に最も進化した大脳皮質部位の一つである前頭極の個体発生過程に影響する可能性が示唆された。統合失調症をはじめ、一卵性双生児での一致率が 50-60%に留まるような精神疾患の病態と成因の解明には、若年者を対象として縦断研究も含んだ“発達双生児法”による大規模研究を進めることが今後必要であろう。

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G．知的財産権の出願・登録状況

1．特許取得

無し。

2．実用新案登録

無し。

3．その他

無し。

厚生労働科学研究費補助金（こころの健康科学研究事業）
（総合）研究報告書

双生児法による精神疾患の病態解明に関する研究
精神疾患における内因性レトロウイルスの関与についての検討

（主任又は分担）研究者　陣野　吉廣　琉球大学（院・医）教授

研究要旨：統合失調症の発症に関して内在性レトロウイルス（HERV）やLINE-1（L1）とDNAメチル化に注目し、網羅的探索を行った。脳での潜在的転写活性を持つ期待の高いHERV４つを同定した。L1の同定には効率の高い画期的方法の開発が必須である。

A．研究目的：環境要因→DNAメチル化→HERV・L1→病像形成遺伝子⇒発症.
模式的に示した作業仮説を検証することが１つの区切りとなる目標である。この分担課題では、ヒトゲノムの全配列を探索して、転写活性能を持っているHERV及びL1を可能なかぎり数多く同定することが目的となった。

B．研究方法：HERV及び(昨年度の)L1のスクリーニングにヒトdbESTを利用した。照合元のQuery には延べ98種類のHERV配列又はL1.3完全長（約6 kb）を用いた。得られたESTをBLASTにより該当するローカスに配置して選出の指標にした。更にL1に関しては、今年度の報告書に記したようにゲノム配列からのアプローチも行った。抽出した候補ローカスは従来の方法で検証を行った（一次検証）。
（倫理面への配慮）
データベース解析と特定不能な1例の核酸を材料とした基礎的実験であるため、特に倫理面の問題はないと判断した。

C．研究結果：転写活性能を保持している可能性が高い４つのHERVローカスを同定した：HERV-K102（1q21-q22），HERV-H（22q12），HERV-P3b（1p36.13），HERV-K9（5p15.33）．L1のESTデータベース スクリーニングから、転写活性能保持型L1候補を254ローカスに絞り込んだ。そのうち染色体２番上のL1ローカスと染色体15番に局在するL1ローカスが一次検証の対象となった。が、二カ所に設定されたプライマーによるRT-PCRのいずれにも検出されなかった。ゲノム配列からの探索では146個のL1が抽出された。同様に、解析した計403クローンに検出されたローカスはわずか７つだった。しかし、サブファミリーの分布はゲノムPCRとRT-PCRで異なっていた。また、同一配列が複数のクローンに検出される頻度にも有意な差が認められた。

D．考察：潜在的転写活性を持つHERV４つを同定した。期待したよりはるかに少ない。L1に至っては一次検証の段階で壁にぶつかった。母集団が大き過ぎる。反復配列解析の困難さを改めて痛感した。また、EST配列の質が低いことも問題である。検証法の開発・Queryの選び方が課題になる。

E．結論：転写活性能をもつHERVおよびL1の探索・同定を試みた。困難な壁があり、Queryの選び方の工夫と検証法の開発が必須である。

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G．知的財産権の出願・登録状況
1．特許取得：なし／2．実用新案登録：なし／3．その他：なし

研究成果の刊行一覧表（平成17～19年度）

加藤　忠史

氏名	タイトル	雑誌／書籍名	巻	頁	年
Kakiuchi C, Ishiwata M, Nanko S, Ozaki N, Iwata N, Umekage T, Tochigi M, Kohda K, Sasaki T, Imamura A, Okazaki Y, Kato T.	Up-regulation of ADM and SEPX1 in the lymphoblastoid cells of patients in monozygotic twins discordant for schizophrenia.	Am J Med Genet B Neuropsychiatr Genet.			2007
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資　　料

AJMB-07-0127.R1(30643)

American Journal of Medical Genetics Part B (Neuropsychiatric Genetics) 9999:1–8 (2007)

Up-Regulation of *ADM* and *SEPX1* in the Lymphoblastoid Cells of Patients in Monozygotic Twins Discordant for Schizophrenia

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The contribution of genetic factors to schizophrenia is well established and recent studies have indicated several strong candidate genes. However, the pathophysiology of schizophrenia has not been totally elucidated yet. To date, studies of monozygotic twins discordant for schizophrenia have provided insight into the pathophysiology of this illness; this type of study can exclude inter-individual variability and confounding factors such as effects of drugs. In this study we used DNA microarray analysis to examine the mRNA expression patterns in the lymphoblastoid (LB) cells derived from two pairs of monozygotic twins discordant for schizophrenia. From five independent replicates for each pair of twins, we selected five genes, which included *adrenomedullin* (*ADM*) and *selenoprotein X1* (*SEPX1*), as significantly changed in both twins with schizophrenia. Interestingly, *ADM* was previously reported to be up-regulated in both the LB cells and plasma of schizophrenic patients, and *SEPX1* was included in the list of genes up-regulated in the peripheral blood cells of schizophrenia patients by microarray analysis. Then, we performed a genetic association study of schizophrenia in the Japanese population and examined the copy number variations, but observed no association. These findings suggest the possible role of *ADM* and *SEPX1* as biomarkers of schizophrenia. The results also support the usefulness of gene expression analysis in LB cells of monozygotic twins discordant for an illness.

KEY WORDS: adrenomedullin; selenoprotein; DNA microarray; gene expression; genetic association study

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INTRODUCTION

Genetic factors in schizophrenia have been shown by family, twin, and adoption studies. A higher concordance rate of schizophrenia in monozygotic twins (41–79%) compared with that in dizygotic twins (0–14%) especially supports the contribution of genetic factors in schizophrenia [Shih et al., 2004]. As the risk genes for schizophrenia, a balanced translocation disrupting disrupted schizophrenia-1 (*DISC1*) [Millar et al., 2000] and a chromosomal deletion at 22q11 [Bassett and Chow, 1999] are well established. As common variants associated with schizophrenia, dystrobrevin-binding protein 1 (*DTNBP1*) [Straub et al., 2002] and neuregulin 1 (*NRG1*) [Stefansson et al., 2002], which were identified from linkage analysis, were reported. However, the association of DTNBP1 haplotype with schizophrenia is not consistent among studies [Mutsuddi et al., 2006]. Further studies to identify the molecular pathology of this illness are needed.

In addition to the traditional genetic approaches, an additional strategy to identify the genetic basis of endophenotypes of schizophrenia is becoming popular. In this approach, endophenotypes, measurable biological variables associated with genetic risk of schizophrenia, are first identified; then their genetic basis is studied [Braff et al., 2007]. Many established endophenotypes, such as eye tracking abnormality [Holzman et al., 1977], ventricular enlargement [Reveley et al.,

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1982], reduced hippocampal volume [Suddath et al., 1990], hypofrontality [Berman et al., 1992], and neuropsychological measures [Goldberg et al., 1995], were validated by the study of monozygotic twins discordant for schizophrenia.

In an attempt to identify molecular endophenotypes, biochemical differences in blood between the monozygotic twins discordant for schizophrenia have been investigated. These studies showed some differences between twins: plasma haptoglobin levels [Vander Putten et al., 1996], DNA methylation status [Tsujita et al., 1998; Petronis et al., 2003], soluble interleukin-2 receptors (SIL-2Rs) [Rapaport et al., 1993], mRNA expression level of a certain transcript [Friedhoff et al., 1995], retrovirus [Deb-Rinker et al., 1999], catecholamine levels [Walker et al., 2002], DNA stability [Nguyen et al., 2003], and lipid metabolism [Tsang et al., 2006]. On the other hand, no difference was found for viral nucleic acids [Sierra-Honigmann et al., 1995], platelet monoamine oxidase activity [Reveley et al., 1983], and genomic sequences [Polymeropoulos et al., 1993; Vincent et al., 1998; McDonald et al., 2003]. If a robust difference between discordant twins is well validated, such a finding will become a clue to identify the cause of this difficult illness [Kato et al., 2005a].

To identify the genes differentially expressed between the twins, one may use peripheral blood cells. However, this method is hampered by the fact that most of the patients are under treatment with drugs such as antipsychotics, which potentially affect the gene expression patterns. One possible method to avoid these confounding factors is to use the lymphoblastoid (LB) cells. Gene expression patterns in LB cells can be assessed with minimum inter-individual variability [Cheung et al., 2003], and the effect of drugs may be avoided or reduced by culturing the cells.

We previously performed DNA microarray analysis and examined the mRNA expression pattern using LB cells of monozygotic twins discordant for bipolar disorder. On the basis of our findings, we suggested the possible contribution of the endoplasmic reticulum stress response pathway to the pathophysiology of the illness [Kakiuchi et al., 2003]. Recently, Matigian et al. [2007] also performed DNA microarray analysis in three pairs of monozygotic twins discordant for bipolar disorder and found that genes related to the WNT signaling pathway were altered in patients. Several other groups have also applied the similar strategy to other illnesses such as autism and rheumatoid arthritis [Haas et al., 2006; Hu et al., 2006].

In this study, we used DNA microarray analysis to examine the mRNA expression pattern in the cells of two pairs of monozygotic twins discordant for schizophrenia. Because one of the problems in this strategy is lack of statistical analysis due to small sample size, we performed five independent experiments for each pair of twins. The expression of five genes commonly was shown to be altered in both of the twins, and two genes survived after the exclusion of three immunoglobulin-related genes. Interestingly, both of the final genes, *adrenomedullin* (*ADM*) and *selenoprotein X1* (*SEPX1*), had been reported to be up-regulated in the cells or plasma of schizophrenic patients. We further tried to identify the genetic basis of up-regulation of *ADM* and *SEPX1* levels in schizophrenia by a case-control association analysis of schizophrenia in the Japanese population. Because copy number variation (CNV) was reported to exist around these loci, CNV was also examined.

MATERIALS AND METHODS

Subjects

For the DNA microarray analysis, two pairs of monozygotic twins discordant for schizophrenia (SZ twins) were recruited. The SZ twins A were 54-year-old males, and SZ twins B were 24-year-old females, who were previously reported elsewhere [Kunugi et al., 2003].

The SZ twins A were diagnosed by the consensus of two senior psychiatrists after independent unstructured interviews. Their family history was obtained from interviews of the twins. They had two healthy sisters, and their parents did not have major mental disorders. The affected twin of this pair (SZ-twin-A1) graduated from a university and worked as an office worker for 2 years. At age 25, he developed disorganized behavior and thought, accompanied by excitation. He also had non-systematic delusion of persecution and auditory hallucination. He was hospitalized in a psychiatric ward for 3 months. After the first episode, he was admitted to psychiatric hospitals 13 times. He began to develop negative symptoms and changed jobs several times because of interpersonal problems. He married at age 32, but divorced 1 year later. After that, he could not continue to work and lived alone, supported by social welfare. His diagnosis according to the International Classification of Diseases, Revision 10 (ICD-10) was schizophrenia, disorganized type. He was also diagnosed to have diabetes mellitus. He had been treated with 150 mg of clocapramine hydrochloride, a typical antipsychotic, and 3 mg of trihexyphenidyl hydrochloride, as an antiparkinsonian drug. It is not known whether his diabetes is a side effect of these drugs. His co-twin had been working at a company for 30 years and had been married. He was not diagnosed to have any major mental disorders or personality disorders. He did not have diabetes.

The proband of SZ twins B was diagnosed by the consensus of two senior psychiatrists after independent unstructured interviews. The diagnosis of the proband according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV; American Psychiatric Association) was schizophrenia. Her co-twin was interviewed with the use of the schedule for affective disorders and schizophrenia (SADS), which revealed no current or past history of affective disorders or psychotic disorders. Their mother was interviewed and found to be healthy. Their father was also healthy, according to the available information. The symptoms of the proband is minutely described elsewhere [Kunugi et al., 2003]. In brief, the proband's symptoms began around the age of 15, with delusion of persecution. After that, she developed auditory hallucination and negative symptoms.

For the case-control association study, the genomic DNA derived from peripheral blood cells of 223 patients with schizophrenia (45.7 ± 14.9 years old, 129 males and 94 females) and 364 controls (50.4 ± 12.5 years old, 184 males and 180 females) in the Japanese population were analyzed. They were diagnosed according to the DSM-IV criteria. Controls were selected from students, nurses, office workers, and doctors in participating institutes, and their friends. A senior psychiatrist interviewed the controls and found no major mental disorders. Only a subset of the controls were interviewed with the use of a structured interview, the mini-international neuropsychiatric interview (M.I.N.I.) [Sheehan[Q3], 1998 #61]. In the Japanese population, no significant population stratification has been repeatedly reported in several studies [Kakiuchi et al., 2003; Arinami et al., 2005; Shimizu et al., 2006].

For the quantitative genomic polymerase chain reaction (PCR), we used genomic DNA derived from LB cells of the two pairs of discordant SZ twins, 46 Japanese unrelated schizophrenia patients (38.6 ± 14.6 years old, 18 males and 28 females), and 11 controls (56.3 ± 11.0 years old, 8 males and 3 females), and 13 schizophrenia patients (55.0 ± 9.9 years old, 9 males and 4 females) obtained from NIMH Genetics Initiative Pedigrees. Written informed consent was obtained from all subjects. The ethics committees of the Brain Science

Institute (RIKEN) and participating institutes approved the study.

Cell Culture

The lymphocytes derived from peripheral blood were transformed by Epstein-Barr (EB) virus and cultured with the use of standard techniques as described before [Kato et al., 2002]. For mRNA quantification by DNA microarray analysis, we extracted the RNA from frozen cells, and thawed and recultured the cells. The culture of the cells and mRNA extraction were performed independently five times for each pair of twins.

DNA Microarray

DNA microarray experiments were performed as described previously with the use of an Affymetrix HU133A chip (Affymetrix, Santa Clara, CA) [Kakiuchi et al., 2006]. We used 5 μg of total RNA for reverse-transcription into cDNA, and biotin-labeled cRNA was synthesized from the cDNA. After testing the integrity of the samples by the Test2Chip (Affymetrix), fragmented cRNA was applied to the HU133A chip. The hybridization signal on the chip was scanned and subjected to image analysis (Affymetrix).

Analysis of DNA Microarray Data

The microarray raw data were processed by MAS5.0 (Affymetrix) and robust multiarray average (RMA) methods [Irizarry et al., 2003], and analyzed with the use of GeneSpring software (SiliconGenetics, Redwood, CA). Data were normalized by the median value. Genes expressed differently in each pair of twins were selected by the following criteria: (1) the genes were called as present in all samples (five samples of affected twin and five samples of control co-twin); (2) both the parametric test and the non-parametric test showed a significant difference ($P < 0.05$) between the five cultures in a patient and five cultures in the co-twin by both normalization methods (MAS5.0 and RMA). Then, the genes commonly changed to the same trend in both SZ twins A and SZ twins B according to these four statistical comparisons: MAS5 and RMA, parametric and non-parametric.

Genetic Association Studies

We selected five SNPs (rs7944706, rs6484148, rs6484147, rs4597056, rs726102) for *ADM* according to the linkage disequilibrium (LD) map database on SNPbrowserTM (Applied Biosystems, Foster City, CA). Although a previous report in the Japanese population hypothesized a possible role of dinucleotide repeat in the 4 kb downstream of *ADM* in the pathophysiology of hypertension, this microsatellite marker was not associated with plasma ADM concentration [Ishimitsu et al., 2001]; thus, this marker was not selected for the analysis. We selected three SNPs (rs9928312, rs9934331, rs1003904) for *SEPX1*, because their TaqMan probes were commercially available and they are polymorphic in Japanese according to the LD map database on HapMap projects accessed with the SNPbrowserTM software. We performed genotyping by TaqMan probes and ABI7900HT according to the protocol recommended by the manufacturer (Applied Biosystems). Assessment of LD patterns by the standardized disequilibrium coefficient (D′) and squared correlation coefficient (r^2), and analysis of haplotypic distribution, and frequencies were performed with the use of the COCAPHASE programs (http://portal.litbio.org/Registered/Option/unphased.html). Global significance was calculated by the random permutation test (10,000 times).

Quantification of Genome Copy Number

The copy number of *ADM* and *SEPX1* was analyzed by the real-time PCR method with the use of SYBR/GREEN dye (Applied Biosystems) as described elsewhere [Kato et al., 2005b]. *MLC1* (megalencephalic leukoencephalopathy with subcortical cysts gene 1) was used as a single copy control gene and the copy number of *ADM* was calculated as a relative ratio to *MLC1*. A minimum of three probes for *ADM* was used. For quality control, a gene on the X chromosome [phosphofructo-2-kinase (*PF2K*)] was also examined by SYBR/GREEN dye, and separation between males and females was confirmed. Sequences of primers and probes for these analyses will be provided upon request.

RESULTS

Microarray Analysis in the Cells of Monozygotic Twins Discordant for Schizophrenia

By the criteria described above, five genes were identified (Table I). Among the up-regulated genes in schizophrenia, two genes (GenBank accession nos. L06101 and Z00008) were immunoglobulin-related genes, and *CD200* (GenBank accession no. AF063591) was also a member of the immunoglobulin superfamily (OMIM 155970). This result possibly reflects transformation of a subset of B-cells by the EB virus rather than a difference in disease state. Surprisingly, both of the finally listed genes [*ADM* (GenBank accession no. NM_001124) and *SEPX1* (GenBank accession no. NM_016332)] have been reported to be altered in schizophrenia. The mRNA expression of *ADM* was reported to be up-regulated in the LB cells derived from schizophrenia patients, and the plasma ADM level was significantly higher in schizophrenic patients than in controls [Zoroglu et al., 2002; Huang et al., 2004; Yilmaz et al., 2007]. *SEPX1* was included in the list of genes up-regulated in the peripheral blood cells of schizophrenia by microarray analysis [Glatt et al., 2005]. Interestingly, the expressions of both genes were up-regulated in all the studies, which was the same trend shown in this study. These results suggested that *ADM* and *SEPX1* were strong candidate genes for schizophrenia.

Association Analysis of *ADM* and *SEPX1* in Schizophrenia

If up-regulation of *ADM* and *SEPX1* is a risk factor for schizophrenia, genetic variations of these genes may contribute to the illness. Thus, we also performed association analysis of *ADM* and *SEPX1* in schizophrenia in the Japanese population. We examined the genotype of five SNPs for *ADM* and three SNPs for *SEPX1*. LD patterns for *ADM* and *SEPX1*, as measured by D′ and r^2, are shown in Figure 1. No significant association was observed in single SNPs (Table II) and haplotypes (Table III) for *ADM*, and in single SNPs for *SEPX1* (Table II).

Quantification of Genome Copy Number

In addition to sequence variations, CNVs may also contribute to the up-regulation of *ADM* and *SEPX1*. Indeed, CNVs were reported for the loci of both genes [*ADM* (RP11-79E12) and *SEPX1* (RP11-451K7 and Variation_5329), http://projects.tcag.ca/variation/]. The CNV may cause altered mRNA expression and may confound the results of association analysis. Thus, we quantified the copy number of *ADM* and *SEPX1* genes by the real-time PCR method in two pairs of discordant SZ twins, 46 Japanese unrelated schizophrenia patients, and 11 controls, and from genetic information for 13 schizophrenia patients obtained from NIMH Genetics Initiative Pedigrees. However, we observed no loss or gain of the genome in the tested loci (data not shown).

TABLE I. The Result of DNA Microarray Analysis in the Lymphoblastoid Cells of Monozygotic Twins Discordant for Schizophrenia

			SZ twin1						SZ twin2					
			MAS			RMA			MAS			RMA		
Probe ID	Genbank	Symbol	FC	*P*(P)	*P*(non-P)	FC	*P*(P)	*P*(non-P)	FC	*P*(P)	*P*(non-P)	FC	*P*(P)	*P*(non-P)
Up-regulation														
211641_x_at	L06101		1.428	0.042	0.028	1.273	0.022	0.009	1.380	0.030	0.028	1.277	0.011	0.016
217977_at	NM_016332	SEPX1	1.183	0.015	0.047	1.175	0.012	0.016	1.124	0.044	0.028	1.129	0.011	0.028
202912_at	NM_001124	ADM	1.622	0.011	0.028	1.564	0.005	0.009	1.966	0.002	0.009	1.922	0.004	0.009
216517_at	Z00008		1.684	0.022	0.028	1.683	0.038	0.028	2.076	0.000	0.009	1.839	0.001	0.009
Down-regulation														
209583_s_at	AF063591	CD200	0.755	0.016	0.009	0.750	0.019	0.009	0.603	0.003	0.016	0.704	0.005	0.016

FC, fold change; *P*(P/non-P), *P*-value calculated by parametric/non-parametric test using GeneSpring software.

DISCUSSION

In this study, we demonstrated that mRNA expressions of *ADM* and *SEPX1* were up-regulated in the LB cells of the two patients with schizophrenia compared with their healthy co-twins. This observation is consistent with the previous reports examined in unrelated patients and controls. Genetic association studies of *ADM* and *SEPX1* for schizophrenia in the Japanese population, however, did not support the association of SNPs in these genes with schizophrenia. Further, we did not observe CNVs in these genes.

ADM is a potent vasodilator peptide consisting of 52 amino acids (OMIM103275), which was initially identified from pheochromocytoma [Kitamura et al., 1993]. ADM is synthesized by many tissues including the central nervous system and is known to bind to calcitonin receptor-like receptor. The reported roles of ADM are variable, such as dilation of blood vessels and increase in urine output. *ADM* is also abundantly expressed in the central nervous system, especially in the thalamus, hypothalamus, and pituitary gland, and it regulates neuroendocrine response to stress [Taylor and Samson, 2004]. Intracerebroventricular administration of ADM is known to affect water intake and salt appetite. A probably reactive increase of ADM in plasma is reported in some diseases such as heart failure, renal diseases, septic shock, and diabetes mellitus [Beltowski and Jamroz, 2004]. This increased level in plasma was first reported in patients with schizophrenia [Zoroglu et al., 2002]. This observation might reflect reactive up-regulation associated with some somatic condition associated with schizophrenia. However, elevated mRNA levels also were reported in LB cells of schizophrenia patients [Huang et al., 2004], which suggested that increase of ADM is intrinsic rather than reactive. In this study, *ADM* mRNA level was increased in the affected co-twins. Thus, intrinsic increase of ADM may be related to the pathophysiology of schizophrenia.

SEPX1 is one of the selenoproteins, which includes selenocystein, and is abundant in liver, leucocytes, and pancreas (OMIM 606216). The function of SEPX1 has not been clarified; however, interestingly, selenium-binding protein1 (*SELENBP1*), which also binds to selenium, was demonstrated to be up-regulated in both the brain and the peripheral blood leukocytes in patients with schizophrenia, and was suggested to be a candidate biomarker of schizophrenia [Glatt et al., 2005]. In the list of genes up-regulated in peripheral blood cells in this report, *SEPX1* was also included. In the present study, *SEPX1* mRNA level was also increased in the affected co-twins. Thus, the up-regulation of *SEPX1* may play a role in the pathophysiology of schizophrenia. Geographical analysis showed that low selenium in soil and food might be associated with schizophrenia [Brown, 1994]. At deficiency selenium is preferentially retained in the brain compared with other organs, and several studies have shown that selenium deficiency is associated with mood [Benton, 2002]. A possible role of selenium transport has been proposed in schizophrenia [Berry, 1993]. Thus, the roles of selenium metabolism in pathophysiology of schizophrenia may merit further study.

Although linkage with schizophrenia and presence of CNVs around the ADM and SEPX loci [Yamada et al., 2004; Moon et al., 2006; Redon et al., 2006] prompted us to perform an association study, no association was found. This result suggests that up-regulation of *ADM* and *SEPX1* might be a phenomenon secondary to schizophrenia. However, in the association study, we studied only 223 schizophrenic patients and 364 control subjects. The number of the subjects and the number of SNPs examined are not large enough to totally exclude a possible association between schizophrenia and the SNPs of *SEPX1* and *ADM*. In addition, the result should be treated with caution, because there was a significant difference in gender between patients with schizophrenia and controls ($P < 0.05$).

TABLE II. The Result[Q4] of Case-Control Studies in Japanese Population

		Genotype			HWE	*P* -value	Allele		*P* -value
ADM									
rs7944706		A/A	A/G	G/G			A	G	
	CT	50	176	138	0.606		276	452	
	SZ	35	102	86	0.604	0.748	172	274	0.823
rs6484148		C/C	C/T	T/T			C	T	
	CT	43	166	155	0.887		252	476	
	SZ	25	84	114	0.121	0.117	134	312	0.106
rs6484147		C/C	C/T	T/T			C	T	
	CT	43	166	155	0.887		252	476	
	SZ	25	84	114	0.121	0.117	134	312	0.106
rs4597056		C/C	C/T	T/T			C	T	
	CT	157	163	44	0.865		477	251	
	SZ	114	84	25	0.121	0.160	312	134	0.116
rs726102		A/A	A/G	G/G			A	G	
	CT	42	165	157	0.892		249	479	
	SZ	25	84	114	0.121	0.147	134	312	0.140
SEPX1									
rs9928312		A/A	A/G	G/G			A	G	
	CT	45	175	144	0.464		265	463	
	SZ	27	102	94	0.934	0.820	156	290	0.622
rs9934331		C/C	C/G	G/G			C	G	
	CT	103	176	85	0.559		382	346	
	SZ	61	109	53	0.752	0.969	231	215	0.821
rs1003904		A/A	A/G	G/G			A	G	
	CT	158	150	56	0.044		466	262	
	SZ	78	105	40	0.653	0.129	261	185	0.060

CT, control; SZ, schizophrenia; HWE, Hardy–Weiberg equilibrium.
P values are calculated by Fisher's exact test.

With regard to endophenotypes of schizophrenia, mainly psychophysiological, neurocognitive, and neuroimaging findings have been proposed [Gottesman and Gould, 2003]. Relatively few studies focused on blood analysis in schizophrenia. Altered mRNA levels in LB cells were reported for *ADM* [Huang et al., 2004] and *PDLIM5* [Iwamoto et al., 2004]. Alterations in peripheral blood leukocytes mRNA were reported for *SELENBP1* and other candidate genes [Glatt et al., 2005], mitochondria-related transcripts [Whatley et al., 1998; Mehler-Wex et al., 2006], dopamine receptors [Ilani et al., 2001; Kwak et al., 2001; Zvara et al., 2005; Boneberg et al., 2006], alpha 7-nicotinic acetylcholine receptor subunit (*CHRNA7*) [Perl et al., 2006], and transforming growth factor beta receptor II (*TGFBR2*) [Numata et al., 2007]. Although none of these candidate mRNA markers in blood cells has been established, it is promising that two genes detected in this study have already been reported in the literature. *ADM* and *SEPX1* are a promising target of further research of biomarkers of schizophrenia.

After our previous report of gene expression analysis in monozygotic twins discordant for bipolar disorder [Kakiuchi et al., 2003], we used the same approach in LB cells [Haas et al., 2006; Hu et al., 2006; Matigian et al., 2007] or different tissues [Zhou et al., 2005; Cutting and Snowden, 2006; Sarkijarvi et al., 2006]. The present results that two previously reported genes were identified in the twins supported the validity of this methodology. It has been difficult to apply statistical analysis to a limited number of twin samples. Thus, in this study, we performed five independent experiments for each pair of twins. Although it is difficult to prove the validity of this method, it is possible that this extensive analysis enabled the successful selection of these two genes.

In this study, the two pairs of twins discordant for schizophrenia did not have other family history. Thus, the dysregulation of genes in the affected twin is not due to a heritable factor such as a genetic polymorphism, but rather to some environmental or epigenetic effect. Thus, lack of association of the two genes with schizophrenia may be reasonable.

Although we focused on *ADM* and *SEPX1* in this study, the change in *CD200* might also be potentially interesting, because several studies reported that the immune system in schizophrenics may be involved in its susceptibility [Nawa and Takei, 2006]. Moreover, *CD200* has a unique expression pattern that is expressed on B-cells and neurons [Wright et al., 2001].

TABLE III. Haplotype Analysis of ADM in Japanese SZ Samples

Haplotype	SZ	CT	χ^2	*P*-value	Global *P*-value
ADM					
A-T-T-C-G	168 (0.380)	270 (0.381)	0.00184	0.965	
G-C-C-T-A	132 (0.298)	238 (0.336)	1.76	0.184	
G-T-T-C-G	142 (0.321)	200 (0.282)	1.94	0.162	0.345

SZ, schizophrenia; CT, control.
Global *P*-value was calculated by a random permutation test (10,000 times) with the use of COCAPHASE program.
Only haplotypes that were verified at least once were analyzed.

A

ADM

r2	D' rs7944706	rs6484148	rs6484147	rs4597056	rs726102
rs7944706		1	1	1	1
rs6484148	0.3172		1	1	1
rs6484147	0.3172	1		1	1
rs4597056	0.3093	1	1		1
rs726102	0.3114	1	1	1	

B

SEPX1

r2	D' rs9928312	rs9934331	rs1003904
rs9928312		1	0.4592
rs9934331	0.6233		1
rs1003904	0.2071	0.5721	

Fig. 1. Intermarker linkage disequilibrium pattern for *ADM* (**A**) and *SEPX1* (**B**). The standardized disequilibrium coefficient (D′) and squared correlation coefficient (r^2) calculated by the COCAPHASE program are shown for Japanese control samples.

CD200 is expressed in developing neuronal cell bodies and axons [Morris and Beech, 1987]. Thus, *CD200* may be a promising target for further study.

In conclusion, we demonstrated the possible pathological contribution of *ADM* and *SEPX1* to schizophrenia and the usefulness of LB cells of monozygotic twins discordant for schizophrenia.

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ORIGINAL ARTICLES

Evidence for Acquired Pregenual Anterior Cingulate Gray Matter Loss from a Twin Study of Combat-Related Posttraumatic Stress Disorder

Kiyoto Kasai, Hidenori Yamasue, Mark W. Gilbertson, Martha E. Shenton, Scott L. Rauch, and Roger K. Pitman

Background: Controversy exists over the nature and origin of reduced regional brain volumes in posttraumatic stress disorder (PTSD). At issue is whether these reductions represent preexisting vulnerability factors for developing PTSD upon traumatic exposure or acquired PTSD signs due to the traumatic stress that caused the PTSD or the chronic stress of having the disorder (or both). We employed a case–control design in monozygotic twin pairs discordant for combat exposure to address the preexisting versus acquired origin of brain morphometric abnormalities in PTSD.

Methods: We used voxel-based morphometry to search for gray matter density reductions in magnetic resonance imaging (MRI) data obtained in a previous study of combat-exposed Vietnam veteran twins with (n = 18) versus without (n = 23) PTSD and their "high-risk" versus "low-risk" (respectively) identical combat-unexposed cotwins.

Results: Compared with the combat-exposed twins without PTSD, the combat-exposed twins with PTSD showed significant gray matter density reductions in four predicted brain regions: right hippocampus, pregenual anterior cingulate cortex (ACC), and left and right insulae. There was a significant PTSD Diagnosis × Combat Exposure interaction in pregenual ACC in which combat-exposed PTSD twins had lower gray matter density than their own combat-unexposed cotwins as well as than the combat-exposed twins without PTSD and their cotwins.

Conclusions: The results point to gray matter volume diminutions in limbic and paralimbic structures in PTSD. The pattern of results obtained for pregenual ACC suggests that gray matter reduction in this region represents an acquired sign of PTSD consistent with stress-induced loss.

Key Words: Anterior cingulate gyrus, brain, magnetic resonance imaging, monozygotic twins, posttraumatic stress disorder

Several structural magnetic resonance imaging (MRI) studies employing anatomic segmentation have found lower gray matter volumes in the hippocampus in posttraumatic stress disorder (PTSD) stemming from various traumatic events (1). One segmentation study found diminished gray matter volumes in pregenual anterior cingulate cortex (ACC) and subcallosal cortex but not dorsal ACC (2), whereas another did find dorsal ACC reduction (3). Decreased pregenual ACC activation in response to trauma-related stimuli is a prominent functional neuroimaging finding in PTSD (4–5).

The technique of voxel-based morphometry (VBM) allows an automated examination of structural brain differences using statistical parametric mapping (SPM) techniques. The validity of the VBM technique for assessing regional gray matter density compared with conventional region of interest measurements has been confirmed in several previous studies (6–9). Employing VBM, the first authors found reduced dorsal ACC gray matter density in victims of an urban terrorist attack with PTSD (10). Another recent study that employed VBM found gray matter density reduction in pregenual ACC but not dorsal ACC, although manual segmentation did not confirm volumetric reduction in the former structure (11). That study also found gray matter density reduction in left insula. Yet another recent PTSD VBM study found gray matter density reductions in hippocampus, pregenual ACC, and insula (12).

Controversy exists over the nature and origin of reduced regional brain volume in PTSD. Thus far the debate has focused on the hippocampus (13). At issue is whether reduced volume represents an acquired PTSD sign, for example, is due to the traumatic stress that caused the PTSD or the chronic stress of having PTSD (or both) or is a preexisting vulnerability factor for developing PTSD upon traumatic exposure. We have been employing a case–control design in monozygotic twin pairs discordant for combat exposure in Vietnam to address the preexisting versus acquired origin of biological abnormalities found in PTSD (14). In a structural MRI study that manually traced the outlines of the right and left hippocampus, we found that lower total hippocampal volume constituted a "familial" vulnerability factor for PTSD because it was found in both the combat-exposed twins with PTSD and their "high-risk" combat-unexposed cotwins whose hippocampal volumes were lower than those of the combat-exposed twins without PTSD and their "low-risk," combat-unexposed cotwins (15). (Note that the term "familial" includes both heredity and shared environment, i.e., environmental experiences that both members of a twin pair have had in common.)

In this study, we applied a VBM analysis to MRI data from the same twin sample to conduct a search throughout the entire brain for regional gray matter structural differences. On the basis of the published structural imaging studies just reviewed, we

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predicted lower gray matter density in combat-exposed twins with PTSD compared with combat-exposed twins without PTSD in the following regions: hippocampus, dorsal ACC, pregenual ACC, subcallosal cortex, and insula. In an attempt to clarify the origin of any such differences, we used the data from the combat-unexposed cotwins. Gray matter diminution that confers familial vulnerability to PTSD would be expected in the high-risk, compared with the low-risk, combat-unexposed twins. In contrast, diminution that reflects acquired damage in PTSD would be expected to be manifest in a Diagnosis × Combat Exposure interaction in which the combat-exposed twins with PTSD had lower gray matter density than their own high-risk, combat-unexposed cotwins as well as the combat-exposed twins without PTSD and their low-risk cotwins.

Methods and Materials

Subjects

The strategy for subject ascertainment and recruitment has been presented elsewhere (16). The sample was described in detail in the report of our previous hippocampus manual tracing study (15). This study reanalyzed the same MRI scans from the same subjects. In the previous study, one combat-exposed twin with PTSD and his combat-unexposed cotwin were removed from the analysis because the former was an extreme, asymmetrical outlier for manually traced hippocampal volume. This subject and his cotwin were included in the current study. (Exclusion of this pair did not alter the conclusions.) The protocol was approved by the institutional review board of the Manchester, New Hampshire, VA Medical Center. All subjects had previously given written informed consent before participation after a complete description of the procedures.

MRI Data Preanalysis

The MRI acquisition techniques were described in the previous report (15). The methods used to analyze these data in the present study were similar to those reported elsewhere (10). Image analysis was performed using ANALYZE PC 3.0 (Mayo Foundation, Rochester, Minnesota) and SPM 99 software (Wellcome Department of Cognitive Neurology, Institute of Neurology, London, United Kingdom) running in MATLAB 6.1 (Mathworks, Sherborn, Massachusetts). In ANALYZE, image data were resampled using an algorithm to make them isotropic, with the sides measuring .9375 mm, and then stored. Resampled images were first spatially normalized into the standard MNI152 template (17,18). Normalized images were then segmented into gray matter, white matter, cerebrospinal fluid, and skull and scalp compartments using an automated, operator-independent process (19). The segmentation step also incorporated an image density nonuniformity correction to address image density variations caused by various positions of cranial structures within the MRI head coil (20). The spatially normalized segments of the gray matter were smoothed with a 12-mm full-width at half-maximum isotropic Gaussian kernel to accommodate individual variability in sulcal and gyral anatomy. For medial temporal regions (e.g., hippocampus), a 4-mm smoothing kernel was used instead, as has been recommended (21). By smoothing the data, the partial volume effect was used to create a spectrum of gray matter densities. Gray matter density is equivalent to the weighted average of the gray matter voxels located in the volume defined by the smoothing kernel. Because previous studies have shown a fair correlation between regional gray matter density determined by VBM and structural volumes measured by conventional, manual tracing (7,9,22), the regional gray matter density can be considered to represent the local volume of gray matter.

Statistical Analyses

Demographic and psychometric data were analyzed by means of a mixed model that treated Diagnosis (in the combat-exposed twin) as a between-pairs fixed effect, Combat Exposure as a within-pairs fixed effect (repeated measure), and pairs as a random effect (16). This model analysis yields a t statistic. Gray matter density was estimated on a voxel-by-voxel basis using SPM 99 (23). Contrasts were made between the 18 combat-exposed twins with PTSD and the 23 combat-exposed twins without PTSD, and separately between their high- and low-risk cotwins, using independent t tests, adjusted for individual intracranial volume. Diagnosis × Exposure interactions were evaluated by means of a mixed, multigroup (Diagnosis), conditions (Combat Exposure), and covariates (intracranial volume) model in which one twin pair was treated as though one subject with two conditions. In this analysis, 82 covariates were entered corresponding to 82 images ([18 + 23] × 2). For each of the foregoing analyses, a statistical parametric map (SPM) of the t statistic (SPM{t}) was created, and the SPM{t} values were transformed to the normal distribution (SPM{z}). The statistical significance threshold was set at $p < .05$ corrected for multiple comparisons using the False Discovery Rate (FDR) (24).

The anatomic locations of peak coordinates were initially defined using the latest version of Talairach Daemon Client (25). These localizations were then confirmed by visually inspecting the coordinates overlaid on the mean structural image of the study sample. For peaks located within predicted brain regions, small volume correction was applied using the following a priori volume approximations from the literature: hippocampus 3.5 mL each side; insula 8 mL each side; dorsal ACC 10 mL bilaterally; pregenual ACC 5 mL bilaterally; and subcallosal cortex 5 mL bilaterally (total volume of predicted brain regions = 43 mL). For peaks located outside predicted regions, correction for whole brain was employed. Because the predictions were directional, namely, lower gray matter density in combat-exposed subjects and PTSD pairs, the tests were one-tailed, and only results in the predicted direction(s) are reported.

Results

Demographics and Psychometrics. Group mean age, Combat Severity Score (26), total Clinician-Administered PTSD Scale (CAPS) score (27), number of potentially traumatic lifetime noncombat events (16), total Michigan Alcoholism Screening Test (MAST) score (28), and Symptom Check List-90-Revised (SCL-90-R) Depression scale score (29), along with statistical analyses, are presented in Table 1. It may be seen that age was similar among subject groups. Combat-exposed twins with PTSD had more severe combat exposure than combat-exposed twins without PTSD. As expected by virtue of selection, the former had greater combat-related symptom severity on the CAPS. Twin pairs with PTSD (i.e., twin pairs in which the combat-exposed twin was diagnosed with current, combat-related PTSD) reported more potentially traumatic lifetime noncombat events than non-PTSD pairs (i.e., twin pairs in which the combat-exposed twin was diagnosed with neither current nor past combat-related PTSD). Combat-exposed twins also reported more potentially traumatic lifetime noncombat events than their combat-unexposed cotwins. Combat-exposed twins with PTSD had more severe alcoholism histories than the other three groups. Combat-exposed twins with PTSD also reported more depression than

Table 1. Group Means (SD) of Combat-Exposed Vietnam Veterans with and without Posttraumatic Stress Disorder (PTSD) and Their Combat-Unexposed, Identical Cotwins

	PTSD Pairs[a]		Non-PTSD Pairs[b]		Mixed Model						Independent *t* Tests			
					Diagnosis		Exposure		Interaction		Exposed		Unexposed	
	Exposed (n = 18)	Unexposed (High Risk) (n = 18)	Exposed (n = 23)	Unexposed (Low Risk) (n = 23)	t 39	p	t 40	p	t 39	p	t 39	p	t 39	p
Age (years)[c]	52.8 (3.4)	52.8 (3.4)	51.8 (2.3)	51.8 (2.3)	—	—	—	—	—	—	1.1	.27	1.1	.27
CAPS[d]	73.3 (16.9)	—	6.2 (7.3)	—	—	—	—	—	—	—	17.2	<.001	—	—
Combat Severity[e]	7.7 (2.1)	—	3.5 (2.6)	—	—	—	—	—	—	—	5.6	<.001	—	—
Traumatic Events[f]	8.1 (2.6)	5.2 (3.7)	5.1 (4.0)	4.2 (3.0)	2.6	.01	2.4	.02	1.3	.20	2.8	.009	1.0	.34
MAST[g]	19.1 (17.6)	6.4 (10.2)	2.4 (4.5)	2.5 (4.0)	4.5	<.001	2.8	.007	2.8	.007	4.4	<.001	1.7	.10
Depression[h]	2.5 (.9)	.3 (.5)	.6 (.7)	.4 (.5)	4.6	<.001	5.3	<.001	6.9	<.001	7.4	<.001	.7	.51

[a]As determined by the presence of current combat-related PTSD in the combat-exposed twin.
[b]As determined by the absence of current or past combat-related PTSD in the combat-exposed twin.
[c]As of October 1, 2000.
[d]Clinician-Administered PTSD Scale (range 0–136).
[e]18-item measure (range 0–18).
[f]Number of potentially traumatic lifetime noncombat events.
[g]Michigan Alcoholism Screening Test (range 0–25).
[h]Symptom Check List-90-Revised Depression Subscale (range 0–4).

the other three groups. The Pearson Correlation Coefficient between total CAPS score and SCL-90-R Depression was large (r = .86) in the PTSD pairs but negligible (r = −.06) in the non-PTSD pairs.

Gray Matter Density

Table 2 presents the results of the contrasts between combat-exposed twins with versus without PTSD. For the sake of a complete exposition of these data, all results statistically significant at the liberal threshold of uncorrected $p < .001$ with spatial extent of $k > 10$ voxels are shown. Of the seven peaks that met this threshold, four were located in predicted brain regions, namely, right hippocampus, pregenual ACC, right midinsula, and left anterior insula (shown in Figure 1). Each of these four peaks also met the statistical significance threshold of $p < .05$ with small volume correction for the a priori size of the structure (as shown in the second column of Table 3). No voxels in nonpredicted brain regions met the threshold of $p < .05$ corrected for whole brain in these, or any other, analyses.

At the right hippocampus, pregenual ACC, left anterior insula, and right midinsula loci shown in Table 2, within the 18 PTSD combat veterans, we examined the correlations between gray matter density and total CAPS score, as well as SCL-90-R Depression score. Because these analyses involved single voxels, the significance threshold was $p < .05$ uncorrected. None of these correlations were significant. We also performed the same correlations for CAPS A (reexperiencing), B (avoidance/numbing), and C (hyperarousal) symptom cluster subscores; for these analyses we applied a Bonferroni correction to the significance threshold, namely, $p < .017$ (.05/3). The only significant correlations were between symptom cluster B (reexperiencing) and gray matter density in pregenual ACC (r = −.57, p = .008), left anterior insula (r = −.53, p = .0130), and right midinsula (r = −.59, p = .006).

Table 2. Loci Showing Gray Matter Density Reductions in Combat-Exposed Twins with versus without Combat-Related Posttraumatic Stress Disorder That Were Significant at Uncorrected $p < .001$

z	p_{svc}	k	[x y z]	Brain Region
4.50	**.001**	**248**	**[44 −2 −14]**	**Right midinsula**
4.47		145	[−62 −56 −4]	Left middle temporal gyrus
4.39	**.001**	**27**	**[34 −28 −16]**	**Right hippocampus**
3.95	**.005**	**236**	**[−36 10 −4]**	**Left anterior insula**
3.71	**.004**	**185**	**[0 46 10]**	**Pregenual anterior cingulate cortex**
3.52		35	[−46 42 −14]	Left inferior frontal gyrus
3.47		22	[−64 −44 10]	Left superior temporal gyrus

k, cluster size; p_{svc}, significance level with small volume correction on the basis of the a priori size of the structure; [x y z] = Montréal Neurological Institute coordinates of peak voxel.
Predicted areas appear in boldface.

The contrasts between the high-risk, combat-unexposed cotwins of the combat-exposed twins with PTSD versus the low-risk, combat-unexposed cotwins of the combat-exposed twins without PTSD did not identify any voxels that met the statistical significance threshold of $p < .05$, even with small volume corrections. The only statistically significant Diagnosis × Exposure interaction was found in pregenual ACC ([8 50 12], z = 3.32, k = 16, p = .02 corrected for the a priori size of this structure). The location of this cluster is shown in Figure 2. Scatterplots of individual subjects' values at the [8 50 12] pregenual ACC locus are shown in Figure 3. There were no significant correlations between gray matter density in the 18 PTSD combat veterans minus gray matter density in their combat-unexposed cotwins, and total CAPS score, SCL-90-R Depression, or (with Bonferroni corrections) any of the CAPS symptom cluster subscores.

The mixed model and t test analyses that yielded the statistically significant results described earlier were repeated entering the following possibly confounding variables into the respective models as covariates: age, combat severity (in the exposed twin), number of potentially traumatic lifetime noncombat events, MAST score, and SCL-90-R Depression score. To control for a possibly confounding role of childhood physical or sexual abuse, the data were re-analyzed deleting pairs within which either member had such a history. All these results are shown in Table 3.

Discussion

Of the seven loci at which combat-exposed twins with PTSD had lower gray matter density than combat-exposed twins without PTSD at a liberal threshold of $p < .001$, four were located in predicted brain regions, namely, right hippocampus, pre-

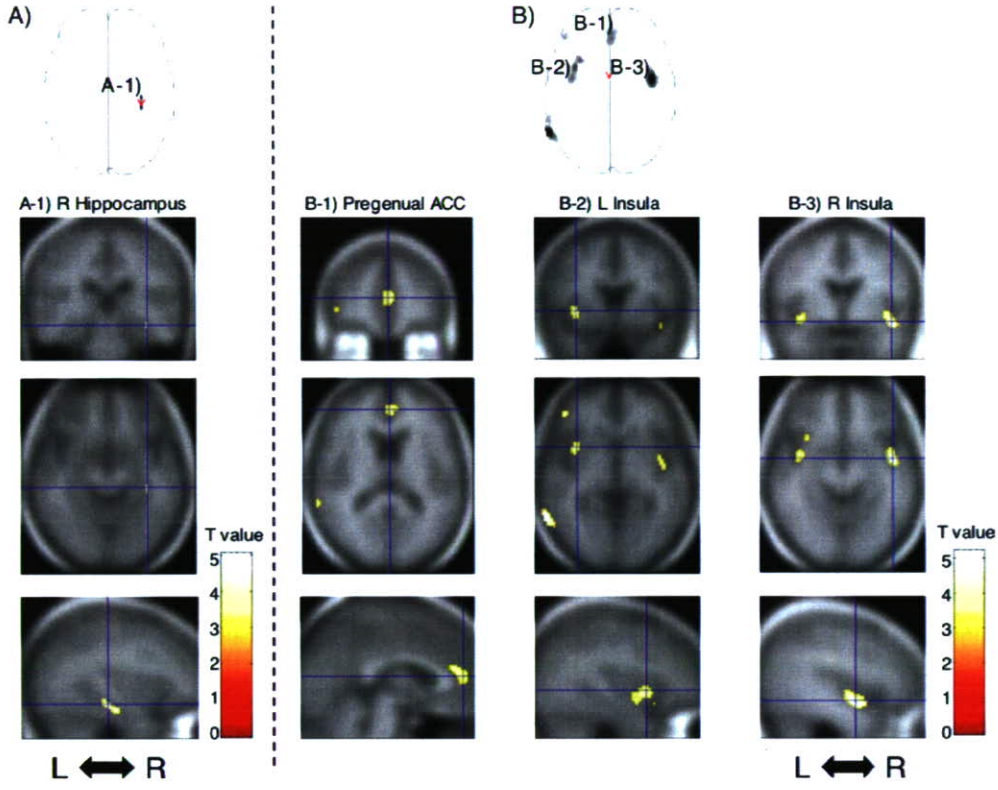

Figure 1. Brain regions showing diminution in gray matter density in combat-exposed twins with posttraumatic stress disorder (PTSD) versus those without PTSD. **(A)** Statistical parametric mapping (SPM) analysis with 4-mm Gaussian smoothing kernel revealed statistically significant reduced gray matter density shown in the axial projection. **(A-1)** Regional gray matter density reduction in the right hippocampus is rendered onto orthogonal slices of the averaged magnetic resonance image of the present study's subjects. **(B)** SPM analysis with 12-mm Gaussian smoothing kernel revealed statistically significant reduced gray matter density shown in the axial projections. Regional gray matter density reductions in the following areas are rendered: **(B-1)** pregenual anterior cingulate cortex; **(B-2)** left insula; **(B-3)** right insula. L, left hemisphere; R, right hemisphere; ACC, anterior cingulate cortex.

genual ACC, and left anterior and right midinsula, even though the predicted brain regions occupy less than 10% of total gray matter volume. This regional specificity supports the validity of our results and implicates limbic and paralimbic structures as the major sites of gray matter density reductions in combat-related PTSD. Gray matter reductions in pregenual ACC and both insulae significantly correlated only with the cluster B "reexperiencing" symptoms of PTSD.

The ACC, especially its pregenual (or "affective") division, and insula are components of the anterior "paralimbic belt," are strongly interconnected to each other and to the amygdala, and are highly involved in emotional aspects of brain function (30–32). Impaired pregenual ACC function is one of the most robust neuroimaging findings in PTSD (4–5). A neurocircuitry model of PTSD posits that the ventromedial prefrontal cortex, including pregenual ACC, inhibits the expression of classically conditioned fear responses by the amygdala (33). Thus impairment in this brain region might be expected to most affect the DSM-IV symptoms that are putatively most closely related to conditioned fear, namely, the cluster B symptoms (especially B.4 and B.5, that is, intense psychological distress (and/or) physiological reactivity on exposure to internal or external cues that symbolize or resemble an aspect of the traumatic event). To the extent that diminished structure implies diminished function, reduced pregenual ACC gray matter density is consistent with this neurocircuitry model.

Table 3. Results Adjusted for Potentially Confounding Variables

Unadjusted	Age	Combat Severity[a]	Traumatic Events[b]	MAST Score[c]	Childhood Abuse[d]	SCL-90-R Depression[e]	
$z\ (p_{svc})$	$z\ (p_{svc})$	$z\ (p_{svc})$	$z\ (p_{svc})$	$z\ (p_{svc})$	$z\ (p_{svc})$	$z\ (p_{svc})$	Brain Region
Combat-Exposed Twins: PTSD vs. non-PTSD							
4.50 (.001)	4.43 (.001)	3.23 (.05)	3.98 (.005)	3.39 (.02)	3.19 (.04)	2.07 (.49)	Right midinsula [44 −2 −14]
4.39 (.001)	4.36 (.001)	3.05 (.11)	4.11 (.004)	4.63 (<.001)	3.56 (.04)	1.52 (.99)	Right hippocampus [34 −28 −16]
3.95 (.005)	3.98 (.005)	3.32 (.03)	3.06 (.07)	3.26 (.04)	3.63 (.01)	2.15 (.44)	Left anterior insula [−36 10 −4]
3.71 (.004)	3.59 (.009)	2.50 (.18)	2.79 (.08)	2.40 (.14)	2.92 (.03)	.93 (.99)	Pregenual anterior cingulate cortex [0 46 10]
Diagnosis × Combat Exposure Interaction							
3.32 (.02)	3.39 (.02)	3.32 (.02)[f]	2.76 (.10)	2.77 (.10)	3.42 (.01)	1.81 (.53)	Pregenual anterior cingulate cortex [8 50 12]

p_{svc}, significance level with small volume correction; PTSD, posttraumatic stress disorder.
[a]18-item measure (range 0–18).
[b]Number of potentially traumatic lifetime noncombat events.
[c]Michigan Alcoholism Screening Test (range 0–25).
[d]Deleting six PTSD pairs and five non-PTSD pairs within which either twin had a history of childhood abuse.
[e]Depression subscale of Symptom Check List 90—Revised.
[f]Covariate is value in combat-exposed twin.

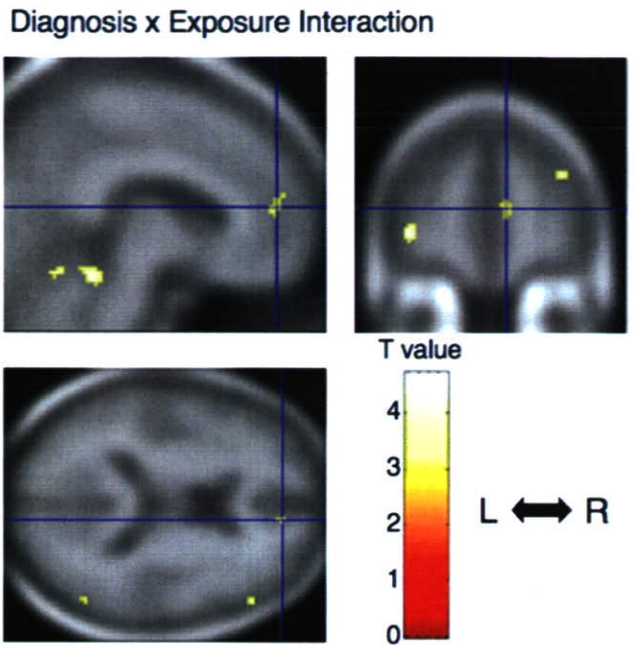

Figure 2. Brain region showing posttraumatic stress disorder Diagnosis × Combat Exposure interaction. Regional interaction for gray matter density in pregenual anterior cingulate cortex is rendered onto orthogonal slices of the averaged magnetic resonance image of the study subjects. Crosshairs indicate the peak coordinate of the interaction [8 50 12]. L, left hemisphere; R, right hemisphere.

Functional neuroimaging studies of the hippocampus in PTSD are less common, but they, too, support impairment in this brain region (34–36). Hippocampal impairment may contribute to PTSD by reducing the ability to construct declarative narratives that bind the affect associated with the traumatic event (37), by the ability to recognize safe contexts (33), or by other unknown mechanisms. The reduced gray matter density found in bilateral insulae is paradoxical in light of studies that have generally found hyperactivity in this brain region in PTSD (38) and other anxiety conditions (39). One model of anterior insula function posits that this structure detects the difference between an observed and expected body state and generates an interoceptive prediction signal that triggers anxiety (39). A structurally compromised insula may be less inhibited in generating such signals in PTSD, but this is in the realm of speculation.

The most interesting result from our study is the significant Diagnosis × Exposure interaction in the pregenual ACC, with combat-exposed PTSD twins having lower gray matter density than their own combat-unexposed cotwins and than the combat-exposed twins without PTSD and their cotwins, supporting the inference that pregenual ACC gray matter reduction is an acquired sign of PTSD. In animals, exposure to chronic stress has been shown to damage not only the hippocampus in rodents (40) and primates (41) but also the ACC in rodents (42,43) and primates (44). It has been hypothesized that such damage may provide a basis for structural changes observed in PTSD (42,45). A recent study of mentally healthy persons that used automated segmentation found that those who reported early life stressors had smaller ACCs than those who did not (46). However, causal inferences are difficult to draw from the cross-sectional study of nontwins.

When the Diagnosis × Exposure interaction at [8 50 12] was adjusted for MAST score, its statistical significance level was reduced to corrected $p = .10$, which falls short of statistical significance. We did not obtain data regarding recent alcohol consumption. This is a limitation considering that imaging findings related to alcohol may be more sensitive to recent as opposed to more remote intake. On the other hand, the likelihood that increased alcohol use by the PTSD veterans accounts for the gray matter density reduction in their ACCs is diminished by the consideration that if the PTSD subjects studied here had consumed enough alcohol to damage their brains, evidence for this should have been found in other brain regions known to be affected by alcohol, including superior, motor, and other areas of the frontal cortex and cerebellum (47,48), none of which (except for a small cluster in left inferior frontal cortex) showed volumetric reduction in the PTSD compared with the non-PTSD combat veterans at even the liberal threshold of uncorrected $p < .001$. Thus the specificity of volumetric diminution to our predicted brain regions argues against a global effect such as alcohol-induced brain damage. Finally, a recent manual tracing study found comparably (and significantly) reduced ACC volume in subgroups of PTSD veterans with and without a history of lifetime alcohol abuse or dependence, in comparison to non-PTSD veterans (49).

When the Diagnosis × Exposure interaction in pregenual ACC was adjusted for number of potentially traumatic lifetime noncombat events, its statistical significance level was also reduced to corrected $p = .10$. This means we cannot be fully confident that stressful events other than military combat do not account for the reduced ACC gray matter density in the PTSD veterans. However, even if such events did contribute, this would still not be inconsistent with stress-induced diminution of this structure. When the Diagnosis × Exposure interaction in pregenual ACC was adjusted for depression, it was no longer significant. This is not surprising given the high association between depression and PTSD in our sample, in which self-

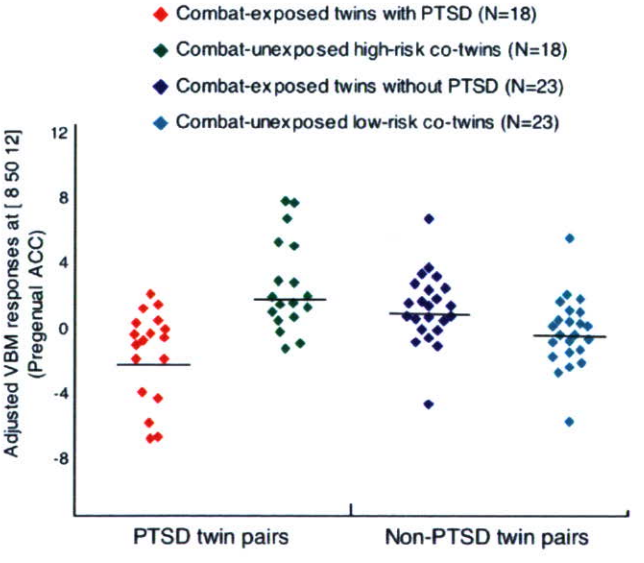

Figure 3. Scatterplots of individual subjects' adjusted voxel-based morphometry responses. Shown at the site of the posttraumatic stress disorder Diagnosis × Combat Exposure interaction in pregenual anterior cingulate cortex [8 50 12]. Means are represented by solid horizontal lines drawn on each group's distribution.

reported depression appears to have been acquired along with PTSD, making the two likely facets of the same posttraumatic psychopathology.

However, because the most salient, common difference in our study was the presence of combat-related PTSD in the former, and because, as noted earlier, the observed effects remained significant or nearly significant after considering the contributions of several important potentially confounding variables, it is reasonable to attribute this lower gray matter density to the presence of combat-related PTSD.

Combat-exposed twins with PTSD also had lower gray matter density than combat-exposed twins without PTSD in right hippocampus and left anterior and right midinsula, as well as at another site within pregenual ACC, replicating previous studies. These results could not be explained by group differences in age, combat severity, number of potentially traumatic lifetime noncombat events, alcoholism, or child abuse. Unfortunately, the analyses that included the data from the combat-unexposed cotwins were unable to shed light on the origin of these gray matter reductions in the combat-exposed twins with PTSD, because they failed to yield either a significant difference between high- and low-risk combat-unexposed cotwins (which would support a pretrauma vulnerability factor) or a significant Diagnosis × Exposure interaction (which would support an acquired abnormality). Finally, these results were unable to replicate previously reported segmentation and voxel-based morphometric findings of gray matter reduction in subcallosal cortex and dorsal ACC.

In the same twin sample studied here, we previously found manual tracing evidence that diminished hippocampal volume represents a pretrauma vulnerability factor for PTSD, rather than an acquired PTSD sign (15). In contrast, our VBM results suggest that diminished volume in pregenual ACC is acquired as a result of the combat exposure that led to PTSD, the PTSD itself, or both. We have no ready explanation as to why diminutions in these two structures should have different origins. As noted earlier, however, the origin of gray matter density reduction in the pregenual ACC site other than the one that showed the significant interaction could not be explicated by our data; it is possible that it represents a PTSD vulnerability factor. Additional techniques that may help to clarify this uncertainty in future studies include cortical parcellation (segmentation) and magnetic resonance spectroscopy.

It has been suggested that VBM may not detect very small, localized gray matter volume reductions because false-negative VBM findings may arise from the changes in the shape or displacement of structures in the course of spatial normalization (7). Additionally, VBM may be biased against finding group differences in areas that are spatially complex (50). Inversely, we cannot rule out the possibility that the abnormalities detected by VBM in our study reflected group differences in the shape of brain structures rather than their volume (11), although even shape differences may have functional consequences. The failure of VBM to find a significant hippocampal gray matter reduction in the high- versus low-risk, combat-unexposed cotwins contrasts with our positive result in the same sample using manual segmentation of hippocampus (15) suggests that the latter technique may be more sensitive to reduced volume in this structure than the voxel-based approach. Similarly, we are unable to rule out the possibility that subtle group differences in other brain regions in this study remained below the sensitivity of VBM or the detection power conferred by our sample.

KK and HY contributed equally to this work.

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