (2002) did not observe the association between *ATP10C* and autism. The contradiction may be attributed to marker density and statistical method; a small number of markers were selected from the *ATP10C* locus in Nurmi et al. (2001) and haplotype analysis was not performed in Kim et al. (2002). The present result suggests that the susceptible variant for autism might exist in the haplotype block consisting of SNPs 20-23 in *ATP10C*. Further analysis of the locus is strongly recommended.

Caution might be needed to interpret the present results. One is the significant deviations from the Hardy-Weinberg equilibrium in SNPs 6 and 8 in the controls. To exclude the possibility of technical error, we confirm the results by genotyping all subjects twice. Although the statistical level of the deviations became insignificant after Bonfferoni correction, the possibility of sampling bias may not be completely denied. Second, the controls in the present study were not age- or sex-matched to the patients. However, this may not be likely to significantly affect the result, considering no major effect of environmental factors in autism (Folstein and Rosen-Sheidley, 2001).

Imbalance in sex ratio between the patients and controls may be overcome by analysis confining the subjects to males considering its higher prevalence in males than in females. Third may be diagnostic issues. Neither the ADOS (Autism Diagnostic Observation Schedule) (Lord et al., 1989) nor the ADI-R (Autism Diagnostic Interview-Revised) (Lord et al., 1994) has been available in Japan to date. The existence of bias might not be denied in the diagnosis of the present subjects, although the effect of which is few if any.

In conclusion, we obtained a weak but significant?? support for the association of the MED or the upstream IC in chromosome 15q11-q13 with autism in Japanese people. Further investigation of the region with larger sample size and denser markers may be needed to confirm the present results.

Figure legend

Figure 1. Schematic representation of 15q11-13 and the MED. The regions of paternal-specific and maternal-specific gene expression are delineated by arrows. Gene positions, the IC location, and the region analyzed in the present study are shown.

Figure 2. Pattern of LD in the MED and the IC in chromosome 15q11-13. Pairwise LD between SNPs, as measured by D', is represented. The D'-values for controls are visualized in the lower left diagonal and those for patients are in the upper right diagonal.

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Table I. Allelic frequencies of 31 SNPs in the MED and the IC

SNP No.	db SNP ID	Location	Alleles (Major/Minor)	Minor allele frequency		Chromosome position (bp)
				Autism*	Control*	
1	rs11161139	*SNRPN*	T/C	0.54 (165)	0.49 (365)	3227367
2	rs7166784	*SNRPN*	A/G	0.17 (167)	0.15 (394)	3228422
3	rs28687287	*SNRPN*	T/A	0.078 (167)	0.063 (411)	3233162
4	rs17178750	*SNRPN*	T/G	0.13 (167)	0.11 (413)	3235145
5	rs4474655	*SNRPN*	C/T	0.11 (166)	0.11 (359)	3237143
6	rs736008	*SNRPN*	T/C	0.37 (166)	0.32 (375)	3256123
7	rs2167926	*SNRPN*	T/C	0.039 (166)	0.054 (409)	3257566
8	rs7164989	*SNRPN*	A/G	0.38 (167)	0.46 (413)	3261084
9	rs12441116	*SNRPN*	T/G	0.039 (166)	0.052 (403)	3264196
10	rs752873	*SNRPN*	A/G	0.16 (165)	0.12 (412)	3265693
11	rs11161149	*SNRPN*	G/A	0.14 (166)	0.15 (369)	3282819
12	rs2201839	*SNRPN*	T/C	0.26 (162)	0.28 (307)	3307419
13	rs705	*SNRPN/SNURF*	T/C	0.48 (166)	0.45 (404)	3381507
14	rs4906699	*SNRPN*	C/T	0.51 (166)	0.49 (416)	3483000
15	rs1549477	*SNRPN/PWCR1*	C/T	0.52 (166)	0.49 (415)	3494089
16	rs1549478	*SNRPN/PWCR1*	C/T	0.46 (165)	0.48 (409)	3494171
17	rs2714751	*SNRPN/PAR4*	G/A	0.021 (167)	0.029 (415)	3612801
18	rs12907375	*UBE3A/SNRPN*	A/G	0.35 (166)	0.38 (397)	3762340
19	rs7496951	*UBE3A/SNRPN*	G/C	0.35 (163)	0.38 (395)	3833303
20	rs8041681	*ATP10C*	A/G	0.42 (166)	0.37 (412)	4091521
21	rs3743438	*ATP10C*	C/T	0.087 (167)	0.079 (414)	4095406
22	rs2066705	*ATP10C*	T/C	0.50 (166)	0.50 (367)	4099315
23	rs4906750	*ATP10C*	T/C	0.21 (165)	0.17 (411)	4108894
24	rs2291355	*ATP10C*	G/A	0.47 (165)	0.49 (361)	4115377
25	rs4906629	*ATP10C*	G/A	0.41 (166)	0.41 (376)	4145788
26	rs11638039	*ATP10C*	T/C	0.23 (166)	0.23 (415)	4172261
27	rs1444623	*ATP10C*	G/A	0.22 (165)	0.23 (394)	4173839
28	rs17637170	*ATP10C*	T/C	0.27 (165)	0.29 (411)	4178794
29	rs11632263	*ATP10C*	C/T	0.48 (166)	0.50 (372)	4189988
30	rs8039801	*ATP10C*	C/T	0.27 (164)	0.28 (415)	4198700
31	rs882406	*ATP10C*	G/A	0.24 (166)	0.22 (416)	4208033

SNRPN; small nuclear ribonucleoprotein polypeptide N, *SNURF*; SNRPN upstream reading frame, *PWCR1*; Prader-Willi syndrome chromosome region 1, *PAR4*; Prader-Willi/Angelman region gene 4, *UBE3A*; ubiquitin-protein ligase E3A, *ATP10C*; ATPase, Class V, type 10C

* Number of genotyped individuals for each SNP is given in parenthesis.

Table 2. Estimated haplotype frequencies of the haplotype consisting of SNPs 20-23

SNP	20	21	22	23	Frequency in all subjects*			Frequency in males**		
					Autism	Control	Permutation p value	Autism	Control	Permutation p value
	A	C	T	T	0.491	0.495	0.846	0.049	0.492	0.932
	G	C	C	C	0.172	0.213	0.132	0.222	0.160	0.087
	A	C	C	T	0.141	0.086	0.00799	0.084	0.158	0.00622
	G	C	C	T	0.119	0.121	0.921	0.121	0.118	1.000
	G	T	C	T	0.077	0.085	0.719	0.083	0.072	0.641

Haplotypes whose frequencies were estimated >1% were described.
* global permutaion p value = 0.0739, ** global permutation p value = 0.00472

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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 directedness	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).

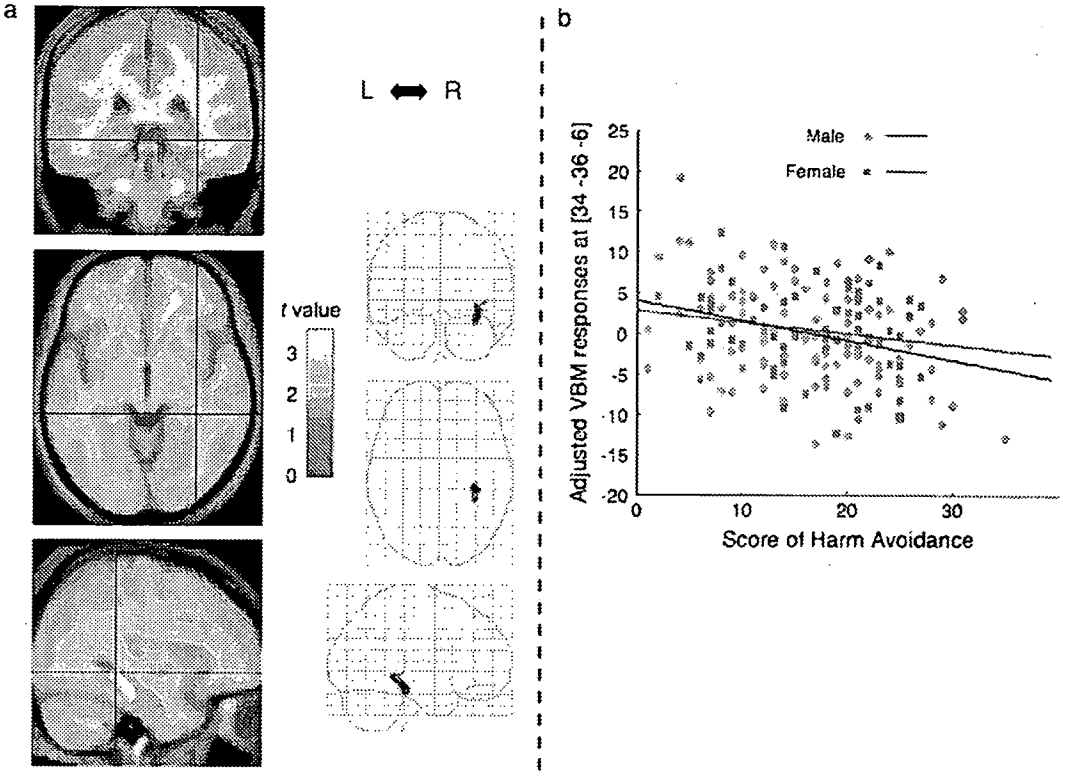

Figure 1. Gender-common negative correlation between HA and regional gray matter volume in the right hippocampus. (*a*) Gray matter regions showing significant correlations with the individual variability of HA were rendered in the Montreal Neurological Institute space. (Right) Statistical parametric 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.

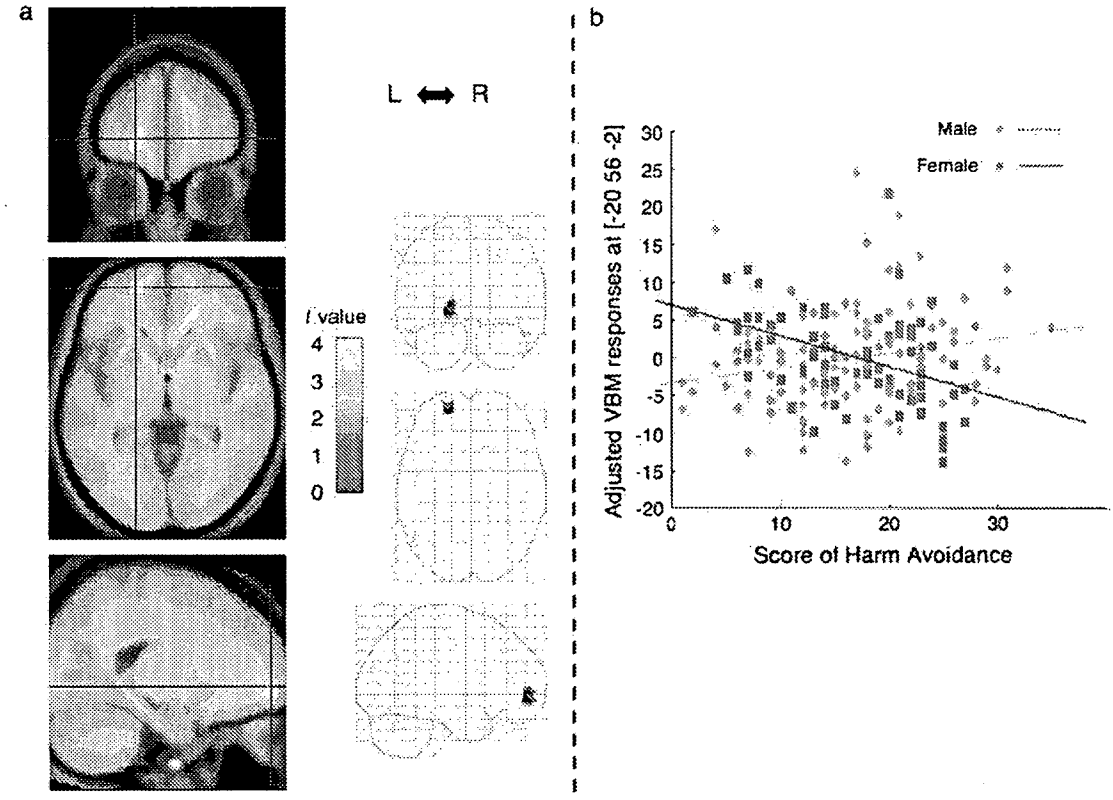

Figure 2. Female-specific negative correlation between HA and regional gray matter volume in the left anterior prefrontal cortex. (*a*) The gray matter regions where interaction between gender and HA was found are rendered in the Montreal Neurological Institute space. (Right) Statistical parametric map 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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