male; 47.0 ± 14.9) and 529 controls (270 female; 39.7 ± 15.4 years: 259 male; 34.9 ± 12.4 years) were genotyped for association analysis of rs175174. Moreover, in additional linkage disequilibrium (LD) mapping around this SNP, 95 schizophrenic patients (50 female and 45 male) and 96 controls (44 female and 52 male), part of each sample used in association analysis, were genotyped for three SNPs. The general characterization of these subjects and a description of their psychiatric assessment according to identical criteria were published elsewhere [13]. After explaining the study to all subjects, written informed consent was obtained from each. This study was approved by the Ethics Committee of the Nagoya University Graduate School of Medicine and Fujita Health University.

Genomic DNA was extracted from peripheral blood of all subjects. For rapid genotyping of SNPs, rs175174 and additional three SNPs for LD mapping (rs175169, rs175175 and rs2292570), polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) assays were developed. The information of PCR primers is available on request. The PCR reactions of all SNPs were carried out in a 10 μl volume containing 10 ng genomic DNA, 0.4 M of each primer, 200 μM of dNTP, 1× PCR Gold Buffer, 1.5 mM $MgCl_2$ and 0.25 U of Amplitaq Gold™ (Applied Biosystems Japan Ltd., Tokyo, Japan), using the GeneAmp™ PCR system 9700 (Applied Biosystems Japan Ltd.). PCR cycling conditions consisted of an initial denaturation step at 95 °C for 9 min, followed by 45 cycles of 95 °C for 15 s, 56 °C for 20 s, 72 °C for 30 s, and ending with a final extension step at 72 °C for 7 min. PCR product was digested using appropriate restriction enzymes according to the manufacture's recommendation (New England Biolabs, England, UK) (Table 1). DNA fragments were resolved by electrophoresis in a 6% acrylamide gel stained with ethidium bromide.

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by χ^2 test. Marker-trait association analysis was also evaluated by χ^2 test (SPSS 10.0J, SPSS Japan Inc., Japan). To evaluate pairwise LD matrices among SNPs (by D' and r^2), we used the software HAPLOVIEW version 2.05 (developed in Mark Daly's lab., URL; http://www.broad.mit.edu/personal/jcbarret/haploview/index.php). This software also defined "LD blocks" by reasonable criteria based on 95% confidential bounds on D' values [4]. Power calculation was performed using a statistical program prepared by Ohashi et al. [10]. The significance level for all statistical tests was 0.05.

In view of the gender differences in gene effects, we included analyses of samples divided according to the gender. Both in cases and controls, genotype frequencies of total, female and male samples were not significantly different from HWE.

In association analysis, we could not find associations of rs175174 with schizophrenia in either male or female (Table 2).

Next, to test whether rs175174 is representative for ZDHHC8 or not, we performed LD mapping using three additional SNPs around ZDHHC8 (Fig. 1). LD matrices between each pair of SNPs showed strong LD both in cases and controls (Table 1). Even after dividing samples according to the gender, all LD patterns showed the same trends (data not shown). These findings may suggest that the LD pattern of ZDHHC8 is a block-like pattern and that rs175174 is the "representative SNP" of this gene.

The power based on genotype relative risk (GRR) was calculated to evaluate the non-significant results due to type II error. When we set the GRR at 1.28, 1.42 and 1.40 in all, female and male samples, respectively (multiplicative model), our sample size provided powers of more than 80%.

We could not replicate an original positive association using TDT of ZDHHC8 with schizophrenia by the present case–control association analysis among Japanese. Nor could we replicate the gender-specific effect of the risk SNP. In this association analysis, our sample sizes provide enough power to deny the hypothesis. We also performed the fine LD mapping of Japanese samples and showed that the LD pattern of ZDHHC8 was the same block-like pattern as one of the samples from the United States and South Africa. The results provide evidence that not only rs175174 but also ZDHHC8 would not be a susceptibility factor for schizophrenia in either Japanese females or males. The discrepancy between Japanese and the samples from the United States and South Africa may derive from ethnic differences.

A couple of limitation should be addressed to discuss the present results. Initially, the mean age of controls is much younger than that of patients in the present study. This means that a number of young controls, although not more than five subjects given a lifetime morbidity risk of 0.8–1.0%, may go on to develop schizophrenia. This confounding factor might weaken the power of the present study. Another limitation

Table 1
SNPs in LD mapping and pairwise LD matrices

SNP ID	D'				Restriction enzyme
	rs175169	rs175174	rs175175	rs2292570	
rs175169		0.97 (0.78)	1.0 (0.29)	1.0 (0.67)	*Bsl*I
rs175174	0.97 (0.80)		1.0 (0.36)	1.0 (0.58)	*Bse*RI
rs175175	1.0 (0.26)	1.0 (0.31)		1.0 (0.21)	*Alw*NI
rs2292570	0.93 (0.76)	0.97 (0.70)	1.0 (0.23)		*Tsp*RI

Upper diagonal figures are D' (r^2) of controls and lower diagonal figures are D' (r^2) of schizophrenia.

Table 2
Association analysis of rs175174

Samples	Number	G/G	G/A	A/A	*P* value (genotype)	MAF[a]	*P* value (allele)
Total							
SCZ	561	238	245	78		0.357	
CON	529	205	259	65	0.213	0.368	0.618
Female							
SCZ	259	114	106	39		0.355	
CON	270	112	130	28	0.133	0.344	0.714
Male							
SCZ	302	124	139	39		0.359	
CON	259	93	129	37	0.457	0.392	0.260

SCZ: schizophrenia; CON: control.
[a] Minor allele frequency.

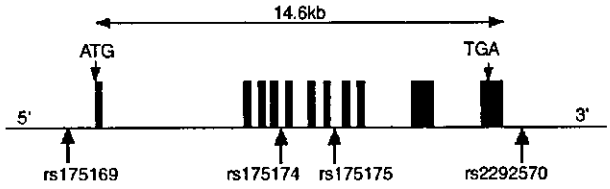

Fig. 1. Genomic structure of ZDHHC8 and SNPs used in association analysis and LD mapping. Vertical bars represent exons of ZDHHC8, and each number under arrows represents SNP ID.

which must be exercised is that the other candidates related to the neurodevelopmental and neuroprotective effect of ZDHHC8 would be in locus heterogeneity [11]. For example, ZDHHC8 encodes a putative transmembrane palmitoyltransferase modulating numerous classes of neuronal proteins including proteins important for neuronal development, neurotransmitter receptors such as NMDA [3]. Thus, the combined effect between ZDHHC8 and the other genes might be a stronger predisposing factor. Further genetic analysis including related candidate genes would definitely be required for a conclusive result.

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ORIGINAL INVESTIGATION

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Fluvoxamine, a selective serotonin reuptake inhibitor, suppresses tetrahydrobiopterin levels and dopamine as well as serotonin turnover in the mesoprefrontal system of mice

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Abstract *Rationale:* Tetrahydrobiopterin (BH_4) is a coenzyme of tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH), rate-limiting enzymes of monoamine biosynthesis. According to the monoamine hypothesis of depression, antidepressants will restore the function of the brain monoaminergic system, and BH_4 concentration. *Objective:* To investigate the effects of fluvoxamine on BH_4 levels and dopamine (DA) and serotonin (5-HT) turnover in the mesoprefrontal system, incorporating two risk factors of depression, social isolation and acute environmental change. *Methods:* Male ddY mice (6W) were divided into two housing groups, i.e. group-housing (eight animals per cage; 35 days), and isolation-housing (one per cage; 35 days), SC injected with fluvoxamine (20 or 40 mg/kg; days 29–35), and exposed to 20-min novelty stress (day 35). The levels of BH_4, DA, homovanilic acid (HVA), 5-HT, and 5-hydroxyindoleacetic acid (5-HIAA) were measured in the prefrontal cortex and midbrain. *Results:* Under the group-housing condition, novelty stress significantly increased BH_4 levels in both regions, and the HVA/DA ratio in the midbrain, whereas it did not change any parameters in either region under the isolation-housing condition. In the prefrontal cortex, fluvoxamine significantly decreased the 5-HIAA/5-HT ratio under the group-housing condition, and BH_4 levels and the HVA/DA ratio under the isolation-housing condition. In the midbrain, fluvoxamine significantly decreased all parameters, except for an increasing in the 5-HIAA/5-HT ratio under the isolation-housing condition. *Conclusion:* Isolation-housing suppressed the increase of BH_4 levels and DA turnover elicited by novelty stress. Fluvoxamine suppressed BH_4 levels, and DA and 5-HT turnover. Fluvoxamine may have altered DA turnover by suppressing BH_4 levels.

Keywords Social isolation · Novelty stress · Animal model · Tetrahydrobiopterin · Dopamine turnover · Serotonin turnover

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Introduction

(6R)-5,6,7,8-tetrahydrobiopterin (BH_4) is a coenzyme of tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH), which are the rate-limiting enzymes of monoamine biosynthesis. BH_4 is also a coenzyme of NO synthase (NOS). NO is known to act as a signaling molecule in the central nervous system (CNS) (Barañano et al. 2001; Kiss and Vizi 2001; Ohkuma and Katsura 2001; Prast and Philippu 2001; Esplugues 2002). Thus, BH_4 plays an important role in CNS activity.

With regard to the cause of human depression, the monoamine hypothesis focuses on impaired function of the monoaminergic system of the brain (Smith et al. 1997; Delgado 2000; Hirschfeld 2000; Leonard 2000). Pharmacological studies of almost all clinically effective antidepressants have supported this hypothesis. Antidepressant-induced suppression in the activity of TH (Nestler et al. 1990) and TPH (Lapierre et al. 1983) may be related to BH_4 levels. Furthermore, recent studies suggest the possibility that alteration of NOS activity may be related to the antidepressant-like effects of NOS inhibitors in animal models (Harkin et al. 1999; Karolewicz et al. 1999; Da Silva et al. 2000). Investigation into the relationship between changes in the activities of these enzymes (TH, TPH, and NOS) and BH_4 levels induced by antidepressants should therefore help to clarify the pathophysiology of human depression.

Concerning environmental risk factors of depression, most patients become ill after adverse life events, such as interpersonal loss (separation, etc.) (Paykel 1994). Further, absence of social support appears to be associated with an

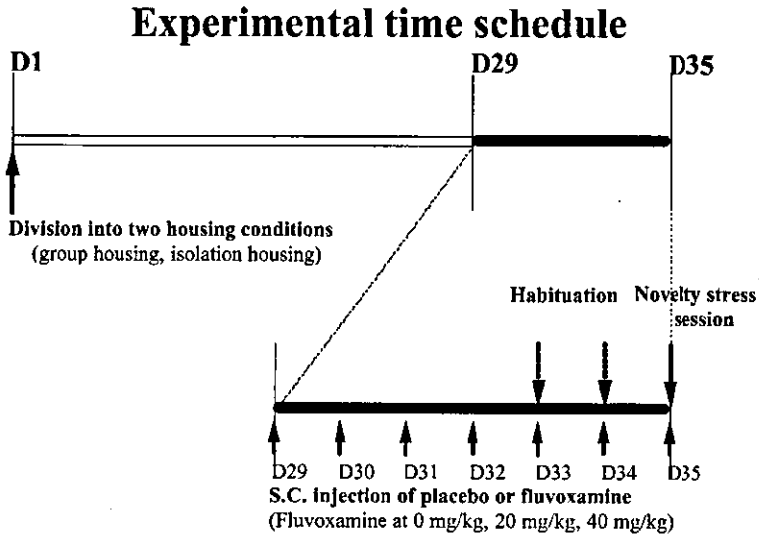

Fig. 1 Experimental time schedule. Mice were divided into 12 groups as described in Materials and methods

onset and relapse of depression (Paykel 1994). Kendler showed that genetic factors cooperate with environmental factors to induce the onset of depression in humans (Kendler et al. 1993). Thus, we proposed an animal model that simulated two of the major environmental risk factors of human depression (Miura et al. 2002a,b; Miura et al. 2004), i.e. social isolation and acute environmental change (Kendler et al. 1993; Paykel 1994).

We propose that changes in brain BH_4 levels play an important role in the pathophysiology of depression, and that antidepressants modulate these changes. Our recent study suggested that fluvoxamine, an SSRI, suppressed BH_4 levels as well as 5-HT turnover in the hippocampus of mice (Miura et al. 2004). In the present study, we further investigated other regions that we suspect are involved in the pathophysiology of human depression, and we measured BH_4, dopamine (DA), and serotonin (5-HT) levels simultaneously. Mesocorticolimbic DA projections (A8, A10) originating from the ventral tegmental area (VTA) of the midbrain (Cooper et al. 2003) have been shown to play an important role in a reward system, i.e. in motivating behavior (Kupferman and Schwartz 1995). We therefore selected two regions of focus, the prefrontal cortex and the midbrain. The aim of the present study was to investigate the effects of fluvoxamine on BH_4 levels and DA and 5-HT turnover in the mesoprefrontal regions, and to clarify the role of BH_4 in our novel animal model simulating two of the major environmental risk factors of human depression (Miura et al. 2002a,b, 2004).

Materials and methods

Animals

A total of 96 male ddY mice were used in the present experiments. The mice were transported from a breeding company at 5 weeks of age to our experimental animal center. After a 1-week habituation period, the mice, all of which had previously been housed in groups (eight per cage), were divided into two different groups according to housing conditions, i.e. a group-housing group (eight per cage; n=48) and an isolation-housing (one per cage; n=48; Fig. 1) group. The cages used for group-housing were 21×31×13 cm, and the cages used for isolation-housing were 17×29×13 cm. After being assigned to one of the two housing conditions, the mice were reared for 35 days (Fig. 1). Cage exchange was performed twice a week. Food and water were provided ad libitum. A 12-h light/dark cycle was maintained and room temperature was maintained at 21–23°C. All efforts were made to minimize both the number of animals used and the degree of their suffering. All of the experiments were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). The experiments also comply with the current laws of Japan.

Fluvoxamine injection

In week 5 (days 29–35) after being assigned to one of the two housing conditions, the mice were SC injected with placebo (distilled water) or low-dose (20 mg/kg) or high-dose (40 mg/kg) fluvoxamine once per day (Fig. 1). The fluvoxamine was kindly donated by Solvay Pharmaceuticals (Brussels, Belgium). The mice were then further divided into three groups: a control (0 mg/kg, n=32), a low-dose (20 mg/kg, n=32), and a high-dose (40 mg/kg, n=32; Fig. 1) group.

Novelty stress test

After being assigned to one of the two housing conditions, the animals were further separated into two groups: a stress group (n=48) in which the animals were exposed to a 20-min novelty stress on day 35 [i.e. the animals were placed into a transparent plastic box (28×35×30 cm) that they had not yet experienced]; and a non-stress group (n=48) in which the animals experienced a habituation

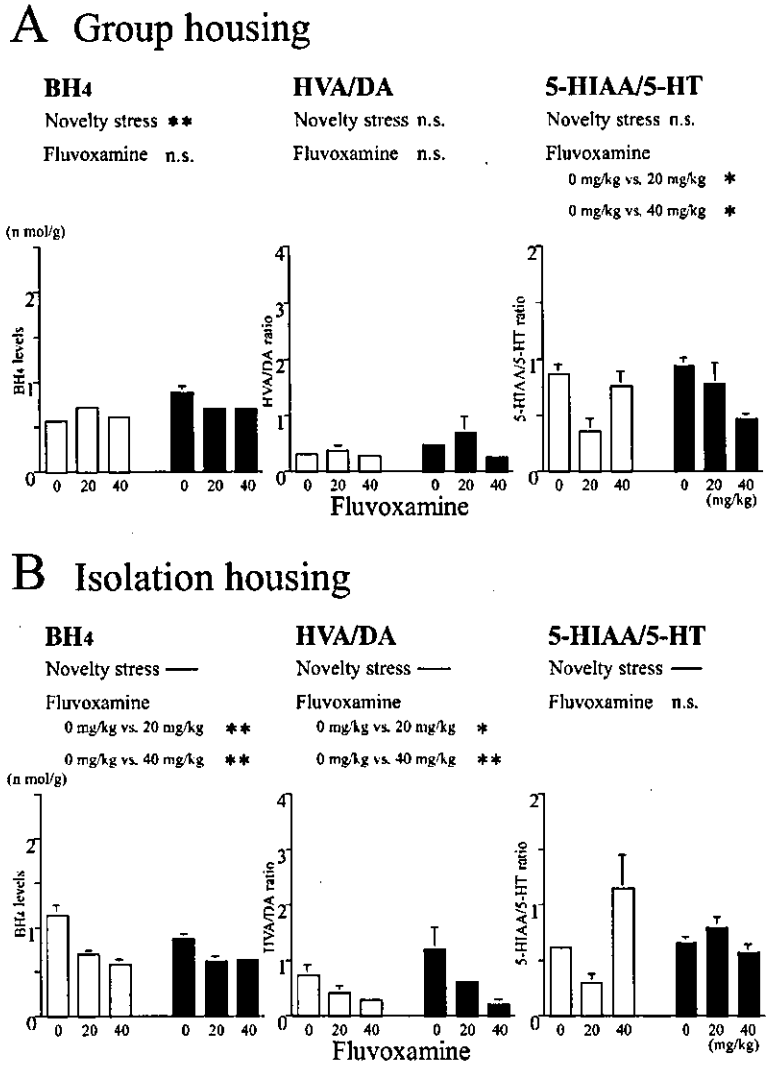

Fig. 2 Changes in BH_4 levels, and HVA/DA and 5-HIAA/5-HT ratios in the prefrontal cortex elicited by novelty stress and by fluvoxamine. **A** group-housing condition (n=48); **B** isolation-housing condition (n=47). *White bars*, non-stress (n=48); *black bars*, novelty stress (n=47, n=95 total). Fluvoxamine: 0.0 mg/kg (n=31); 20.20 mg/kg (n=32); 40.40 mg/kg (n=32, n=95 total). Each *bar* indicates the final group division. The number of animals used for each group was eight, except in the case of the isolation-housed, stress, 0 mg/kg group (n=7). Values are shown as the mean ±SEM. *Asterisks* indicate the results of the Tukey-Kramer test for novelty stress and fluvoxamine in each housing and stress condition: $*P<0.05$, $**P<0.01$, *n.s.* not significant. In the isolation-housing condition, the post hoc test for novelty stress was not performed because the MANOVA result was not significant

session (i.e. the animals were placed into the transparent plastic box for 10 min on days 33 and 34 before the 20-min session on day 35; Fig. 1). The habituation session was performed in the room in which the mice had been reared, whereas the novelty stress test was performed in a dark room that was separated from the breeding room. By combining the above conditions, the mice were divided into 12 groups.

Sample preparation

Mice were killed by decapitation immediately after the 20-min stress session. The brains were removed and, as quickly as possible, the prefrontal cortex and midbrain were dissected out on glass plates over ice. The samples were weighed and treated with 1000 µl of an ice-cold 0.2 M perchloric acid (PCA) solution containing 0.2 mM sodium pyrosulfite, 0.01% EDTA-2Na, and 0.5 µM isoproterenol (ISO) as an internal standard per 100 mg wet tissue. The solution was sonicated and then centrifuged at 10,000 g for 20 min at 4°C. The supernatant was filtered through a Millipore HV filter (0.45 µm pore size) and then subjected to both high-performance liquid chromatography (HPLC) with electrochemical detection (ECD) of monoamines (DA, 5-HT) and their metabolites (homovanilic acid, HVA; 5-hydroxyindoleacetic acid, 5-HIAA), and HPLC with fluorimetric detection (FD) of BH_4.

HPLC-ECD determination of brain levels of monoamines and their metabolites

The levels of DA, HVA, 5-HT, and 5-HIAA in the brain extracts were measured by HPLC with ECD. The system employed for HPLC-ECD consisted of a CMA/200 autosampler (CMA/Microdialysis AB, Stockholm, Sweden), a micro LC pump (BAS, West Lafayette, Ind., USA), an LC-4C ECD (BAS), a Bio-Phase ODS-4 51-6034 column (4.0×110 mm; BAS), a CR-6A recorder (Shimadzu, Kyoto, Japan), an LC-26A vacuum degasser (BAS),

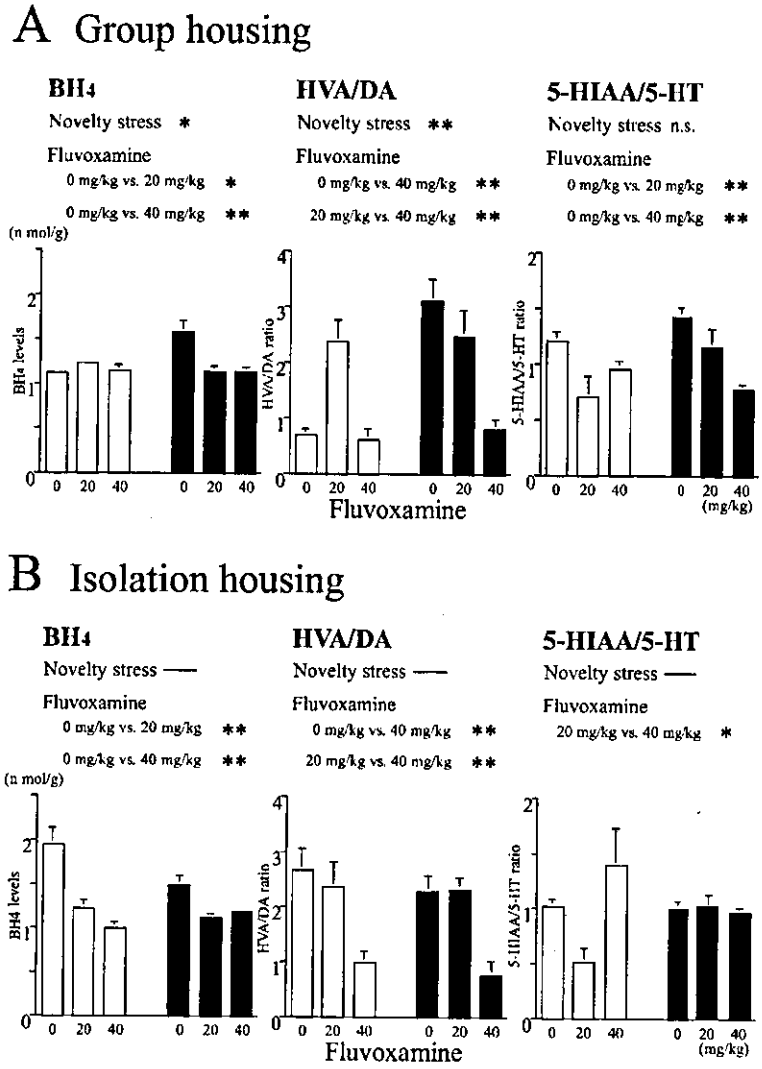

Fig. 3 Changes in BH_4 levels, and HVA/DA and 5-HIAA/5-HT ratios in the midbrain elicited by novelty stress and by fluvoxamine. A group-housing condition (n=48); B isolation-housing condition (n=45). *White bars*, non-stress (n=46); *black bars*, novelty stress (n=47, n=93 total). Fluvoxamine: 0:0 mg/kg (n=29); 20:20 mg/kg (n=32); 40:40 mg/kg (n=32, n=93 total). Each *bar* indicates the final group division. The number of animals used for each group was eight, except in the case of the isolation-housed, stress, 0 mg/kg group (n=7), and isolation-housed, non-stress, 0 mg/kg group (n=6). Values are shown as the mean±SEM. *Asterisks* indicate the results of the Tukey-Kramer test for novelty stress and fluvoxamine in each housing and stress condition: $^*P<0.05$, $^{**}P<0.01$, n.s. not significant. In the isolation-housing condition, the post hoc test for novelty stress was not performed because the MANOVA result was not significant

and a CTO-10A column heater set at 35°C (Shimadzu). The mobile-phase solution consisted of 0.1 M tartaric acid–0.1 M sodium acetate buffer, pH 3.2, containing 0.5 mM EDTA-2Na, 555 μM sodium 1-octane sulfonate, and 5% acetonitrile. The flow rate was 700 μl/min. The concentration of each compound was calculated by comparison with both the internal and the external standards.

HPLC-FD by post-column sodium nitrite oxidation for the determination of brain levels of BH_4

Tani and Ohno developed a method for the direct measurement of BH_4, the active form of biopterin (Tani and Ohno 1993), and we used this method to measure BH_4 levels in the present study. BH_4 (SIGMA) was stored in 0.1 M HCl (20 mM), and was prepared in 0.01 M HCl as an external standard (0.25 μM) immediately before sample injection. This system consisted of two LC-10AD pumps (Shimadzu), a CMA/200 autosampler, a Cosmosil 5C18 column (4.6×250 mm), a CR-6A recorder (Shimadzu), an LC-26A vacuum degasser, and a PF-10A FD (Shimadzu). The excitation wavelength was 350 nm, and the emission wavelength was 440 nm. The temperature of the reaction coil was set at 80°C using a column heater. The concentration of BH_4 was calculated by comparison with an external standard. The mobile phase was 0.1 M sodium phosphate buffer (pH 2.9) containing 5% methanol, 3 mM sodium 1-octane sulfonate, 0.1 mM EDTA-2Na, and 0.1 mM ascorbic acid (to prevent oxidation). The flow rate was 1.0 ml/min. Reduced pterins were oxidized by $NaNO_2$ (5 mM; flow rate: 1.0 ml/min) in the reaction coil (80°C).

Statistical analyses

To examine differences in the levels of BH_4, and in the ratios of HVA/DA and 5-HIAA/5-HT, three-way MANOVA (Wilks's lambda) for housing condition, novelty stress, and fluvoxamine was conducted on dependent measures in each brain region. Further analyses were performed to

consider the interactions. In each housing condition, i.e. group-housing and isolation-housing, two-way MANOVA (Wilks's lambda) for novelty stress and fluvoxamine was conducted on dependent measures in each brain region, followed by the Tukey-Kramer test. There were some missing values: in both regions, isolation-housing/stress/0 mg/kg (n=7, due to undetected DA in one animal), and in the midbrain, isolation-housing/non-stress/0 mg/kg (n=6, due to undetected DA in two animals).

Results

Prefrontal cortex

Three-way MANOVA (Wilks's lambda) for housing condition, novelty stress, and fluvoxamine was conducted for BH_4 levels, and to determine the HVA/DA and 5-HIAA/5-HT ratios. Housing condition [F(3, 81)=3.630, P=0.0163)]and fluvoxamine [F(6, 162)=12.013, P<0.0001] significantly altered the dependent measures, whereas novelty stress [F(3, 81)=1.663, P=0.1814] did not. The interactions between housing condition and novelty stress [F(3, 81)=4.932, P=0.0034], housing condition and fluvoxamine [F(6, 162)=9.153, P<0.0001], and novelty stress and fluvoxamine [F(6, 162)=4.527, P=0.0003] were significant. The interaction among housing condition, novelty stress, and fluvoxamine [F(6, 162)=2.749, P=0.0143] was also significant.

In the group-housing condition, two-way MANOVA for novelty-stress and fluvoxamine was conducted on dependent measures. Novelty stress [F(3, 40)=7.011, P=0.007] and fluvoxamine [F(6, 80)=4.722, P=0.0004] significantly altered the dependent measures. The interaction between novelty stress and fluvoxamine was significant [F(6, 80) =4.526, P=0.0005]. The post hoc test revealed that novelty stress significantly increased BH_4 levels (P<0.01, Fig. 2A), and fluvoxamine significantly decreased the 5-HIAA/5-HT ratio (0 mg/kg versus 20 mg/kg, P<0.05; 0 mg/kg versus 40 mg/kg, P<0.05; Fig. 2A). In the isolation-housing condition, two-way MANOVA for novelty stress and fluvoxamine was conducted on dependent measures. Novelty stress [F(3, 39)=1.363, P=0.2683] did not alter these measures, whereas fluvoxamine [F(6, 78)=11.442, P<0.0001] significantly altered them. The interaction between novelty stress and fluvoxamine was significant [F(6, 78)=3.419, P=0.0048]. The post hoc test revealed that fluvoxamine significantly decreased BH_4 levels (0 mg/kg versus 20 mg/kg, P<0.01; 0 mg/kg versus 40 mg/kg, P<0.01; Fig. 2B) and HVA/DA ratio (0 mg/kg versus 20 mg/kg, P<0.05; 0 mg/kg versus 40 mg/kg, P<0.01; Fig. 2B).

Thus, in the group-housing condition, novelty stress was found to increase BH_4 levels and fluvoxamine to decrease 5-HT turnover. In the isolation-housing condition, novelty stress did not alter dependent measuresand fluvoxamine decreased BH_4 levels and DA turnover.

Midbrain

Three-way MANOVA (Wilks's lambda) for housing condition, novelty stress, and fluvoxamine was conducted for BH_4 levels, and to determine the HVA/DA and 5-HIAA/5-HT ratios. Neither housing condition [F(3, 79) =2.251, P=0.0889] nor novelty stress [F(3, 79)=1.646, P=0.1854] altered dependent measures, whereas fluvoxamine [F(6, 158)=22.222, P<0.0001] significantly altered them. The interactions between housing condition and novelty stress [F(3, 79)=4.513, P=0.0057], housing condition and fluvoxamine [F(6, 158)=4.790, P=0.0002], and novelty stress and fluvoxamine [F(6, 158)=4.470, P=0.0003] were significant. The interaction among housing condition, novelty stress, and fluvoxamine [F(6, 158)=4.807, P=0.0002] was also significant.

In the group-housing condition, two-way MANOVA for novelty stress and fluvoxamine was conducted on dependent measures. Novelty stress [F(3, 40)=5.011, P=0.0048] and fluvoxamine [F(6, 80)=9.868, P<0.0001] significantly altered the dependent measures. The interaction between novelty stress and fluvoxamine was significant [F(6, 80) =6.807, P<0.0001]. The post hoc test revealed that novelty stress significantly increased BH_4 levels (P<0.05, Fig. 3A) and the HVA/DA ratio (P<0.01, Fig. 3A), and fluvoxamine significantly decreased BH_4 levels (0 mg/kg versus 20 mg/kg, P<0.05; 0 mg/kg versus 40 mg/kg, P<0.01; Fig. 3A), and decreased the HVA/DA (0 mg/kg versus 40 mg/kg, P<0.01; 20 mg/kg versus 40 mg/kg, P<0.01; Fig. 3A) and 5-HIAA/5-HT (0 mg/kg versus 20 mg/kg, P<0.05; 0 mg/kg versus 40 mg/kg, P<0.05; Fig. 3A) ratios. In the isolation-housing condition, two-way MANOVA for novelty stress and fluvoxamine was conducted on dependent measures. Novelty stress [F(3, 37)=1.044, P=0.3845] did not significantly alter the dependent measures, whereas fluvoxamine [F(6, 74) =13.336, P<0.0001] did significantly alter them. The interaction between novelty stress and fluvoxamine was significant [F(6, 74)=3.264, P=0.0067]. The post hoc test revealed that fluvoxamine significantly decreased BH_4 levels (0 mg/kg versus 20 mg/kg, P<0.01; 0 mg/kg versus 40 mg/kg, P<0.01; Fig. 3B), decreased the HVA/DA ratio (0 mg/kg versus 40 mg/kg, P<0.01; 20 mg/kg versus 40 mg/kg, P<0.01; Fig. 3B), and significantly increased 5-HIAA/5-HT ratio (20 mg/kg versus 40 mg/kg, P<0.05; Fig. 3B).

Thus, in the group-housing condition, novelty stress increased BH_4 levels and DA turnover, and fluvoxamine decreased BH_4 levels, as well as the DA and 5-HT turnover. In the isolation-housing condition, novelty stress did not alter dependent measures and fluvoxamine decreased BH_4 levels and DA turnover, whereas fluvoxamine increased 5-HT turnover.

Discussion

Human depression has a number of etiological risk factors. Both environmental and genetic factors have been

associated with the pathogenesis of the disease (Kendler et al. 1993). Among environmental factors, adverse life events such as interpersonal loss (e.g. separation) or an absence of social support (e.g. that occurring with a loss of social contact) appear to play important roles in the onset and relapse of depression (Paykel 1994). In the present study, we investigated the effects of fluvoxamine on BH_4 levels and on the DA and 5-HT turnover ratio in the mesoprefrontal system of mice using an animal model incorporating these two environmental risk factors. Isolation-housing is known to change the activities in the mesoprefrontal monoaminergic system. Isolation-housing suppresses TPH activity in the midbrain (Yanai and Sze 1983), whereas it increases the accumulation of the monoamine precursor, dihydroxyphenylalanine (DOPA) and 5-hydroxytryptophan (5-HTP) in the cerebral cortex (Miachon et al. 1993). Isolation has also been shown to increase the DA level and decrease the 5-HIAA/5-HT ratio in the prefrontal cortex (Jones et al. 1992), and to increase the KCl-induced release of 5-HT and DA from slices of prefrontal cortex (Jaffe 1998). Finally, Crepsi et al. (1992) showed by in vivo voltammetric analysis of the prefrontal cortex that isolation-housing prolonged 5-HT release and increased DA release following KCl or fenfluramine treatment.

In the prefrontal cortex and midbrain in the present study, novelty stress increased BH_4 levels under the group-housing condition (Figs 2A, 3A), whereas these levels were not altered under the isolation-housing condition (Figs 2B, 3B). Our recent study also suggested that novelty stress increases BH_4 levels in the hippocampus, although they did not change under the isolation-housing condition (Miura et al. 2004). According to these results, it appears likely that BH_4 plays a role in stress response mechanisms, and that isolation-housing attenuates these changes. A recent review demonstrated that intracellular concentrations of BH_4, which are mainly determined by GTP cyclohydrolase 1(GCH), probably regulate the activity of TH, TPH, and also NOS (Nagatsu and Ichinose 1999). Thus, the increase in BH_4 levels elicited by novelty stress may have been related to the activities of TH, TPH, and NOS. Previous studies have shown elevations in brain TH and TPH activities elicited by physiological stress (Boadle-Biber et al. 1989; Serova et al. 1998; Chamas et al. 1999). Physiological stress has also been shown to increase NOS I mRNA levels in the hypothalamic paraventricular nucleus (Kishimoto et al. 1996). Thus, novelty stress may have elevated GCH activity, increased the BH_4 concentration, and differentially regulated TH, TPH, and NOS I activities in each brain region. Although the mechanisms of the BH_4 elevation elicited by novelty stress and the effect of isolation-housing on this type of elevation remain unknown, our results suggest that isolation-housing suppressed the elevation of BH_4 levels elicited by novelty stress. Further in vivo study using GCH inhibitors will help to clarify the mechanisms of these stress responses of BH_4.

In the midbrain, novelty stress enhanced DA turnover under the group-housing condition (Fig. 3A), whereas it did not alter DA turnover under the isolation-housing condition (Fig. 3B). Novelty stress did not alter DA turnover in the prefrontal cortex (Fig. 2A,B). Thus, isolation-housing may have attenuated the increase in DA turnover elicited by novelty stress in the midbrain. Our previous study suggested that isolation-housing suppressed the elevation of monoamine (DA and 5-HT) turnover elicited by novelty stress (Miura et al. 2002a), although the regions in which such suppression was evident in that study, i.e. the prefrontal cortex and the nucleus accumbens (Miura et al. 2002a), differed from the regions of the present study. Although in our previous studies we reported that novelty stress significantly changed the levels of monoamines and their metabolites (Miura et al. 2002a,b), novelty stress was not found to significantly alter either of these levels in the present study. These differences in the stress response between our present and previous studies may be attributable, at least in part, to differences in the stress-session procedures. Here, we employed a non-stress condition in which animals were habituated twice to the novel environment before final exposure, and a stress condition in which they experienced the novel environment without prior habituation. In our previous studies, animals in the non-stress group were killed without exposure to the novel environment. We cannot rule out the possibility that these habituation sessions influenced CNS activity in the non-stress group. The differences between housing cages may also have had an influence. In the previous studies we used hanging-type cages with wire-mesh bottoms to minimize the influence of handling and social experience (Holson et al. 1991; Krebs-Thomson et al. 2001). However, the use of these cages may itself have constituted a chronic stressor. Finally, the difference in species used (rats versus mice) may also have contributed to the difference in results. Despite these differences, however, isolation-housing was clearly shown to attenuate the elevation of DA turnover elicited by novelty stress.

Fluvoxamine was also shown to suppress BH_4 levels in this study (Figs 2B, 3A,B), with the exception of the group-housing condition in the prefrontal cortex (Fig. 2A). In both regions, two-way MANOVA revealed significant interactions between novelty stress and fluvoxamine in each housing condition; thus fluvoxamine attenuated the elevation of BH_4 levels elicited by novelty stress. Our recent study demonstrated that fluvoxamine decreased BH_4 levels in the hippocampus of mice (Miura et al. 2004). Because chronic antidepressant treatment has been shown to suppress TH (Nestler et al. 1990) as well as TPH activity (Lapierre et al. 1983), the decrease in BH_4 levels elicited by fluvoxamine would seem to have attenuated the activities of these enzymes. Recently, NOS inhibitors have been shown to exhibit antidepressant-like effects in animal models (Harkin et al. 1999; Karolewicz et al. 1999; Da Silva et al. 2000). Paroxetine, a selective serotonin reuptake inhibitor (SSRI), is known to act as an NOS inhibitor (Finkel et al. 1996). Thus, antidepressants may possess clinical potency by inhibiting NOS activities. Fluvoxamine, an inhibitor of cytochrome P450 isozymes

that are structurally homologous to NOS (Richelson 1997), may influence NOS activity by decreasing BH_4 levels, although the mechanism of BH_4 suppression remains unknown. However, further research into the relation between alterations in BH_4 levels and GCH activity, and that between changes in BH_4 levels and NOS I activity elicited by fluvoxamine will help to clarify the role of NOS I in the clinical efficacy of the drug.

Fluvoxamine was found to inhibit DA turnover (Figs 2B, 3A,B), with the exception that there was no change in DA turnovers in the prefrontal cortex under the group-housing condition (Fig. 2A). In both regions, two-way MANOVA revealed significant interactions between novelty stress and fluvoxamine in each housing condition, and thus fluvoxamine attenuated the elevation of DA turnover elicited by novelty stress. To our knowledge, this is the first study showing a decrease in BH_4 levels and a simultaneous inhibition of DA turnover elicited by fluvoxamine. The mechanism of the effects of fluvoxamine on the DA system remains unknown. We suspect that the inhibition of 5-HT transporter (SERT) activity elicited by fluvoxamine cannot solely account for the inhibition of DA turnover. It is likely that other pharmacological effects related to the changes in BH_4 levels also played a role in inducing these changes. We propose here two possible explanations for these findings, although these are only speculation at present. The first explanation involves the regulation of DA neuron activity by the innervation of 5-HT neurons. The 5-HT innervations of the DA system are thought to attenuate the activity of DA neurons, and thus fluvoxamine may have potentiated the attenuation by increasing 5-HT levels (Di Mascio et al. 1998; Dong et al. 1999). The second possibility is that fluvoxamine may have suppressed TH activity via the decrease in BH_4 levels, and thereby suppressed DA biosynthesis. A study using 6-pyruvoyltetrahydropterin synthase-knockout mice (i.e. mice in which the second step of BH_4 biosynthesis is blocked) showed that the suppression of TH and NOS activities in the brain did not affect TPH activity (Sumi-Ichinose et al. 2001). In a study by Flatmark (2000), TH activity was highly dependent on the intracellular concentration of BH_4.

Fluvoxamine decreased the 5-HIAA/5-HT ratio under the group-housing condition in both regions (Figs 2A, 3A). Under the isolation-housing condition fluvoxamine did not alter the 5-HIAA/5-HT ratio in the prefrontal cortex (Fig. 2B), whereas it increased this ratio in the midbrain (Fig. 3B).

Although the original monoamine hypothesis has advanced our understanding of the etiology and pathophysiology of human depression, it does not address several major issues. The hypothesis has evolved to include adaptive changes in receptors to explain why there should be only a gradual clinical response to antidepressant treatment when the increase in availability of monoamine is rapid (Hirschfeld 2000). On the other hand, the dysfunction of SERT is the target of some of the newest forms of antidepressant pharmacotherapy, including the SSRIs (Leonard 2000). Activity of the SERT in platelets is reduced in patients with depression (Owens and Nemeroff 1994), and changes in the SERT in platelets have been found to correlate with response to treatment (Leonard 2000). A study using single photon emission computed tomography with [^{123}I]-2β-carbomethoxy-3β-(4-iodophenyl) tropane, the radiolabeled tracer binding with high affinity to SERT in the midbrain, revealed a reduction in the activity of the transporter in patients with depression (Malison et al. 1998). In the present study, fluvoxamine may have modified the activity and/or expression of SERT. The discrepancy of fluvoxamine-induced changes in 5-HT turnover between isolation and group-housing might be attributable to the difference in the responses of SERT activities to fluvoxamine between the two housing conditions, although the underlying mechanism remains to be clarified. Further, impaired activity of enzymes essential for monoamine synthesis may play a role in depression, although reports on this subject are few (Leonard 2000). Our results suggest the possibility that the decreased BH_4 levels elicited by fluvoxamine suppressed TH and/or TPH activity.

In the present study, both novelty stress and fluvoxamine induced changes in BH_4 levels, DA turnover, and 5-HT turnover in the mesoprefrontal system. In the group-housing animals, novelty stress significantly increased BH_4 levels. The suppression of BH_4 levels by fluvoxamine may have in turn been related to the suppression of DA turnover. As mentioned above, our results suggest the possibility that the clinical efficacy of fluvoxamine may be due to its influence on BH_4 levels as well as due to its effect on SERT. Further investigation of these potential mechanisms will help clarify the pathophysiology and pathogenesis of human depression.

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Short Communication

Association of the *XBP1* –116C/G polymorphism with schizophrenia in the Japanese population

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Abstract Schizophrenia and bipolar disorder share some clinical features and linkage studies have shown that several loci are common. Recently, the authors found that the –116C→G substitution in the promotor region of *XBP1*, a pivotal gene in endoplasmic reticulum (ER) stress response, causes the impairment of ER stress response, and that the –116C/C genotype is a protective factor; in other words the presence of the G allele increases the risk for bipolar disorder. The gene is located on 22q12.1, which is also linked with schizophrenia. The polymorphisms were investigated in 234 schizophrenic patients as compared with controls. Significant difference of genotype distribution was observed, which suggested that the –116C/C genotype is a protective factor for both of the major mental disorders.

Key words association study, rheumatoid arthritis, schizophrenia, XBP1.

INTRODUCTION

Schizophrenia is a severe mental illness affecting 1% of the population. Although genetic factors have been shown in family, twin and adoption studies, susceptibility genes or molecular mechanisms of the illness are controversial.[1] Schizophrenia and bipolar disorder are known to share a common genetic background evidenced by linkage and family studies. A case of monozygotic twins, of which one had schizophrenia and the other had bipolar disorder, has been reported.[2] Several loci (18p11, 13q32, 10p14, 22q11–13) are reported to be linked with both of these disorders.[3] We have recently identified that the XBP1 gene is associated with a genetic risk of bipolar disorder.[4]

It is known that XBP1 is a pivotal gene in endoplasmic reticulum (ER) stress response.[5] When unfolded proteins are accumulated in the ER, ER chaperons such as HSPA5 (also called GRP78 or BiP) assist in the re-folding of them. When HSPA5 proteins are consumed and dissociated from ATF6, ATF6 protein is cleaved. Cleaved ATF6 protein induces the expression of target genes harboring ER stress-response element (ERSE), such as *XBP1* and *HSPA5*. In parallel with ATF6 protein cleavage, IRE1 proteins on the ER membrane are dimerized by dissociation of HSPA5 and subsequently splice *XBP1* mRNA. The spliced mRNA encodes active-form XBP1 that strongly induces the expression of chaperons including *HSPA5*, as well as *XBP1* itself. This sequential response is referred to as ER stress response.[5–7] We found that the –116C→G substitution in the promotor region of the *XBP1* gene, losing the binding motif of XBP1 itself, impaired the XBP1 loop in the ER stress response, and that the –116C/C genotype was a protective factor; in other words, having the –116G allele was a risk factor for bipolar disorder.[4]

The *XBP1* gene is located on 22q12.1, one of the common susceptibility loci for schizophrenia and bipolar disorder.[8] In this report we examined the association of this polymorphism with schizophrenia to examine whether this polymorphism is a common

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Table 1. *XBP1* –116C/G polymorphism

	Genotype			Allele	
	C/C *n* (%)	C/G *n* (%)	G/G *n* (%)	C *n* (%)	G *n* (%)
Schizophrenia	17 (0.08)	111 (0.47)	106 (0.45)	145 (0.31)	323 (0.69)
Control	65 (0.14)	195 (0.43)	191 (0.42)	325 (0.36)	577 (0.64)

Genotype frequencies ($P = 0.019$) but not allele frequencies ($P = 0.062$), differ significantly between patients and controls.

protective or risk factor for these two major mental disorders.

METHODS

We genotyped the *XBP1* –116C/G polymorphism in Japanese case–control samples. The patients consisted of 234 unrelated patients followed up at the hospitals or clinics participating in the present study and diagnosed by the authors after repeated clinical interviews according to the *Diagnostic and Statistical Manual of Mental Disorders* (4th edn; DSM-IV) criteria. Written informed consent was obtained from all the subjects. The Ethics Committees of the Brain Science Institute (RIKEN) and participating institutes approved the study.

The *XBP1* –116C/G polymorphism was genotyped by polymerase chain reaction (PCR) amplification with the primers 5′-CGACAGAAGCAGAACTTTAG and 5′-CTGAGGTAATTCTCTGTTAG in a 12.5 mL volume containing LA Taq, deoxynucleotide triphosphates (dNTP) and 2 × GC buffer I (Takara, Shiga, Japan). Amplification conditions consisted of an initial 2 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 54°C and 1 min at 72°C, followed by a final extension of 2 min at 72°C. Sequencing was performed using a commercial kit (BigDye terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Foster City, CA, USA). The genotype and allele frequencies in patients with schizophrenia were compared with that in 451 unrelated healthy control subjects reported elsewhere.[3] Differences in genotype and allele frequencies were examined by Fisher's exact test.

RESULTS

Genotype distribution was within the Hardy–Weinberg Equilibrium in both groups. There was a significant difference of –116C/G genotype distribution between patients and controls. A similar tendency was also found for allele frequency (Table 1). The C/C genotype was significantly less common (8%) in schizophrenia patients compared with controls (14%). The odds ratio for having the G allele was 2.15 (95%CI: 1.23–3.76). These results suggest that the –116C/C genotype, the protective genotype for bipolar disorder, is also a protective factor for schizophrenia, although the significance is modest and the odds ratio is lower than that for bipolar disorder (4.6; 95%CI: 2.1–10.2).[4]

DISCUSSION

In the present report we have suggested that the –116C/C genotype, a protective factor for bipolar disorder, is also protective for schizophrenia. The molecular function of XBP1 in the brain is not known, but the XBP1 gene is expressed in the brain at relatively high levels.[4,9] The ER stress response cascade or other unidentified downstream genes of XBP1 may be associated with the pathophysiology of both of the mental disorders. Further studies of this cascade have a potential to clarify the molecular mechanism of both disorders.

In contrast, *XBP1* is not only pivotal in ER stress response, but also plays a critical role in plasma cell differentiation of B cells and is highly expressed in the plasma cells of inflammatory synovium of rheumatoid arthritis patients.[10,11] Negative association between schizophrenia and rheumatoid arthritis is a classical and well-replicated finding and a meta-analysis suggested that the prevalence of rheumatoid arthritis in patients with schizophrenia is only 10–30% of the general population.[12] However, the mechanism is unknown.[13] Although the association of schizophrenia with the XBP1 –116C/G polymorphism itself was modest, other polymorphisms in this cascade, in addition to the reduced promotor activity due to –116C/G, may explain this classic but unexplained finding.

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Association Between Chromogranin B Gene Polymorphisms and Schizophrenia in the Japanese Population

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Background: *We found in previous work a significant association between schizophrenia and D20S95 on chromosome 20p12.3. In this study, we analyzed 10 microsatellite markers and found an association of schizophrenia with D20S882 and D20S905 that flank D20S95. The chromogranin B gene (*CHGB*) is 30 kb from D20S905. The chromogranin B (secretogranin I) belongs to a series of acidic secretory proteins that are widely expressed in endocrine and neuronal cells, and its cerebrospinal fluid levels have been reported to decrease in patients with chronic schizophrenia.*

Methods: *We screened for polymorphisms in* CHGB *with polymerase chain reaction direct sequencing methods in 24 Japanese schizophrenic patients and identified a total of 22 polymorphisms. Allelic and genotypic distributions of detected polymorphisms were compared between unrelated Japanese schizophrenic patients (*n = *192) and healthy control subjects (*n = *192).*

Results: *Statistically significant differences in the allelic distributions were found between schizophrenic patients and control subjects for 1058C/G (A353G) (corrected* $p = 7.7 \times 10^{-5}$*) and 1104A/G (E368E) (corrected* $p = 8.1 \times 10^{-6}$*). The 1058C/G and 1104A/G alleles were in almost complete linkage disequilibrium and were in linkage disequilibrium with D20S95.*

Conclusions: *Results suggest that the* CHGB *variations are involved in the susceptibility to schizophrenia in our study population.*

Key Words: Schizophrenia, chromogranin B, association study, neuropeptide

The region of 20p12.3-p11 may contain a locus of predisposition to schizophrenia (Lewis et al 2003). In our previous screening association study, we observed a significant association with schizophrenia at the locus of D20S95 ($p = 5 \times 10^{-6}$, corrected p value after Bonferroni correction, .00035) on 20p12.3 (Kitao et al 2000). The marker D20S95 is approximately 2 megabases (Mb) outside the 21.2 to 47.5 cM region of chromosome 20 highlighted in the meta-analysis of Lewis et al (2003), though quite possibly within its confidence bounds. In the individual genome scans, Moises et al (1995) reported a p-value of .009 with marker D20S40 and Hovatta et al (1998) found a maximum lod score of 1.22 with marker D20S172. These markers are located approximately 7 Mb and 12 Mb centromeric to D20S95.

The only known gene within 180 kb from D20S95 is the gene encoding chromogranin B, a tyrosine-sulfated secretory protein found in a wide variety of peptidergic endocrine cells. The granins (secretogranins/chromogranins) belong to a family of soluble proteins stored and released from the large dense-core secretory vesicles of the synapse (Benedum et al 1987; Winkler and Fischer-Colbrie 1992). A number of studies have compared the chromogranin levels in cerebrospinal fluid (CSF) between healthy control subjects and schizophrenic patients at various stages of this disease (Landen et al 1999; Miller et al 1996; van Kammen et al 1991, 1992, 1994). Landen et al (1999) reported that levels of chromogranin A and chromogranin B were lower in chronic schizophrenic patients and pointed out the possibility that an acute increase of chromogranin levels reflects an active disease process and a chronic decline indicates an advanced neurodegenerative process. Reduction of chromogranin B-like immunoreactivity in distinct subregions of the hippocampus from individuals with schizophrenia was reported (Nowakowski et al 2002). An association between some *CHGB* polymorphisms and schizophrenia was reported in the Chinese Han population (Zhang et al 2002).

In the present study, we performed additional dense-mapping analyses using 10 microsatellite markers close to D20S95 and the mutation search of the *CHGB*, followed by the case-control association studies on its detected polymorphisms in schizophrenic patients, using the same sample set as we previously used in the screening study in which a significant association with schizophrenia was detected at D20S95.

Methods and Materials

Ethical Considerations

The present study was approved by the Ethical Committee of the Kohnodai area, National Center of Neurology and Psychiatry, University of Tsukuba, and Nagoya University. Written informed consent was obtained from all subjects.

Subjects

The DNA samples were all selected from those of the subjects enrolled in our previous screening study (Kitao et al 2000). The subjects consisted of 192 schizophrenic patients and 192 healthy control subjects. The schizophrenic patients (91 men, 101 women; aged 19–90 years [mean 57.2 years]) were all inpatients recruited from several psychiatric facilities located around the Tokyo area. They were interviewed several times during a hospitalization period of 6 months or more by experienced psychiatrists who were familiar with the structured clinical interview for DSM-III-R (SCID) rating system (Spitzer et al 1992). The diagnosis of schizophrenia was assigned on the basis of clinical interviews and chart review of medical records, accord-

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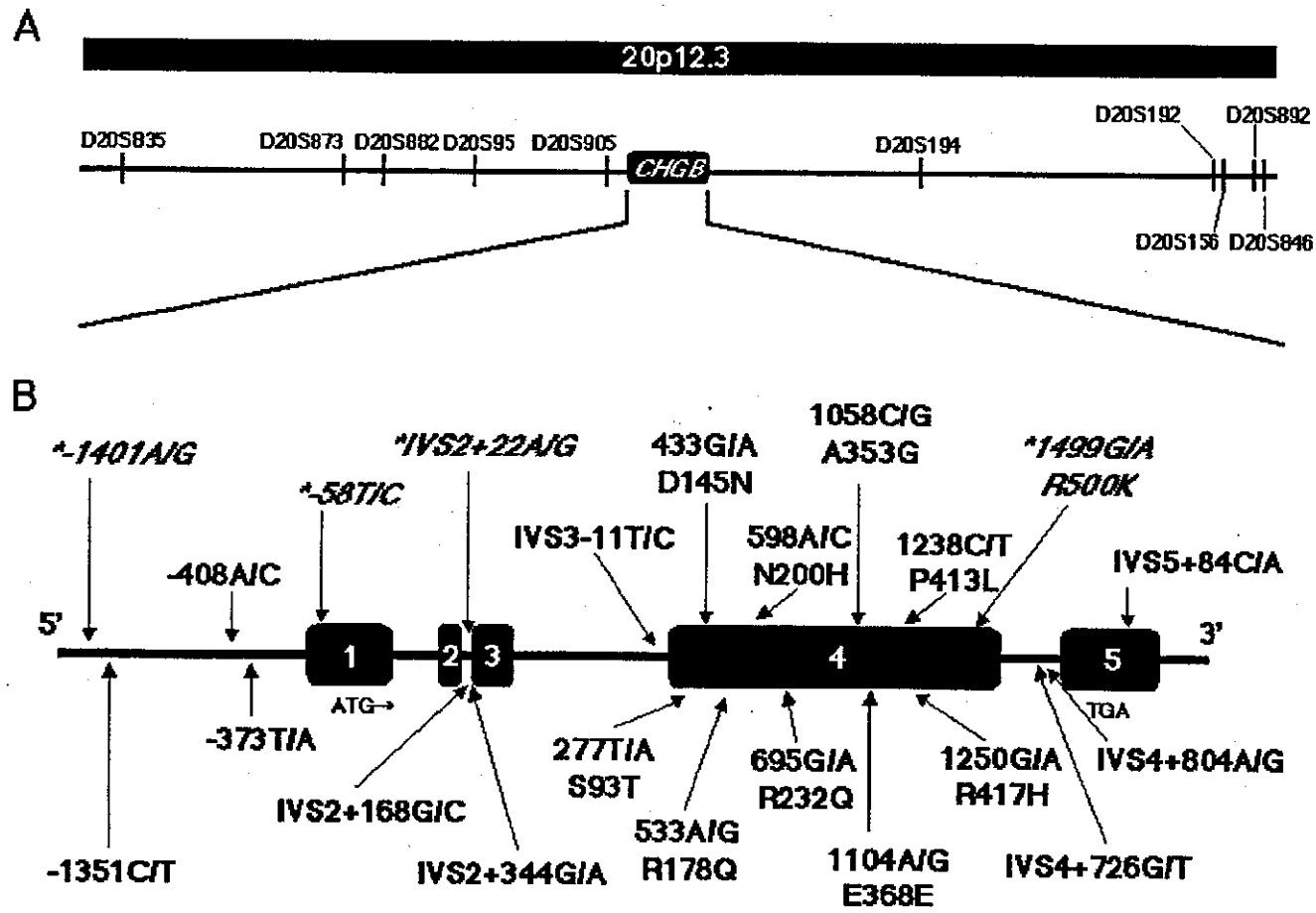

Figure 1. (A) The positions of the microsatellite markers in relation to chromogranin B gene (*CHGB*). **(B)** Genomic organization and positions of the SNPs in *CHGB*. *Italics are newly identified SNPs. SNP, single nucleotide polymorphism.

Table 1. Results of the Additional Dense-Mapping Analyses Using 10 Microsatellite Markers Close to D20S95

Microsatellite Markers[a]	Distance from *CHGB* (kb)	Number of Subjects		Statistics[b]	
		Schizophrenia	Control	H-W[c]	S vs. C[d]
D20S835	−577	172	192	ns	ns
D20S873	−295	175	191	ns	ns
D20S882	−257	203	196	.02	.02
D20S95	−176	230	217	ns	.002
D20S905	−29	184	190	ns	.04
CHGB	0				
D20S194	251	164	135	ns	ns
D20S192	805	171	192	ns	ns
D20S156	809	171	192	ns	ns
D20S892	858	174	192	ns	ns
D20S846	873	176	192	ns	ns

Details are shown in Appendix 1.

CHGB, chromogranin B gene; kb, kilobase; H-W, Hardy-Weinberg equilibrium; S, schizophrenia; C, control.

[a]These markers are listed in the order from telomeric (D20S835) to centromeric (D20S846) site. The average distance between the markers is about 120 kb.

[b]*p* value is denoted when $p < .05$.

[c]Deviations from the Hardy-Weinberg equilibrium.

[d]Comparison between schizophrenia vs. control (empirical *p*).

ing to the DSM-III-R criteria (American Psychiatric Association 1987). We actually did not use SCID itself at the clinical interview because: (1) a validated Japanese translation version of SCID was not available at the time of clinical evaluation in this study, and (2) we considered that diagnosis by repeated interviews by trained attendant physicians seems to be more reliable than SCID performed by a nonattendant physician only at once. Control subjects (96 men, 96 women; aged 24–87 years [mean 49.8 years]) were recruited mostly from the medical staff working in the psychiatric facilities and had no history of psychoses. All subjects were of Japanese descent, born to Japanese parents. The sampling methods of the subjects were satisfied with the criteria of the proposed checklists for gene-disease association study (Colhoun et al 2003; Little et al 2002).

Genomic Procedure

Genomic DNA was extracted from leukocytes in venous blood samples. Ten microsatellite markers on chromosome 20p12.3, D20S835 (Genome Database [GDB]:603368), D20S873 (GDB:609813), D20S882 (GDB:610170), D20S905 (GDB:612744), D20S916 (GDB:614700), D20S194 (GDB:200327), D20S192 (GDB:200199), D20S156 (GDB:198517), D20S892 (GDB:611556),

Table 2. Pair-Wise Linkage Disequilibrium in the *CHGB*

		4 −1401A/G	5 −1351C/T	6 −408A/C	7 −373T/A	8 −58T/C	9 IVS3-11T/C	10 277T/A	11 433G/A	12 533A/G	13 598A/C	14 695G/A	15 1058C/G	16 1104A/G	17 1238C/T	18 1250G/A	19 1499G/A
4	−1401A/G		1.00	1.00	.73	.51	.60	1.00	1.00	1.00	1.00	.50	.48	.37	.69	.84	.60
5	−1351C/T	.10		.90	.72	.43	1.00	.89	1.00	.98	.85	.92	.64	.64	.57	.58	.93
6	−408A/C	.01	.35		1.00	.96	.77	.86	.45	.88	1.00	.67	.84	.84	.77	.83	.90
7	−373T/A	.00	.09	.27		.58	1.00	.72	.85	.76	.53	.51	.68	.70	.55	1.00	1.00
8	−58T/C	.00	.02	.45	.01		.50	.36	.44	.42	.74	.43	.64	.63	1.00	.66	1.00
9	IVS3-11T/C	.00	.07	.07	.02	.00		1.00	1.00	1.00	1.00	.98	.61	.62	.83	.74	.51
10	277T/A	.01	.70	.23	.07	.01	.06		.99	.99	1.00	.90	.58	.57	.56	.51	.92
11	433G/A	.01	.38	.20	.08	.08	.17	.37		1.00	1.00	.92	.47	.46	.23	.43	.67
12	533A/G	.02	.58	.49	.14	.04	.11	.60	.60		1.00	.94	.63	.62	.32	.42	.83
13	598A/C	.13	.06	.10	.02	.04	.01	.08	.07	.12		1.00	.79	.79	.54	.43	.90
14	695G/A	.00	.06	.06	.01	.02	.87	.06	.16	.09	.01		.58	.59	.74	.69	.48
15	1058C/G	.00	.13	.57	.16	.29	.05	.08	.15	.21	.04	.02		1.00	1.00	.98	.77
16	1104A/G	.00	.13	.57	.16	.29	.05	.08	.15	.21	.04	.04	1.00		1.00	.98	.77
17	1238C/T	.02	.04	.08	.02	.04	.00	.04	.00	.01	.29	.00	.10	.10		.75	.81
18	1250G/A	.01	.25	.34	.11	.19	.04	.16	.12	.17	.02	.03	.66	.66	.03		.82
19	1499G/A	.01	.05	.05	.02	.03	.31	.04	.09	.07	.01	.24	.10	.10	.01	.04	

Upper diagonal figures are *D'* and lower diagonal figures are r^2.
CHGB, chromogranin B gene.

and D20S846 (GDB:606163), were amplified by polymerase chain reaction (PCR). The positions of these markers in relation to *CHGB* is shown in Figure 1A. The PCR primers are available on the GDB (http://gdbwww.gdb.org). Genotyping to determine the lengths of the microsatellite alleles was done with an ABI PRISM 3100 DNA sequencer (Applied Biosystems, Foster City, California). The sequence and genomic structure of *CHGB* were obtained from the high throughput genome sequences (HTGs) database of the Blast server at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/). All exons containing exon-intron junctions and a 5′-side regulatory region of *CHGB* were amplified by the PCR method. The primers are available on request. Polymorphisms were screened in 24 schizophrenic patients by direct sequencing of PCR products with the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California) and on the ABI PRISM 3100 DNA sequencer. All nucleotide variants detected in this study were genotyped by direct sequencing after PCR amplification. Trace data were aligned by Sequencher software (Gene Codes Corp, Ann Arbor, Michigan) and scanned for polymorphisms.

Statistical Procedures

Microsatellite Marker Analyses. Assessing the deviations from Hardy-Weinberg equilibrium and the case-control comparison of the distribution of the microsatellite allele frequencies between control subjects and schizophrenic patients were performed by the program implemented in MEGA2 (Guo and Thompson 1992).

Single Nucleotide Polymorphism Analyses. Deviations of the genotype distributions from the Hardy-Weinberg equilibrium were assessed with the 2×3 χ^2 test. Case-control comparisons of genotype and allele frequencies of polymorphisms were done with the Armitage Trend Test and 2×2 χ^2 tests. We examined associations by permutation procedure in COCAPHASE ver 2.4 to determine the empirical significance of our findings. We did not use -EM and -missing options. The χ^2 value, the *p*-value, and the odds ratio (OR) were calculated with the SPSS computer package for Windows Release 11.0J (SPSS Japan Inc., Tokyo, Japan). Linkage disequilibrium analyses were conducted with the Cocaphase program: unbiased application of the transmission/disequilibrium test to multilocus haplotypes (Dudbridge et al 2000).

Results

Results of the additional dense mapping association analyses using 10 microsatellite markers close to D20S95 on 20p12.3 with schizophrenia are shown in Table 1 and supplemental data table 1. The genotype frequencies of these microsatellite markers did not significantly deviate from Hardy-Weinberg equilibrium except for D20S882 ($P = 0.02$). As shown in Table 1, possible association with schizophrenia was detected in the loci of D20S882 and D20S905, in addition to D20S95. The 109-bp allele of D20S95 was more frequent in the patient group (.10) than in the control group (.03, uncorrected $p = 7.2 \times 10^{-5}$, empirical $p = .002$). The 80-bp allele of D20S882 was less frequent in the patient group (.14) than in the control group (.22, uncorrected $p = .0009$, empirical $p = .02$). The 90-bp allele of D20S905 was less frequent in the patient group (.01) than in the control group (.05, uncorrected $p = .002$, empirical $p = .004$). Although there was a global significance for the haplotype comprising the three microsatellites ($p = 1 \times 10^{-9}$), no specific individual haplotype of these microsatellite markers was significantly associated with schizophrenia after correction for the number of haplotypes.

Table 3. Genotype and Allelic Distribution of the *CHGB* SNPs in Japanese Control Subjects and Patients with Schizophrenia

		Genotype					Allele			
					p Value[a]					
Polymorphisms Group	*N*	Genotype Count			H-W[c]	S vs. C[d]	Count (frequency)		Odds Ratio (95% CI)	*p* Value[b] S vs. C[d]
−1401A/G		AA	AG	GG			A	G		
Control	192	186	6	0	1.00	.79	378 (.98)	6 (.02)	1.22 (.40–3.65)	.73
Schizophrenia	185	178	7	0	1.00		363 (.98)	7 (.02)		
−1351C/T		CC	CT	TT			C	T		
Control	192	78	77	37	.30	.87	233 (.61)	151 (.39)	.92 (.69–1.23)	.58
Schizophrenia	186	72	89	25	.98		233 (.63)	139 (.37)		
−408A/C		AA	AC	CC			A	C		
Control	96	14	45	37	1.00	.47	73 (.38)	119 (.62)	.79 (.53–1.19)	.26
Schizophrenia	95	15	53	27	.67		83 (.44)	107 (.56)		
−373T/A		TT	TA	AA			T	A		
Control	94	71	21	2	.99	.43	163 (.87)	25 (.13)	.99 (.55–1.79)	.97
Schizophrenia	95	70	25	0	.33		165 (.87)	25 (.13)		
−58T/C		TT	TC	CC			T	C		
Control	185	106	67	12	.96	.24	279 (.75)	91 (.25)	1.22 (.88–1.69)	.24
Schizophrenia	181	94	71	16	.95		259 (.72)	103 (.28)		
IVS3-11T/C		TT	TC	CC			T	C		
Control	187	154	32	1	.84	.32	340 (.91)	34 (.09)	.66 (.38–1.14)	.14
Schizophrenia	186	164	21	1	.99		349 (.94)	23 (.06)		
277T/A (S93T)		TT	TA	AA			T	A		
Control	189	73	86	30	.91	.82	232 (.61)	146 (.39)	.94 (.70–1.26)	.68
Schizophrenia	187	73	89	25	.98		235 (.63)	139 (.37)		
433G/A (D145N)		GG	GA	AA			G	A		
Control	187	73	90	24	.96	.78	236 (.63)	138 (.37)	.99 (.73–1.33)	.94
Schizophrenia	187	77	83	27	.91		237 (.63)	137 (.37)		
533A/G (R178Q)		AA	AG	GG			A	G		
Control	188	51	90	47	.92	.35	192 (.51)	184 (.49)	.87 (.65–1.16)	.34
Schizophrenia	187	42	94	51	1.00		178 (.48)	196 (.52)		
598A/C (N200H)		AA	AC	CC			A	C		
Control	189	149	39	1	.82	.59	337 (.89)	41 (.11)	1.12 (.72–1.76)	.61
Schizophrenia	187	143	43	1	.57		329 (.88)	45 (.12)		
695G/A (R232Q)		GG	GA	AA			G	A		
Control	186	157	28	1	1.00	.45	342 (.92)	30 (.08)	.87 (.50–1.50)	.61
Schizophrenia	184	161	20	3	.50		342 (.93)	26 (.07)		
1058C/G (A353G)		CC	CG	GG			C	G		
Control	192	49	89	54	.80	1.3×10^{-5}	187 (.49)	197 (.51)	1.96 (1.46–2.63)	5.9×10^{-6}
Schizophrenia	190	21	82	87	.97		124 (.33)	256 (.67)		
1104A/G (E368E)		AA	AG	GG			A	G		
Control	192	49	89	54	.80	2.0×10^{-6}	187 (.49)	197 (.51)	2.11 (1.57–2.84)	6.2×10^{-7}
Schizophrenia	187	20	76	91	.89		116 (.31)	258 (.69)		
1238C/T (P413L)		CC	CT	TT			C	T		
Control	192	155	36	1	.82	.54	346 (.90)	38 (.10)	1.15 (.72–1.83)	.55
Schizophrenia	187	147	38	2	1.00		332 (.89)	42 (.11)		
1250G/A (R417H)		GG	GA	AA			G	A		
Control	192	71	84	37	.67	.006	226 (.59)	158 (.41)	1.51 (1.13–2.01)	.005
Schizophrenia	187	42	98	47	.90		182 (.49)	192 (.51)		
1499G/A (R500K)		GG	GA	AA			G	A		
Control	191	160	29	2	.84	.270	349 (.91)	33 (.09)	.67 (.38–1.16)	.15
Schizophrenia	186	166	18	2	.75		350 (.94)	22 (.06)		

CHGB, chromogranin B gene; H-W, Hardy-Weinberg equilibrium; S, schizophrenia; C, control; CI, confidence interval.
[a]Armitage's trend test.
[b]Chi-squared test.
[c]H-W: Observed genotype vs. expected genotype according to Hardy-Weinberg equilibrium.
[d]S vs. C: Genotype comparison between schizophrenia vs. control.

All exons containing exon-intron junctions and a 5′-side regulatory region of *CHGB* were screened for polymorphisms in 24 schizophrenic patients. A total of 22 single nucleotide polymorphisms (SNPs) were identified (Figure 1B). Eighteen SNPs, −1351C/T (rs236139), −408A/C (rs236140), −373T/A (rs236141), IVS2+168G/C (rs236145), IVS2+344G/A (rs236146), IVS3−11T/C (rs6139872), 277T/A(S93T) (rs6085324), 433G/A(D145N) (rs6133278), 533G/A(R178Q) (rs910122), 598A/C(N200H) (rs881118), 695G/A(R232Q) (rs6139873), 1058G/C(A353G) (rs236152), 1104G/A(E368E) (rs236153), 1238C/

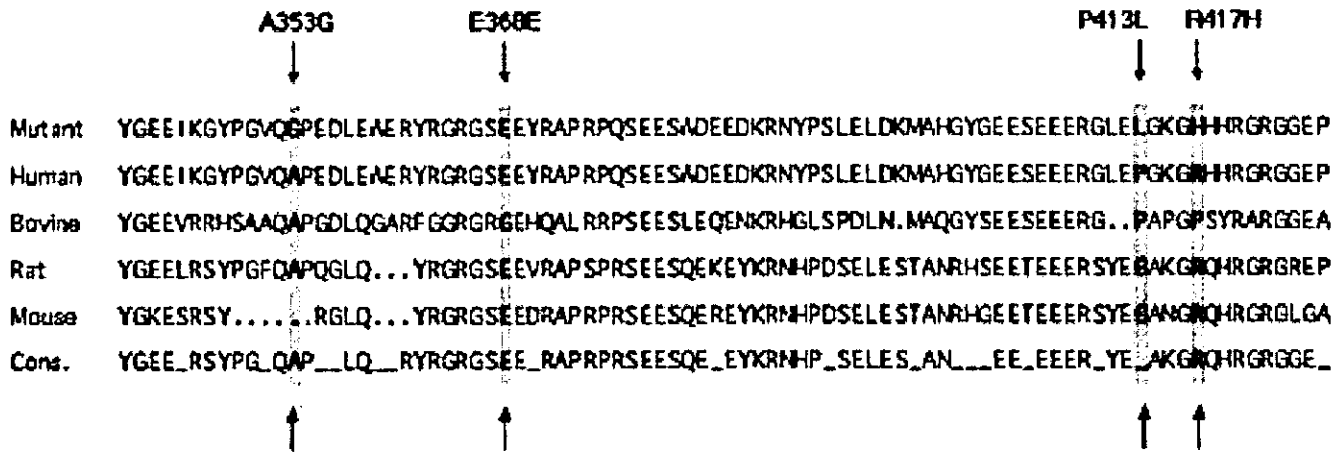

Figure 2. Comparison of the amino acid sequences of chromogranin B from human, bovine, rat, and mouse. Cons. is calculated consensus sequence. Dots (.) denote deletions in the sequence.

T(P413L) (rs742710), 1250G/A(R417H) (rs742711), IVS4+726G/T (rs236154), IVS4+804G/A (rs236155), and IVS5+84C/A (rs2821), are listed in the database for single nucleotide polymorphisms (dbSNP) in the NCBI (http://www.ncbi.nlm.nih.gov/SNP/). Three SNPs, −1401A/G, IVS2+22A/G, and 1499G/A(R500K), not listed in the dbSNP database were identified in the mutation search of *CHGB* in the Chinese Han population (Zhang et al 2002). Seven SNPs, −1095G/C (rs989462), −1042G/A (rs6085321), 402G/C(K177N) (rs236150), 727G/A(T423A) (rs236151), 1466C/T(L489L) (rs6107717), IVS4+9T/C (rs617000), and IVS5+277A/T (rs 236156), which are listed in the dbSNP database, were not detected in the Japanese population. One SNP, −58T/C, was newly identified in this study. Among these SNPs, 16 at the coding or regulatory regions were selected for further association study.

Linkage disequilibrium was found in many pairs of the 16 SNPs (Table 2). The genotype and allele frequencies of all SNPs are shown in Table 3. The genotype distributions did not deviate significantly from those expected according to the Hardy-Weinberg equilibrium for polymorphisms. Statistically significant differences in allele distributions were found between schizophrenic patients and healthy control subjects for 1058C/G ($p = 5.9 \times 10^{-6}$, empirical $p = 8.2 \times 10^{-5}$, OR = .510, 95% confidence interval [CI] = .38–.68) and 1104A/G ($p = 6.2 \times 10^{-7}$, empirical $p = 1 \times 10^{-5}$, OR = .474, 95% CI = .35–.64), and a trend of association was found between 1250G/A and schizophrenia ($p = .005$, empirical $p = .05$, OR = 1.51, 95% CI = 1.13–2.01) (Table 2). The genotype distributions of these three SNPs suggest the presence of a gene dose-response effect: a linear trend in disease risk with number of copies of the high-risk allele (uncorrected $p = 1.3 \times 10^{-5}$, uncorrected $p = 2.0 \times 10^{-6}$, and uncorrected $p = .006$, respectively). The frequency of the risk GG genotype of A353G in the control subjects was .28 and its OR for schizophrenia was 2.1 (95% CI = 1.4–3.3) compared with other genotypes. Two SNPs, 1058C/G and 1104A/G, were in almost complete linkage disequilibrium (Table 2), and almost the same *p*-value was obtained for the haplotype associations with schizophrenia (uncorrected $p = 6.1 \times 10^{-7}$). Other haplotypes are also associated with schizophrenia; however, they are mainly due to 1058C/G and 1104A/G polymorphisms (Table 4). Linkage disequilibrium was significant ($p < .05$) between SNPs associated with schizophrenia, namely 1058C/G ($D' = .43$), 1104A/G ($D' = .44$), and 1250G/A ($D' = .37$) in the *CHGB* and the 109-bp allele of D20S95.

Although it has been reported that the significant association with schizophrenia ($p < .001$) was observed at two SNPs of 433G/A and 533A/G in the Chinese Han population, these findings were not replicated in our present study. The power of this study to replicate the findings from the Chinese-Han population was 90%.

Discussion

Our hypothesis that the chromogranin B gene (*CHGB*) is a plausible positional candidate for association with schizophrenia can be strongly supported by the results of our additional dense mapping analyses examined in the present study. This hypothesis is further supported by the findings in our subsequent association study between *CHGB* and schizophrenia: a significant association of 1058C/G and 1104A/G, and a trend of association of 1250G/A. These SNPs were in linkage disequilibrium with the 109-bp allele of D20S95. Association study may lead to false-positive and false-negative signals because of genetic stratification or population subdivision. Our subjects were all of Japanese descent living in the same area and we have no evidence of population stratification in our case or control samples. As shown in a previous report, Japanese is a relatively homogeneous population with no stratification (Daimon et al 2003; Kakiuchi et al 2003). However, unknown population stratification would not be excluded.

There is another Chinese study showing a significant association between some SNPs of *CHGB* and schizophrenia. Although our study and a study of Zang et al (2002) found significant associations between *CHGB* SNPs and schizophrenia, the associated SNPs differed between the two studies. Significant associations with schizophrenia were found at two SNPs, 433G/A ($p = .004$, the G allele was associated with schizophrenia, OR = 1.60, 95% CI = 1.17–2.18) and 533A/G ($p = .0005$, the A allele was associated with schizophrenia, OR = 1.73, 95% CI = 1.28–2.18), in the Chinese Han population (Zhang et al 2002). We failed to find these associations in the present study, 433G/A (OR = 1.01, 95% CI = .75–1.36) and 533A/G (OR = .87, 95% CI = .65–1.16). The ORs between the two studies significantly differed ($p = .04$ for 433G/A and $p = .001$ for 533G/A, respectively). The alleles

Table 4. Haplotypes Showing Most Significant Association with Schizophrenia per Each Window

Haplotype Window	433 G/A	533A/G	598A/C	695G/A	1058C/G	1104A/G	1238C/T	1250G/A	1499G/A	Haplotype Frequency (%) Schiz	Control	*p* Values Global	Permutation Global	Individual
Window 2					C	A				.31	.49	6.6×10^{-6}	.00009	6.6×10^{-6}
Window 3					C	A	C			.31	.49	5.9×10^{-5}	.0007	1.0×10^{-5}
Window 4				G	C	A	C			.27	.43	.0007	.01	2.0×10^{-5}
Window 5			A	G	C	A	C			.27	.40	.003	.04	.0001
Window 6		A	A	G	C	A	C			.03	.12	.005	.04	.002

Schiz, schizophrenia

that found to be associated with schizophrenia in the Chinese population, the G allele of the 433G/A polymorphism and the A allele of the 533A/G polymorphism, were on the haplotypes negatively associated with schizophrenia in our Japanese population. Linkage disequilibrium between 1058C/G and 433G/A or 533A/G were not observed (D′ = .47, D′ = .63, respectively). Our Japanese study tested more SNPs, and the most interesting SNPs were not tested in the larger Chinese sample. The SNPs significantly associated with schizophrenia in the Chinese population, however, also were nonsynonymous and of potential interest. Although it is possible that the apparent discrepancy between our study and that of Zhang et al (2002) occurred by chance, it is also possible that the discrepancy is due to ethnic differences or differences in the clinical characteristics of schizophrenic patients. We have observed that *CHGB* SNPs are associated with extrapyramidal side effects and clinical symptoms (unpublished data). Therefore, further studies are necessary to address the discrepancy between the Japanese and Chinese studies. Associations between *CHGB* and schizophrenia have been found in two independent studies of East Asian populations, indicating that *CHGB* is a plausible association with susceptibility to schizophrenia and is one of the genes contributing to the linkage of 20p12.3-p11 to schizophrenia found by meta-analysis (Lewis et al 2003).

The granin family plays an important role in the sorting and aggregation of secretory products in the trans-Golgi network (Ozawa and Takata 1995). Chromogranin A, chromogranin B (secretogranin I), and chromogranin C (secretogranin II) are well known. Chromogranin B also localizes to the nucleus and controls transcription of many genes, including those for transcription factors (Yoo et al 2002). Recently, chromogranin B was reported to have an important role in the intracellular calcium signaling in neurons by interacting with IP3 receptor in a pH-dependent manner (Thrower et al 2003). Human chromogranin B consists of 657 amino acids (Benedum et al 1987). Since the function of chromogranin B is still a matter of debate, it is acceptable that no functional analysis was performed for the 1058G/C(A353G). However, the amino acid 353A and 417R are relatively conserved among species, suggesting that the substitution has some impact on the protein function (Bauer and Fischer-Colbrie 1991) (Figure 2).

Because two of three SNPs we found to be associated with schizophrenia were nonsynonymous, they might be related directly to the susceptibility to schizophrenia. It will be important to replicate the finding in independent samples of schizophrenia of the Japanese population. However, it is possible that these SNPs are just in linkage disequilibrium with other polymorphisms responsible for giving the disease susceptibility. Our findings represent an initial step toward an understanding of the possible etiologic role that *CHGB* plays in schizophrenia. It will be interesting to consider the functional changes in the chromogranin B proteins derived from its genetic variations that may have roles in the clinical phenotypes of schizophrenia. Further research is needed with transgenic mice to clarify the exact role of chromogranin B in the pathophysiology of schizophrenia, especially to identify the functional changes derived from the genetic variations of *CHGB*. Clinical studies are also required to evaluate the psychopathological features in schizophrenic patients who have biochemical changes in chromogranin B levels and *CHGB* variations.

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