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Possible impact of *ADRB3* Trp64Arg polymorphism on BMI in patients with schizophrenia

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ABSTRACT

Background: The β3-adrenoceptor (*ADRB3*) gene Trp64Arg polymorphism has been shown to be associated with obesity as well as type 2 diabetes and cardiovascular disease. The incidence of overweight and the risks of type 2 diabetes and cardiovascular disease are also increased in major depression and schizophrenia. We hypothesized that the Trp64Arg polymorphism may be associated with increased risk of schizophrenia and depression.
Methods: The Trp64Arg was genotyped in 504 patients with schizophrenia, 650 with major depressive disorder (MDD), and 1170 healthy controls. Of these participants, body mass index (BMI) data were available for 125 patients with schizophrenia, 219 with MDD, and 261 controls.
Results: No significant difference in genotype or allele distribution was found across the diagnostic groups. No significant difference in BMI was observed between the Arg allele carriers and the non-carriers in the MDD and the control groups. However, patients with schizophrenia carrying the Arg allele had significantly higher BMI (Mean (*SD*): Arg carriers: 26.5 (6.9), Arg non-carriers: 23.8 (4.3); $P=0.019$) and a higher rate of being overweight (BMI of 25 or more) compared to their counterparts (Trp/Trp group) (% overweight (*SE*): Arg carriers: 52.3 (7.5), Arg non-carriers: 32.1 (5.2); $P=0.027$).
Conclusions: We obtained no evidence for the association of *ADRB3* Trp64Arg with the development of MDD or schizophrenia. However, the Arg allele was found to be associated with higher BMI and being overweight in patients with schizophrenia. This may imply that genotyping *ADRB3* is of clinical use to detect schizophrenic individuals at risk for developing obesity.

1. Introduction

The β3-adrenoceptor is mainly expressed in adipose tissue and mediates the physiologic actions of endogenous catecholamines. Its actions include enhancement of lipolysis in the white adipose tissue and increase of thermogenesis in the brown adipose tissue. Trp64Arg is a missense polymorphism in the β3-adrenoceptor (*ADRB3*) gene and is associated with lower lipolytic activities (Umekawa et al., 1999). The Arg allele of this polymorphism has been shown to be associated with obesity as well as type 2 diabetes and cardiovascular disease (Clement et al., 1995; Gjesing et al., 2008; Iwamoto et al., 2011; Oizumi et al., 2001; Walston et al., 1995; Widen et al., 1995).

Adipocytes in the white adipose tissue secrete a variety of adipocytokines such as leptin, adiponectin, and resistin. These adipocytokines have a central role in the regulation of insulin resistance, as well as in many aspects of inflammation and immunity (Tilg and Moschen, 2006). Adipocytes also secrete chemokines, particularly monocyte chemoattractant protein 1 (MCP-1). MCP-1 attracts leukocytes such as monocytes, T lymphocytes, and dendritic cells (Carr et al., 1994; Xu et al., 1996), which then secrete inflammatory cytokines such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor-α. MCP-1 is also known to play a critical role in the development of cardiovascular disease and obesity-induced insulin resistance (Niu and Kolattukudy, 2009).

These inflammatory factors are also implicated in the pathogenesis of psychiatric disorders including schizophrenia and depression. An increased IL-6 level is one of the most robust findings in the study of inflammatory markers in schizophrenia (Potvin et al., 2008;

Abbreviations: ADRB3, β3-adrenoceptor; ANCOVA, analysis of covariance; BMI, body mass index; HWE, Hardy–Weinberg equilibrium; IL, interleukin; MCP-1, monocyte chemoattractant protein 1; MDD, major depressive disorder; SD, standard deviation; SE, standard error.

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Sasayama et al., 2011b) and in depression (Dowlati et al., 2010). Increased levels of IL-1β in the cerebrospinal fluid of patients with first-episode schizophrenia (Soderlund et al., 2009) and depression (Levine et al., 1999) have also been reported. Furthermore, some studies reported significant associations of *IL-1β* polymorphisms with schizophrenia (Hanninen et al., 2008; Papiol et al., 2004; Sasayama et al., 2011a; Zanardini et al., 2003) and with depression (Borkowska et al., 2011).

The incidence of overweight, as well as the risks of type 2 diabetes and cardiovascular disease, is increased in major depressive disorder (MDD) and schizophrenia (De Hert et al., 2009). A recent meta-analysis showed that depression was found to be predictive of developing obesity and that obesity also increases the risk of depression (Luppino et al., 2010). The activation of inflammatory factors related to obesity may be one of the possible explanations for the relationship between obesity and psychiatric illnesses. Based on these findings, we examined whether the Trp64Arg polymorphism in *ADRB3* confers susceptibility to developing schizophrenia and depression. Furthermore, the association of the Trp64Arg polymorphism with being overweight in these disorders was examined.

2. Materials and methods

2.1. Subjects

Trp64Arg was genotyped in 504 patients with schizophrenia (274 men and 230 women; mean age (standard deviation: *SD*): 43.1 (14.0) years), 650 patients with MDD (309 men and 341 women; 45.1 (14.5) years), and 1170 healthy controls (395 men and 775 women; 46.0 (16.2) years). Self-reported body weight and height were obtained from a portion of the participants. Thus, body mass index (BMI) data were available for 125 patients with schizophrenia (74 men and 51 women; mean age: 39.8 (11.7) years), 219 patients with major depressive disorder (MDD) (97 men and 122 women; 42.0 (12.4) years), and 261 healthy controls (71 men and 190 women; 48.5 (15.4) years). Most of the patients with schizophrenia were on chronic treatment of antipsychotic medication; the average (*SD*) chlorpromazine equivalent converted from daily doses of antipsychotics (American Psychiatric Association, 1997; Inagaki et al., 1999) was 574.5 (509.9) mg/day, and the average duration of treatment was 14.1 (10.7) years. All subjects were biologically unrelated Japanese and were recruited from the outpatient clinic of the National Center of Neurology and Psychiatry Hospital, Tokyo, Japan or through advertisements in free local information magazines and by our website announcement. Consensus diagnosis by at least 2 psychiatrists was made for each patient according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition criteria (American Psychiatric Association, 1994), on the basis of unstructured interviews and information from medical records. The controls were healthy volunteers with no current or past histories of psychiatric treatment and were screened using the Japanese version of the Mini International Neuropsychiatric Interview (Otsubo et al., 2005; Sheehan et al., 1998) by a research psychiatrist to eliminate the possibility of any axis I psychiatric disorders. Participants were excluded if they had prior medical histories of central nervous system diseases or severe head injury or if they met the criteria for substance abuse or dependence or mental retardation. None of the participants were under treatment for cardiovascular diseases or diabetes at the time of assessment. The study protocol was approved by the ethics committee at the National Center of Neurology and Psychiatry, Japan. After describing the study, written informed consent was obtained from every subject.

2.2. Genotyping

Genomic DNA was prepared from venous blood according to standard procedures. The Trp64Arg polymorphism was genotyped using the TaqMan 5′-exonuclease allelic discrimination assay (assay ID: C___2215549_20). The thermal cycling conditions for polymerase chain reaction were as follows: 1 cycle at 95 °C for 10 min followed by 50 cycles of 92 °C for 15 s and 60 °C for 1 min. The allele-specific fluorescence was measured with ABI PRISM 7900 Sequence Detection Systems (Applied Biosystems, Foster City, CA). Ambiguous genotype data were not included in the analysis.

2.3. Statistical analysis

Deviations of genotype distributions from Hardy–Weinberg equilibrium (HWE) were assessed using the χ^2 test for goodness of fit. Genotype and allele distributions were compared between patients and controls by using the χ^2 test for independence. Comparison of BMI between genotypes was analyzed using two-way analysis of covariance (ANCOVA) with genotype and diagnosis as independent variables and age and gender as covariates. For patients with schizophrenia, ANCOVA was also performed adding the chlorpromazine equivalent dose as a covariate to control for use of antipsychotics. Because the frequency of Arg/Arg homozygotes in the general population is low, we combined the heterozygotes and variant homozygotes into one group to assess the effect of the polymorphism on the degree of obesity, as in previous studies (Clement et al., 1995; Kurokawa et al., 2003; Widen et al., 1995). Statistical analyses were performed using the Statistical Package for the Social Sciences version 11.0 (SPSS Japan, Tokyo, Japan). All statistical tests were two-tailed, and $P<0.05$ indicated statistical significance.

Power calculation for the genetic association analysis was performed using the power calculator for genetic studies (http://www.sph.umich.edu/csg/abecasis/CaTS/). Assuming a genotype relative risk of 1.3 under an additive model, a disease prevalence of 1% for schizophrenia and 10% for MDD, and a minor allele frequency of 20%, our sample size had 79% and 91% power, respectively, to detect disease associations with an alpha of 0.05. Similarly, assuming a relative risk of 1.3 under a multiplicative model, the power to detect disease associations was 83% and 94% for schizophrenia and depression, respectively.

Power calculation for ANCOVA in subjects with BMI data was performed using G*Power 3.1.3 (Faul et al., 2007). Assuming that frequency of Arg/Arg homozygotes is 0.671 as in the HapMap data (http://www.hapjmap.org/), the present study provided a power of greater than 0.80 to detect an effect size of 0.38, 0.41, and 0.55 for healthy controls, MDD patients, and schizophrenic patients, respectively.

3. Results

The genotype and allele distributions of the Trp64Arg are shown in Table 1. The genotype and allele distributions did not significantly deviate from the HWE. No significant difference in genotype or allele distribution was found across the three diagnostic groups.

BMI was compared between Arg carriers and non-carriers using ANCOVA with Arg allele carrier status and diagnosis as independent variables and age and gender as covariates. The results showed a significant interaction effect between genotype and diagnosis ($F(2,597)=5.34$, $P=0.005$). Therefore, we further compared the BMI between Arg carriers and non-carriers in each diagnostic group separately, with Arg allele carrier status as the independent variable and age and gender as covariates. No significant difference in BMI was observed between the Arg carriers and the non-carriers in the MDD and the control groups. However, patients with schizophrenia carrying the Arg allele had significantly higher BMI compared to their Trp/Trp homozygous counterparts (mean (*SD*): Arg carriers: 26.5 (6.9), Arg non-carriers: 23.8 (4.3); $F(1,121)=5.69$, $P=0.019$). The difference remained significant even after including the chlorpromazine equivalent dose as a covariate ($F(1,120)=4.97$, $P=0.028$). The categorical analysis also showed that schizophrenic patients carrying the Arg allele were more likely to be

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Table 1
The results of the association analysis of theTrp64Arg polymorphism.

Subjects	N	Genotype			Allele		χ^2 test		HWE P-value	Allelic OR versus controls (95% CI)
		Arg/Arg	Trp/Arg	Trp/Trp	Arg	Trp	Genotype	Allele		
Schizophrenia	504	21 (0.04)	148 (0.29)	335 (0.66)	190 (0.19)	818 (0.81)	$\chi^2=1.89$ $df=4$	$\chi^2=0.87$ $df=2$	0.37	1.06 (0.87–1.28)
MDD	650	26 (0.04)	198 (0.30)	426 (0.66)	250 (0.19)	1050 (0.81)	$P=0.76$	$P=0.65$	0.62	1.08 (0.91–1.29)
Controls	1170	36 (0.03)	350 (0.30)	784 (0.67)	422 (0.18)	1918 (0.82)			0.68	

HWE: Hardy–Weinberg equilibrium; OR: odds ratio; CI: confidence interval; MDD: major depressive disorder; *df*: degree of freedom. Numbers in parentheses in the genotype and the allele columns represent the frequencies of genotypes and alleles.

overweight (BMI of 25 or more) than their Trp/Trp homozygous counterparts (% overweight (standard error: *SE*): Arg carriers: 52.3 (7.5), Arg non-carriers: 32.1 (5.2); $\chi^2=4.87$, $df=1$, $P=0.027$; odds ratio = 2.32 (95% confidence interval: 1.09 to 4.92); sensitivity of 0.47 and specificity of 0.72). As shown in Table 2, no significant difference between Arg carriers and non-carriers in age, gender rate, antipsychotic equivalent dose, or treatment duration was observed in patients with schizophrenia. Fig. 1 shows the rate of being overweight for the Arg allele carriers and the non-carriers in each diagnostic group.

4. Discussion

The present study had sufficient power to detect a relatively modest effect of the *ADRB3* gene Trp64Arg on the development of schizophrenia and MDD. Thus, our findings suggest that the Trp64Arg polymorphism is unlikely to have a major role in the development of schizophrenia or MDD. The hypothesized effect of Trp64Arg variant on the development of schizophrenia and MDD, however, was due to an indirect action, mediated by the inflammatory process. Therefore, the association may have been too weak to be detected by the sample size used in this study. The observed effect of the Trp64Arg on BMI was not significant in healthy controls and patients with MDD. In patients with schizophrenia, however, the Arg allele was associated with higher BMI, which was in line with the evidence that Arg allele is associated with lower lipolytic activities (Umekawa et al., 1999).

Previous studies carried out in Japanese (Oizumi et al., 2001) and in Finnish subjects (Widen et al., 1995) reported that the Arg allele of the Trp53Arg polymorphism was associated with obesity. However, some studies failed to find such an association (Buettner et al., 1998; Gagnon et al., 1996; Gjesing et al., 2008; Oeveren van-Dybicz et al., 2001). The inconsistency between studies may be partially explained by the population differences between samples. A meta-analysis suggests that the effect of this polymorphism on BMI is greater in East Asians than in Europeans (Kurokawa et al., 2008). The Trp64Arg may play a particularly important role in the Japanese population, since the minor allele frequency is higher in Japanese than in other populations in the HapMap data (http://www.hapjmap.org/).

Table 2
Demographic and clinical characteristics of Arg carriers and non-carriers in patients with schizophrenia.

	Arg carriers	Arg non-carriers	Analysis
Age (years)	39.9 (11.4)	39.8 (11.9)	ANOVA: $F(1,123)=0.0$, $P=0.98$
Gender (M/F)	31/13	43/38	χ^2 test: $\chi^2=3.56$, $df=1$, $P=0.06$
CP equivalent dose (mg/day)	663.2 (613.2)	526.3 (440.7)	ANOVA: $F(1,123)=2.1$, $P=0.15$
Treatment duration (years)	15.4 (11.2)	13.2 (10.5)	ANOVA: $F(1,123)=1.2$, $P=0.27$

Values are shown as mean (standard deviation). BMI: body mass index; CP: chlorpromazine; ANOVA: analysis of variance; *df*: degree of freedom.

The genetic homogeneity of the Japanese population was a major strength of the present study. However, contrary to the results of the meta-analysis in Japanese subjects (Kurokawa et al., 2008), our results showed no significant association between the Trp64Arg and the BMI in healthy subjects. These negative results may have arisen by the small number of subjects in the present study. Intriguingly, however, the patients with schizophrenia carrying the Arg allele had significantly higher BMI compared to their Trp/Trp homozygous counterparts. Obesity is highly prevalent in patients with schizophrenia due to illness-related factors and use of antipsychotic medications (Kolotkin et al., 2008). Our results suggest that schizophrenic patients carrying the Arg allele especially have a greater tendency to gain weight. Clement et al. (1995) demonstrated that although the frequency of the Arg allele was similar in the morbidly obese patients and the normal subjects, the obese patients with Arg allele had higher capacity to gain weight. Taken together, the Trp64Arg variant may enhance weight gain in individuals already at risk for obesity.

The major limitation of this study was that the effects of medication could not be fully controlled due to the variability in types and doses. Particularly, antipsychotic medications are known to induce metabolic abnormalities such as obesity, hyperglycemia, and metabolic syndrome (De Hert et al., 2011). Therefore, the use of antipsychotics in patients with schizophrenia may have confounded the results. However, the chlorpromazine equivalent dose did not differ between Arg carriers and non-carriers in patients with schizophrenia. Furthermore, using the chlorpromazine equivalent dose as a covariate in an ANCOVA still resulted in significantly higher BMI in Arg carriers of schizophrenic patients. Thus, controlling for total chlorpromazine equivalent dose did not affect the findings of the present study. Nevertheless, the influence of the Trp64Arg on BMI may differ in non-medicated patients or may depend on the type of antipsychotics used. Further investigations are required to elucidate the effects of

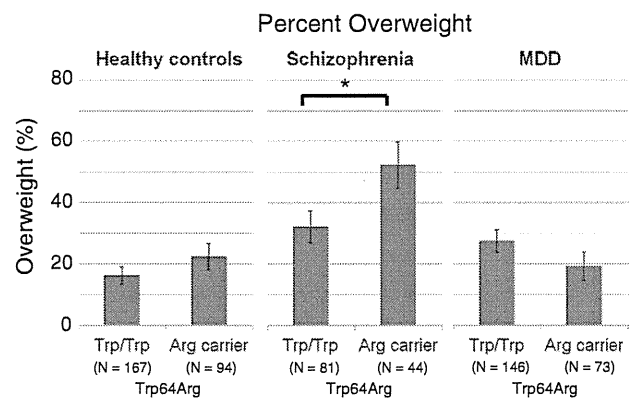

Fig. 1. Percentage overweight in Arg carriers and non-carriers of the Trp358Ala. The rate of being overweight (BMI of 25 or more) is shown for the Arg carriers and the non-carriers in healthy controls and in patients with schizophrenia and MDD. Error bars indicate 1 standard error. In patients with schizophrenia, Arg carriers were significantly more likely to be overweight than the non-carriers. * $P<0.05$.

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antipsychotics. Another limitation of the study is that BMI data relied on self-reports of the participants. However, previous studies show that self-reported BMI is satisfactorily accurate for the assessment of the prevalence of overweight (Craig and Adams, 2009; Dekkers et al., 2008).

In conclusion, we obtained no evidence for the association of *ADRB3* Trp64Arg with the development of MDD or schizophrenia. However, the Arg allele of the Trp64Arg polymorphism was found to be associated with higher BMI in patients with schizophrenia. This may imply that genotyping *ADRB3* is of clinical use to detect schizophrenic individuals at risk for developing obesity, which is an important issue in the antipsychotic medication. Further studies are warranted to elucidate the influence of the *ADRB3* gene variation on the development of psychiatric disorders and also to understand the factors that contribute to the risk of obesity in patients with psychiatric disorders.

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Relationships between psychological distress, coping styles, and HPA axis reactivity in healthy adults

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ABSTRACT

Psychological distress and coping styles have been suggested to relate to altered function in the hypothalamic-pituitary-adrenal (HPA) axis, although there remains much to be understood about their relationships. High and low cortisol levels (or reactivity) both represent HPA axis dysfunction, with accumulated evidence suggesting that they are linked to different types of psychopathology. The dexamethasone (DEX)/corticotropin-releasing hormone (CRH) test has been extensively used to identify HPA axis abnormalities in various psychiatric conditions including mood disorders; however, the possible associations of psychological distress and coping styles with HPA axis function have not been well documented using this test. Here, we examined the relationships of HPA axis reactivity as measured by the DEX/CRH test with subjectively perceived psychological distress and coping styles, both of which were assessed with self-report questionnaires, in 121 healthy volunteers. Subjects were divided into three groups by the cortisol suppression pattern, namely the incomplete-suppressors (DST-Cortisol $\geq$ 5 μg/dL or DEX/CRH-Cortisol $\geq$ 5 μg/dL), moderate-suppressors (DST-Cortisol $<$ 5 μg/dL and 1 μg/dL $\leq$ DEX/CRH -Cortisol $<$ 5 μg/dL), and enhanced-suppressors (DST-Cortisol $<$ 5 μg/dL and DEX/CRH-Cortisol $<$ 1 μg/dL). The enhanced-suppressors showed significantly higher scores in obsessive-compulsive, interpersonal sensitivity and anxiety symptoms and significantly more frequent use of avoidant coping strategy, compared to the other two groups. These results point to the important role of enhanced suppression of cortisol, or blunted cortisol reactivity, in non-clinical psychopathology such as avoidant coping strategy and greater psychological distress.

1. Introduction

A wide variety of stress is associated with alteration in the hypothalamic-pituitary-adrenal (HPA) axis function. Studies looking at cortisol as the main output substance of the HPA axis have thus been critical to advancing our understanding of psychobiological underpinnings of various stress-related conditions (de Kloet et al., 2005; Heim et al., 2000). For instance, perceived stress in everyday life (Pruessner et al., 1999), stressful situations such as academic examinations and seafaring (Droogleever Fortuyn et al., 2004; Liberzon et al., 2008), self-reported symptoms (Van den Bergh et al., 2008), psychological coping styles (Nicolson, 1992; O'Donnell et al., 2008), rejection sensitivity (Tops et al., 2008), sleep status (Backhaus et al., 2004; Lasikiewicz et al., 2008; Wright et al., 2007) and personality profile (Tyrka et al., 2007) have been reported to be associated with alteration in cortisol levels. These studies in healthy subjects have investigated HPA axis function using several different cortisol measures including diurnal cortisol profiles, cortisol awakening response, and cortisol reactivity to psychosocial challenge tests such as Trier Social Stress Test (Kirschbaum et al., 1993).

On the other hand, HPA axis function in clinical populations, particularly in patients with major depression, has been investigated with pharmacological challenge tests including dexamethasone (DEX) suppression test (DST, Carroll et al., 1976) and DEX/corticotropin-releasing hormone (CRH) test (Heuser et al., 1994a;

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Holsboer et al., 1987). The DEX/CRH test is an integrated challenge test for HPA axis function that combines DEX-pretreatment with CRH administration on the following day; thus, it is essentially a DST followed by CRH challenge. The merit of this combined test is that at the moment of CRH infusion the HPA axis is downregulated due to negative feedback induced by the DEX. In the DEX/CRH test a relatively high dose (i.e., 1.5 mg) of DEX is usually used, whereas DST studies, in particular those which examine the HPA function of post-traumatic stress disorder (PTSD), have used a lower dose (i.e., 0.5 mg or 1 mg) of DEX (e.g., Grossman et al., 2003; Yehuda et al., 2004). Sensitivity of the DEX/CRH test in depressed patients has been shown to be high in prior studies including ours (Heuser et al., 1994a; Kunugi et al., 2004, 2006; Watson et al., 2006b). Moreover, this test has revealed altered HPA axis function in those individuals with specific characteristics: dampened cortisol reactivity in healthy adults reporting childhood emotional abuse (Carpenter et al., 2009), increased cortisol responses in healthy adults reporting childhood parental loss with the exception of attenuated cortisol responses in those with parental desertion and low levels of care (Tyrka et al., 2008b), increased cortisol responses in healthy adults with certain personality traits (Tyrka et al., 2006, 2008a), and attenuated cortisol responses in depressed women on job-stress-related longterm sickleave (Rydmark et al., 2006; Wahlberg et al., 2009). On the other hand, the possible associations of more commonly presented psychopathology such as perceived distress in everyday life and coping styles with HPA axis function have not been well documented using the DEX/CRH test. However, these psychological measures are suggested to relate to altered cortisol level (Heim et al., 2000, 2002; Nicolson, 1992; O'Donnell et al., 2008; Pruessner et al., 1999; Van den Bergh et al., 2008). For instance, severity of daily hassles in the past month was negatively related to cortisol concentrations (Heim et al., 2002). Perceived stress was positively, and burnout was negatively, associated with cortisol levels after DEX administration (Pruessner et al., 1999). Passive coping is suggested to relate to hypocortisolism (Heim et al., 2000). Healthy adults scoring high in either problem engagement or seeking social support showed lower cortisol levels (O'Donnell et al., 2008). Given these findings, it would be of interest to examine HPA axis function in relation to psychopathology at a non-clinical level such as psychological distress and coping styles by using the DEX/CRH test.

Various kinds of psychiatric disorders have been shown to be associated with HPA axis hyperactivity as reflected by the high cortisol levels and impaired negative feedback inhibition due to an impaired corticosteroid receptor function (Holsboer, 2000). On the other hand, a number of psychoneuroendocrinological studies have demonstrated that a variety of conditions are associated with hypocortisolism, including low basal cortisol levels, enhanced sensitivity to the negative feedback, and blunted reactivity of provoked cortisol. Examples of psychiatric conditions characterized by hypocortisolism include PTSD, chronic fatigue syndrome, fibromyalgia and atypical depression (Fries et al., 2005; Heim et al., 2000). Together, while both of these two extremes of cortisol activity can represent HPA axis dysfunction, they are likely to be linked to different types of psychopathology. Concerning hypocortisolism, there remains much to be clarified as to its natural course and meaning. Although hypocortisolism is considered to represent the result of prolonged stress exposure (Fries et al., 2005; Heim et al., 2000; Ising et al., 2005), a condition so-called "allostasis" (McEwen, 2003), there also exists some evidence suggesting that this state could be a preexisting vulnerability to stress-related disorders (Delahanty et al., 2000; Wahlberg et al., 2009; Yehuda et al., 2000).

Arginine vasopressin (AVP), in addition to CRH, is an HPA axis secretagogue. AVP produced in parvocellular neurons of hypothalamic paraventricular nucleus (PVN) and secreted into pituitary portal vein system plays an important role in stress response (Herman, 1995; Romero and Sapolsky, 1996). It is reported that, in chronic stress paradigms, the expression of AVP in parvocellular neurons increases and pituitary V1b receptor, through which AVP stimulates the ACTH secretion, up-regulates (Aguilera et al., 1994; Aguilera and Rabadan-Diehl, 2000). There also exist clinical studies that support this notion. For example, de Kloet et al. (2008) have recently reported elevated plasma AVP levels in veterans with PTSD. Watson et al. (2006a) measured plasma AVP levels after pre-treatment of DEX in patients with chronic depression and those with bipolar disorder, and found significantly higher post-DEX AVP levels in the patient groups than in healthy controls, suggesting that post-DEX AVP levels could be more sensitive than baseline AVP levels in detecting HPA axis abnormalities. These findings raise the possibility that the post-DEX AVP measure may help understand whether the hypocortisolism, if present, is a result of chronic HPA axis overactivity or a preexisting vulnerability factor for psychopathology.

In this context, the present study sought to examine the relationships between subjectively perceived psychological distress, psychological coping styles and the cortisol suppression pattern to the DEX/CRH test in non-clinical volunteers. We also examined the relationships of these psychological measures with the post-DEX AVP level to see whether the possible low cortisol levels would reflect allostatic shift or preexisting vulnerability. The study hypothesis was that the higher cortisol levels (or less suppression of cortisol) and/or lower cortisol levels (or more suppression of cortisol) would be related to greater distress and a unique pattern of coping strategies. If the low cortisol, together with elevation of AVP, is related to these psychological measures, it would indicate allostatic shift; while if the low cortisol, together with no elevation of AVP, is related to such psychological measures, it may indicate preexisting vulnerability.

2. Materials and methods

2.1. Participants

From February 2006 to December 2008, 121 healthy volunteers (age range: 20–70; male, 28, female, 93) were recruited from the community, through advertisements in free local information magazines which contained a wide variety of information including healthcare-related information and by our website announcement. Participants were interviewed using the Japanese version of the Mini-International Neuropsychiatric Interview (MINI, Otsubo et al., 2005; Sheehan et al., 1998) by research psychiatrists (H.H., Y.O., T.T. and H.K.), and only those who demonstrated no current Axis I psychiatric disorders, including PTSD, were enrolled in this study. In addition, those who demonstrated one or more of the following conditions during a non-structured interview by an experienced psychiatrist were excluded from this study: past or current contact to psychiatric services, taking psychotropic drugs or had a history of regular use of psychotropics, and the other obvious self-reported signs of past primary psychotic and mood disorders as well as PTSD. Additional exclusion criteria were as follows: having a prior medical history of central nervous system disease or severe head injury, having major systemic medical illnesses, having a history of substance dependence or abuse, or taking corticosteroids or antihypertensive medication. No subjects reported that they were on oral contraceptives or estrogen replacement therapies. The present experiments on our subjects were conducted in accordance with the Declaration of Helsinki. After the nature of the study procedures had been fully explained, written informed consent was obtained

from all subjects. The study was approved by the ethics committee of the National Center of Neurology and Psychiatry, Japan.

2.2. DEX/CRH test procedure and presentation for neuroendocrine data

The DEX/CRH test was administered to all subjects by a single examiner (H.H.) according to a protocol proposed in a previous report (Kunugi et al., 2006). First, they took 1.5 mg of DEX (Banyu Pharmaceutical Corporation, Tokyo, Japan) orally at 2300 h. On the next day, they attended our laboratory and sat on a comfortable couch in a calm room. A vein was cannulated at 1430 h to collect blood at 1500 and 1600 h via an intravenous catheter. Human CRH (100 μg) (hCRH 'Mitsubishi', Mitsubishi Pharma Corporation, Tokyo, Japan) was administered intravenously at 1500 h, immediately after the first blood collection. Subjects fasted and rested semi-supine throughout the testing. Blood samples were immediately centrifuged and stored at −20 °C. Plasma concentrations of cortisol and AVP were measured by radioimmunoassay at SRL Corporation (Tokyo, Japan). The detection limits for cortisol and AVP were 1.0 μg/dL and 0.2 pg/mL, respectively (SRL Corporation, Tokyo, Japan). Cortisol and AVP values under the detection limits were treated as 0 μg/dL and 0 pg/mL, respectively. For cortisol, the intra-assay coefficients of variation at 2.37 μg/dL, 13.02 μg/dL, and 36.73 μg/dL were 6.90%, 4.94%, and 5.78%, respectively. The inter-assay coefficients of variation at 2.55 μg/dL, 13.04 μg/dL, and 34.17 μg/dL were 8.91%, 6.03%, and 6.44%, respectively. For AVP, the intra-assay coefficients of variation at 0.97 pg/mL, 1.64 pg/mL, and 2.88 pg/mL were 1.7%, 7.2%, and 3.5%, respectively. The inter-assay coefficients of variation at 0.94 pg/mL, 1.59 pg/mL, and 2.88 pg/mL were 3.9%, 10.3%, and 6.9%, respectively (SRL Corporation, Tokyo, Japan). Outcome measures of this neuroendocrine test were the DST-Cortisol (i.e., the concentration of cortisol [μg/dl] at 1500 h), DEX/CRH-Cortisol (i.e., the concentration of cortisol at 1600 h) and DST-AVP (i.e., the concentration of AVP [pg/ml] at 1500 h). To further dissect the extent to which the subject's HPA axis responded to the CRH challenge, the magnitude of change from DST-Cortisol to DEX/CRH-Cortisol, namely ΔCortisol, was calculated for each subject. For DST-AVP, data were available for only 106 of the total 121 subjects. This reduction of subjects was because we started to collect the AVP data on May 2006, which was about 3 months after the study initiation.

A cut-off criterion for suppression status was considered as follows; 'Incomplete-suppressors' were defined *a priori* to be individuals where either or both of DST- and DEX/CRH-Cortisols were equal to or more than 5 μg/dL. This cut-off value was based on our previous studies (Kunugi et al., 2004, 2006), where the cortisol value of 5 μg/dL was shown to sensitively distinguish depressed patients from healthy controls. Based on these reports of ours, recent studies (Ising et al., 2007; Schüle et al., 2009) also used the same cut-off value of cortisol. Given these findings, in the present study we assumed that the cortisol value of 5 μg/dL would be also useful in detecting those participants whose negative feedback of cortisol was "incomplete". On the other hand, 'Enhanced-suppressors' were defined as those individuals whose DST-Cortisol was less than 5 μg/dL and DEX/CRH-Cortisol was less than 1 μg/dL, because this DEX/CRH-Cortisol value corresponded to its detection limit and can therefore be regarded as an extremely low cortisol level. The remaining individuals were considered to be 'Moderate-suppressors'. We would like to note that the 'Incomplete-suppressors' were the sum total of the 'Intermediate suppressors' and 'Non-suppressors' as defined in our previous studies (Kunugi et al., 2004, 2006). This slight modification on the grouping criterion was because it was expected that very few subjects would fall into the 'Non-suppressors' group since the present study included only healthy subjects.

2.3. Psychological assessment

To assess subjectively perceived psychological distress and psychological coping styles, the following two self-report questionnaires were distributed to the participants.

2.3.1. The hopkins symptom checklist (HSCL)

Subjectively perceived psychological distress during one week preceding the neuroendocrine test was assessed via the Hopkins Symptom Checklist (HSCL, Derogatis et al., 1974), a self-report questionnaire consisting of 58 (or 54) items which are scored on 5 underlying symptom dimensions, i.e., somatization, obsessive-compulsive, interpersonal sensitivity, anxiety, and depression symptoms. In the present study, a validated Japanese version of the HSCL (Nakano, 2005) comprising 54 items was used. In this questionnaire, subjects were instructed to rate each item based on the distress perceived during the previous week, using a four-point scale of frequency, with "not-at-all" being scored 1, "occasionally", 2, "sometimes", 3, and "frequently", 4. All of the 121 participants answered this questionnaire.

2.3.2. The ways of coping checklist (WCCL)

Psychological coping can be defined as the thoughts and behaviors used to manage the internal and external demands of situations that are appraised as stressful (Folkman and Moskowitz, 2004). Coping styles of the participants were assessed using the Japanese version of the Ways of Coping Checklist (WCCL) (Folkman and Lazarus, 1985; Nakano, 1991), a self-report questionnaire consisting of 47 items which measure each participant's preferred coping styles using a four-point scale of frequency, with "not used" being scored 0, "not frequently used", 1, "sometimes used", 2, and "regularly used", 3. The 47 items were grouped into 6 coping strategies, namely planful problem-solving, positive reappraisal, seeking social support, self-blame, wishful thinking, and escape-avoidance (Nakano, 1991), thus measuring both problem-focused and emotion-focused coping strategies. The WCCL data were obtained from 102 of the total 121 participants. This reduction of participants was because we started to collect the WCCL data on June 2006.

2.4. Statistical analysis

Averages are reported as means ± SD (standard deviation). Categorical variables were compared using the χ^2 test. Mann–Whitney *U*-test was used to compare hormonal measures between two groups. The analysis of variance (ANOVA) or Kruskal–Wallis test was used to examine differences between three groups. Pearson's r was used to examine correlations among psychological measures, while Spearman's ρ was used to examine correlations among hormonal data or between hormonal data and psychological measures. To examine the difference between DST- and DEX/CRH-Cortisols, Wilcoxon signed rank test was performed. The analysis of covariance (ANCOVA), controlling for confounding variables, was performed to compare the scores of questionnaires between the three participant groups. Since age and sex have been shown to significantly influence the cortisol levels (e.g., Heuser et al., 1994b; Kunugi et al., 2006; Kunzel et al., 2003), these variables were considered as confounders regardless of the present data. Statistical significance was set at two-tailed $p < 0.05$. Analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 11.0 (SPSS Japan, Tokyo).

Table 1
Correlations between coping styles and psychological distress (Pearson's r).

	Somatization	Obsessive-compulsive	Interpersonal sensitivity	Anxiety	Depression
Problem-solving	−0.10	−0.22*	−0.24*	−0.17	−0.25*
Positive reappraisal	−0.08	−0.16	−0.26**	−0.20*	−0.32**
Social support	0.11	0.14	0.14	0.16	−0.03
Self-blame	0.29**	0.47***	0.52***	0.49***	0.48***
Wishful thinking	0.22*	0.42***	0.37***	0.40***	0.31**
Escape-avoidance	0.08	0.30**	0.33***	0.30**	0.20*

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3. Results

3.1. Demographic characteristics of the subjects

The numbers of incomplete-suppressors, moderate-suppressors, and enhanced-suppressors were 55, 54, and 12, respectively, indicating that the enhanced-suppressors corresponded to approximately the bottom 10% of total subjects for cortisol levels. The mean ages of incomplete-suppressors, moderate-suppressors, and enhanced-suppressors were 46.8 ± 14.3, 42.7 ± 15.1, and 44.4 ± 14.7, respectively. These three suppression groups did not significantly differ in age [$F(2,118) = 1.49$, $p = 0.23$]. Male/female ratios of incomplete-suppressors, moderate-suppressors, and enhanced-suppressors were 6/49, 15/39, and 7/5, respectively. There was a significant difference in sex distribution [$\chi^2(2) = 13.6$, $p = 0.001$]; males demonstrated significantly greater suppression than females.

3.2. Correlations between coping styles and psychological distress

Table 1 shows the correlations between coping styles assessed with the WCCL and psychological distress assessed with the HSCL. Significant negative correlations were seen between problem-focused coping strategies (i.e., problem-solving and positive reappraisal) and greater psychological symptoms including interpersonal sensitivity and depression. In contrast, significant positive correlations were observed between emotion-focused coping strategies (i.e., self-blame, wishful thinking and escape-avoidance) and most of the symptom dimensions. Social support was not significantly related to any of the symptom dimensions.

3.3. Relationships between hormonal measures

The cortisol values for the three suppressor groups on the three cortisol indices are provided in Table 2. DST-Cortisol of 64 subjects and DEX/CRH-Cortisol of 12 subjects fell under the detection limit, while DST-AVP did not fall under the detection limit in any subjects. DEX/CRH-Cortisol was significantly higher than DST-Cortisol in the whole sample, as expected ($p < 0.001$; Wilcoxon signed rank test). There was no significant correlation of DST-AVP with DST-Cortisol ($\rho = 0.07$, $p = 0.50$), DEX/CRH-Cortisol ($\rho = 0.03$, $p = 0.75$), or ΔCortisol ($\rho = 0.02$, $p = 0.83$). The three suppression groups did not significantly differ in DST-AVP [Kruskal–Wallis $\chi^2(2) = 0.14$, $p = 0.93$].

3.4. Correlations between hormonal and psychological measures

No significant correlations were seen between DST-Cortisol and any measures of the two questionnaires (all $p > 0.2$). No significant correlations were seen between DEX/CRH-Cortisol and any measures of the two questionnaires (all $p > 0.2$) except for interpersonal sensitivity ($\rho = -0.20$, $p = 0.03$) in the HSCL. Similarly, no significant correlations were observed between ΔCortisol and any measures of the two questionnaires (all $p > 0.2$) except for interpersonal sensitivity ($\rho = -0.21$, $p = 0.02$) in the HSCL. No significant correlation was found between DST-AVP and any of the outcomes of the two questionnaires (all $p > 0.1$).

3.5. Relationships between psychological measures and DEX/CRH outcomes

3.5.1. Psychological distress and DEX/CRH outcomes

Fig. 1 shows the relationships between 5 symptom dimensions of the HSCL and DEX/CRH suppression status. The ANCOVA on 5 symptoms controlling for age and sex showed significant main effects of the suppression status on obsessive-compulsive [$F(2,114) = 5.19$, $p = 0.007$], interpersonal sensitivity [$F(2,114) = 5.43$, $p = 0.006$], and anxiety [$F(2,114) = 5.86$, $p = 0.004$], symptoms. Post-hoc analyses with Bonferroni correction revealed that the enhanced-suppressors, compared to the other two groups or to moderate-suppressors alone, had significantly greater scores on these three symptom dimensions, while no significant differences were seen between incomplete- and moderate-suppressors (Fig. 1).

However, a considerable portion of the subjects fell into the incomplete-suppressors and thus we considered that this group would not necessarily represent those individuals whose cortisol levels were abnormally high. Therefore, to confirm the results obtained by the main analysis, the same ANCOVA was repeated with another grouping criterion as follows: 'incomplete-suppressors' to be individuals where either or both of DST- and DEX/CRH-

Table 2
Plasma cortisol concentrations (mean ± SD (range)) for the three subject groups, based on the suppression pattern.

	Incomplete-suppressors ($n = 55$)[d]	Moderate-suppressors ($n = 54$)[e]	Enhanced-suppressors ($n = 12$)[f]
DST-Cortisol[a]	1.4 ± 1.5 (0 ~ 5.8)	0.4 ± 0.7 (0 ~ 1.9)	0.1 ± 0.3 (0 ~ 1.1)
DEX/CRH-Cortisol[b]	10.0 ± 4.6 (5.0 ~ 25.1)	2.5 ± 1.1 (1.1 ~ 4.9)	0 ± 0 (0 ~ 0)
ΔCortisol[c]	8.6 ± 4.4 (2.3 ~ 20.2)	2.1 ± 1.3 (−0.3 ~ 4.8)	−0.1 ± 0.3(−1.1 ~ 0)

[a] The concentration of cortisol [μg/dl] at 1500 h (i.e., immediately before the CRH challenge).
[b] The concentration of cortisol [μg/dl] at 1600 h (i.e., 1 h after the CRH challenge).
[c] Defined as "DEX/CRH-Cortisol minus DST-Cortisol".
[d] Defined as "DST-Cortisol ≥ 5 or DEX/CRH-Cortisol ≥ 5".
[e] Defined as "DST-Cortisol < 5 and 1 ≤ DEX/CRH -Cortisol < 5".
[f] Defined as "DST-Cortisol < 5 and DEX/CRH-Cortisol < 1".

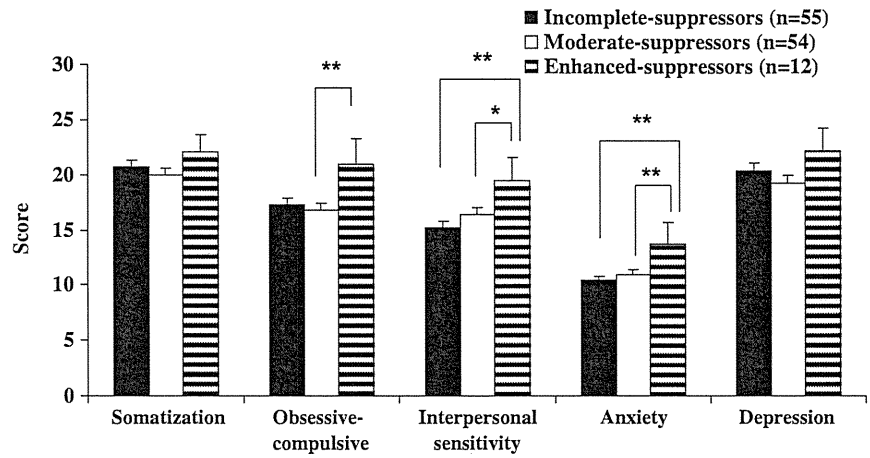

Fig. 1. Comparisons of scores on the 5 dimensions of the Hopkins Symptom Checklist (HSCL) between the three suppression groups. Black, white, and borderline bars are incomplete-suppressors (defined as "DST-Cortisol ≥5 or DEX/CRH-Cortisol ≥5"; $n = 55$), moderate-suppressors (defined as "DST-Cortisol <5 and 1 ≤ DEX/CRH -Cortisol <5"; $n = 54$), and enhanced-suppressors (defined as "DST-Cortisol <5 and DEX/CRH-Cortisol <1"; $n = 12$), respectively. $^{*}p < 0.05$; $^{**}p < 0.01$. Error bars represent standard errors of the mean.

Cortisols were equal to or more than 13 μg/dL, 'enhanced-suppressors' to be those whose DST-Cortisol was less than 13 μg/dL and DEX/CRH-Cortisol was less than 1 μg/dL, and 'moderate-suppressors' to be the remaining individuals. The reason why we here used the cortisol level of 13 μg/dL, instead of the original 5 μg/dL, as the cut-off value for the 'incomplete-suppressors' was that this value corresponded to approximately the top 10% of total subjects for cortisol levels. This 10% derived from the fact that the cortisol value of "enhanced-suppressors" corresponded to approximately the bottom 10% of total subjects. Using this new grouping, additional ANCOVA on the 5 symptoms controlling for age and sex was performed, again showing significant main effects of the suppression status on obsessive-compulsive [$F(2,114) = 4.63$, $p = 0.012$], interpersonal sensitivity [$F(2,114) = 5.50$, $p = 0.005$] and anxiety [$F(2,114) = 5.81$, $p = 0.004$] symptoms. Post-hoc analyses with Bonferroni correction revealed that the enhanced-suppressors, compared to the other two groups or to moderate-suppressors alone, scored significantly higher on these three symptom dimensions, while no significant differences were seen between incomplete- and moderate-suppressors (data not shown).

3.5.2. Coping styles and DEX/CRH outcomes

The relations between the 6 different coping styles of WCCL and suppression status are provided in Fig. 2. The ANCOVA on the 6 coping subscales controlling for age and sex showed a significant main effect of the suppression status on escape-avoidance [$F(2,95) = 5.26$, $p = 0.007$]. Post-hoc analyses with Bonferroni correction revealed that the enhanced-suppressors, compared to the other two groups, had significantly greater scores on this coping strategy, while no significant differences were found between incomplete- and moderate-suppressors (Fig. 2).

The additional ANCOVA with the other grouping criterion of suppression status on the 6 coping subscales showed significant main effects of the suppression status on wishful thinking [$F(2,95) = 3.31$, $p = 0.041$] and escape-avoidance [$F(2,95) = 5.56$, $p = 0.005$]. Post-hoc analyses with Bonferroni correction revealed that the enhanced-suppressors, compared to the other two groups or to incomplete-suppressors alone, scored significantly higher on these two coping strategies, while no significant differences were seen between incomplete- and moderate-suppressors (data not shown).

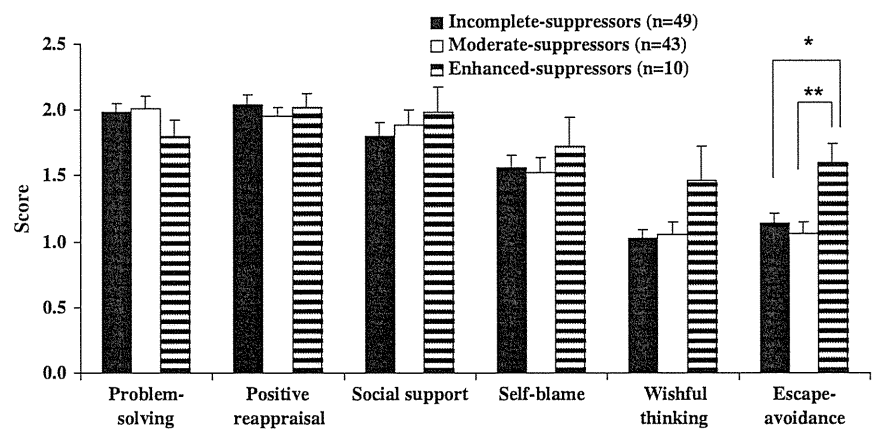

Fig. 2. Comparisons of scores on the 6 subscales of the Ways of Coping Checklist (WCCL) between the three suppression groups. Black, white, and borderline bars are incomplete-suppressors (defined as "DST-Cortisol ≥5 or DEX/CRH-Cortisol ≥5"; $n = 49$), moderate-suppressors (defined as "DST-Cortisol <5 and 1 ≤ DEX/CRH-Cortisol <5"; $n = 43$), and enhanced-suppressors (defined as "DST-Cortisol <5 and DEX/CRH-Cortisol <1"; $n = 10$), respectively. $^{*}p < 0.05$; $^{**}p < 0.01$. Error bars represent standard errors of the mean.

4. Discussion

We examined the relationships of cortisol reactivity to the DEX/CRH test with subjectively perceived psychological distress and psychological coping styles as assessed with the self-report questionnaires in a non-clinical population. The most salient finding was that the enhanced cortisol suppression to the DEX/CRH test, or blunted cortisol response to CRH challenge, was significantly related to various psychological distress and avoidant coping style.

Besides the well-established relation between acute stress and elevated cortisol levels, numerous studies have linked low cortisol levels to various kinds of stress, in particular to chronic stress (reviewed in Heim et al., 2000). In line with this, a large number of DST studies using a low dose of DEX have observed enhanced suppression of cortisol in a variety of psychiatric conditions including PTSD (Grossman et al., 2003; Yehuda et al., 1993). To our knowledge, however, the present study is the first DEX/CRH study where the overtly defined enhanced suppression, in addition to the incomplete suppression, was examined in the context of non-clinical psychological distress and coping styles. Based on the previous literature, it was hypothesized that both incomplete and enhanced suppression of cortisol due to the negative feedback by DEX administration would be related to greater distress and/or a unique pattern of coping strategies. The significant associations of enhanced suppression with greater distress and more frequent use of avoidant coping strategy supported the hypothesis, while contrary to our prediction incomplete suppression was not significantly related to any of the psychological measures.

We observed significant negative correlations between interpersonal sensitivity in the HSCL and cortisol values, namely DEX/CRH-Cortisol and ΔCortisol. A significant relation between interpersonal sensitivity and enhanced cortisol suppression was also found. These results were in line with a recent study showing the association between higher rejection sensitivity and lower cortisol awakening responses in community women (Tops et al., 2008). In addition, the significant relationships of enhanced cortisol suppression with obsessive-compulsive and anxiety symptoms, but not depressive symptom, point to the possibility that enhanced suppression is more related to anxiety symptoms than depressive symptoms in healthy populations. Several studies investigated cortisol levels as measured by DST in patients with obsessive-compulsive disorder (OCD), and the majority of these studies found that OCD patients did not show non-suppression or their baseline cortisol levels did not differ from healthy controls (e.g., Kuloğlu et al., 2007; Lieberman et al., 1985). These previous findings, combined with the present result, might suggest that obsessive-compulsive symptoms are associated with normal cortisol suppression to DEX and subsequent blunted response to CRH challenge.

Several lines of research have documented the relationship of coping styles with psychobiological measures including cortisol levels (Frecska et al., 1988; Nicolson, 1992; O'Donnell et al., 2008). While cortisol activity has been considered to reflect the effectiveness of coping strategies (Nicolson, 1992), how differential coping styles in everyday settings relate to cortisol reactivity has not been well documented using pharmacological challenge tests. The present study found that blunted, but not exaggerated, cortisol reactivity was significantly associated with the avoidant coping style. This finding is consistent with the previous study reporting the association between passive coping and low cortisol levels (Heim et al., 2000). However, the findings to date on the association between coping styles and cortisol activity have not been unequivocal. A 1-mg DST study (Frecska et al., 1988), for example, observed an association between high post-DEX cortisol levels and denial and passivity. Furthermore, O'Donnell et al. (2008) found no significant association between the avoidant coping style and cortisol levels in healthy older adults. Instead, they found that individuals who scored higher in either problem engagement or seeking social support had lower cortisol output over the day. These inconsistent findings might be due to different instruments for the measurement of coping styles and/or to different measures for the assessment of HPA axis function (e.g., DST vs. DEX/CRH and high- vs. low-dose of DEX) between studies. Still, the present finding of the association between blunted cortisol reactivity and more frequent use of avoidant coping style might be intriguing, taking into account that atypical depression, a disorder known to relate to down-regulation of HPA axis (Gold and Chrousos, 2002), has been reported to be associated with avoidant personality (Alpert et al., 1997; Parker et al., 2005). Similarly, PTSD has been found to be associated with avoidant coping styles (Bryant and Harvey, 1995; Krause et al., 2008) as well as with low cortisol levels, including somewhat low baseline cortisol levels (Meewisse et al., 2007) and enhanced suppression of cortisol to the low dose of DEX (Grossman et al., 2003; Yehuda et al., 1993).

The associations between coping styles and psychological distress, more specifically the significant positive correlation between more frequent use of avoidant coping strategy and greater psychological distress, were in line with previous studies (Goossens et al., 2008; Spira et al., 2004). Taken together, we observed significant relationships between greater distress, avoidant coping style, and blunted cortisol response. A feasible scenario for this relation would be that psychological distress and avoidant coping style result from the failure to mobilize cortisol to adequately cope with stressors. Alternatively, persistent psychological distress and/or avoidant coping style may end up in blunted cortisol reactivity.

The potential mechanism by which the enhanced suppression was related to the psychological distress and avoidant coping style could be discussed as follows. Since the negative feedback by DEX occurs mainly at the level of the pituitary (Cole et al., 2000), the excessively suppressed cortisol response to the DEX/CRH challenge is likely to stem from high sensitivity of pituitary glucocorticoid receptor. In line with this, enhanced negative feedback inhibition at the level of the pituitary caused by the low dose of DEX (0.5 mg) is considered to underlie the enhanced suppression of cortisol and ACTH in PTSD (Yehuda et al., 2004). The present study, using a higher dose of DEX (1.5 mg), was not primarily aimed to test the association of enhanced feedback inhibition by DEX itself with psychological measures, and actually cortisol levels of a considerable portion of our subjects fell under detection limit. A DST with the higher dose of DEX pre-treatment is optimized for the detection of decreased HPA axis feedback sensitivity whereas a DST with the lower dose of DEX is more sensitive for the detection of increased HPA axis feedback sensitivity. Indeed, using a 0.5-mg DST, a number of studies have found individuals with PTSD to display enhanced suppression of cortisol relative to those without PTSD (e.g., Grossman et al., 2003; Yehuda et al., 2004). Nevertheless, the fact that no significant correlational relationships were seen between DST-Cortisol and the psychological measures might indicate that DST is not very sensitive in detecting HPA axis alteration in relation to the psychopathology at a non-clinical level. Instead, the significant associations between the enhanced suppression to the combined DEX/CRH challenge and the unfavorable psychological outcomes may suggest that HPA axis alteration in relation to the psychopathology in healthy populations would be accounted for, at least in part, by the down-regulation of CRH receptors on the level of the pituitary rather than by the enhanced feedback inhibition detectable by the DST. However, to draw any conclusions as to where in the HPA axis the alteration exists, more adequate dose of DEX for pre-treatment should be further explored.

Based on the preclinical and clinical evidence that elevation of AVP occurs as a consequence of chronic stress (De Goeij et al., 1992; Watson et al., 2006a), we measured the post-DEX AVP level to investigate whether the blunted cortisol response would reflect allostatic shift toward elevation of AVP or preexisting vulnerability, as stated earlier. Actually, no significant association was seen between the post-DEX AVP level and psychological measures, suggesting that the relation of blunted cortisol response with several psychological measures would not be attributable to the allostatic shift. Rather, the blunted cortisol response observed here could be considered to relate to preexisting non-clinical psychopathology, including the psychological distress and avoidant coping style. This corresponds well to the evidence that low cortisol levels may be a risk factor for psychopathological conditions, in particular PTSD (Yehuda et al., 2000). Simeon et al. (2007) also demonstrated the positive association between resilience and higher urinary cortisol levels in healthy adults. However, caution should be exercised in accepting this argument because evidence from clinical studies that the peripheral AVP level is elevated after chronic stress has not been sufficient to date.

Findings reported here should be interpreted in the context of a number of limitations. First, since the DEX/CRH test used here was based on a simple test protocol (i.e., measuring hormones only twice and omitting the ACTH measures), it may have provided less information on HPA axis hormones than the standard DEX/CRH test measuring both cortisol and ACTH levels at 5 time points between 1500 h and 1615 h. Moreover, we did not measure baseline levels of cotisol or AVP, i.e., those before the DEX challenge, which precluded us from knowing the extent to which each participant suppressed his/her cortisol and AVP secretions in response to the 1.5 mg of DEX. Second, the criteria for the suppression pattern employed here do not have sufficient empirical basis of the literature; however, the consistency between the *a priori* defined grouping (where cut-off values of cortisol were 1 μg/dL and 5 μg/dL) and the other grouping (where cut-off values of cortisol were 1 μg/dL and 13 μg/dL) in terms of their associations with the psychological measures might justify the grouping criteria. Third, this cross-sectional study cannot provide information as to whether the psychological outcomes assessed with the questionnaires were temporary or prolonged ones, nor can it address the natural history of the alteration in HPA axis function. Fourth, we cannot determine from the peripheral AVP measures alone whether they originated from the parvocellular or magnocellular system of PVN. Fifth, we did not collect data on menstrual cycle in the female participants, which may have affected HPA axis function. Sixth, one might think that there would be some biases in our sampling because none of the 93 female subjects reported that they were on oral contraceptives or hormone replacement therapies at the time of the neuroendocrine test. However, this issue should be considered in the context of considerable ethnic differences in the prevalence of these medications; some data show that approximately 1% and 4% of Japanese women were on low-dose oral contraceptives and hormone replacement therapies, respectively (Katanoda et al., 2003; Matsumoto et al., 2003), while that approximately 35–60% and 20–30% of women in Western countries were on low-dose oral contraceptives and hormone replacement therapies, respectively (Mishra et al., 2006; Tanis et al., 2003; Terry et al., 2002). Therefore, the absence of the use of such medications in the present sample could be attributed to the very low prevalence of these medications in Japan, unlike in most Western countries. Finally, as we did not collect data on the history of childhood trauma or maltreatment, which has been repeatedly reported to lead to HPA axis dysfunction in adulthood (Carpenter et al., 2007, 2009; Heim et al., 2008), some findings of the present study (e.g., the association of avoidant coping strategy with enhanced suppression of cortisol) might be confounded by such a history of early-life adversity. However, even if this is the case, our purpose of investigating the cross-sectional relations between HPA axis function and its psychological correlates in non-clinical adults will not be hampered.

In conclusion, the present study found that enhanced suppression, or blunted response, of cortisol in the DEX/CRH test was associated with greater psychological distress and avoidant coping style in a healthy population. This finding further suggests that impaired ability to mount an adequate cortisol response to pharmacological challenge may serve as a biomarker to define certain psychopathology in a non-clinical population. Such a biomarker might be useful to better understand the etiology of mental disorders and risk for symptom development.

Conflict of interest

All authors declare no conflict of interest.

Contributors

HH and HK conceptualized and designed the study, including the literature searches and analyses. HH, YO, TT, JM, YumK, YukK, SS and HK collected the data. HH performed the neuroendocrine testing, undertook the statistical analyses, and wrote the first draft of the manuscript, under the supervision of HK. ST and TH gave critical comment on the manuscript. All authors contributed to and have approved the final manuscript.

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Possible association of the semaphorin 3D gene (*SEMA3D*) with schizophrenia

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ABSTRACT

Semaphorins are ligands of plexins, and the plexin–semaphorin signaling system is widely involved in many neuronal events including axon guidance, cell migration, axon pruning, and synaptic plasticity. The plexin A2 gene (*PLXNA2*) has been reported to be associated with schizophrenia. This finding prompted us to examine the possible association between the semaphorin 3D gene (*SEMA3D*) and schizophrenia in a Japanese population. We genotyped 9 tagging single nucleotide polymorphisms (SNPs) of *SEMA3D* including a non-synonymous variation, Lys701Gln (rs7800072), in a sample of 506 patients with schizophrenia and 941 healthy control subjects. The Gln701 allele showed a significant protective effect against the development of schizophrenia ($p = 0.0069$, odds ratio = 0.76, 95% confidence interval 0.63 to 0.93). Furthermore, the haplotype-based analyses revealed a significant association. The four-marker analysis (rs2190208–rs1029564–rs17159614–rs12176601), in particular, not including the Lys701Gln, revealed a highly significant association ($p = 0.00001$, global permutation), suggesting that there may be other functional polymorphisms within *SEMA3D*. Our findings provide strong evidence that *SEMA3D* confers susceptibility to schizophrenia, which could contribute to the neurodevelopmental impairments in the disorder.

1. Introduction

The first discovered semaphorin, collapsing-1 (now Sema3A), was originally reported as a repulsive cue in axon guidance (Luo et al., 1993). To date, more than 20 semaphorins of secreted or membrane forms have been identified in various species ranging from nematodes to humans (Luo et al., 1993; Fujii et al., 2002; Yazdani and Terman, 2006). Semaphorins act as ligands for plexins, and the plexin–semaphorin signaling system has been widely investigated in nervous systems (Mann et al., 2007). Class 3 semaphorins (SEMA3A-G) have been well-studied and generally act as secreted ligands for the heterodimerized complex of the plexin A family members and neuropilins (Fujisawa, 2004). For example, Sema3A binds to neuropilin-1 and activates plexin A1 or plexin A2 to transduce a repulsive axon guidance signal (Takahashi and Strittmatter, 2001). Many studies of the plexin–semaphorin signaling system have concentrated on their roles in neuronal development and plasticity (reviewed in (Kruger et al., 2005; Halloran and Wolman, 2006; Waimey and Cheng, 2006; Mann et al., 2007)).

Recently, the relationship between schizophrenia and molecules in the plexin–semaphorin signaling system has begun to receive much attention, for several reasons (Mann et al., 2007). An increase in levels of SEMA3A was noted in the cerebellum in postmortem brains of schizophrenia patients, as measured by immunoreactivity in the inner molecular layer and by the enzyme-linked immunosorbent assay (ELISA) in cerebellar protein extract (Eastwood et al., 2003). A genome-wide association study using 25,494 single nucleotide polymorphisms (SNPs) revealed that an intronic SNP of *PLXNA2* was most consistently associated with schizophrenia in European–American populations (Mah et al., 2006). Our replication study in a Japanese sample failed to confirm such an association (Fujii et al., 2007); however, a meta-analysis combining data from previous studies of *PLXNA2* yielded a positive association with schizophrenia (Allen et al., 2008), in which it was reported that the C allele of the SNP rs752016 of *PLXNA2* showed a nominally significant protective effect (odds ratios (OR) = 0.82, 95%

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confidence interval (CI) = 0.69–0.99), and association of the SNP rs841865 approached statistical significance (OR = 0.84, 95% CI = 0.69–1.01) when samples of Mah et al. and Fujii et al. were combined (Mah et al., 2006; Fujii et al., 2007). Furthermore, in the updated online database, "SchizophreniaGene (http://www.schizophreniaforum.org/)," association of the SNP rs1327175 approached statistical significance (OR = 0.76, 95% CI = 0.57–1.00) (Mah et al., 2006; Fujii et al., 2007; Takeshita et al., 2008; Budel et al., 2008). Therefore, genes of the plexin family, the semaphorin family, and neuropilins, are intriguing candidates for schizophrenia susceptibility genes. We then focused on *SEMA3D* as a candidate gene for schizophrenia. *SEMA3D* was mapped to chromosome 7q21 (Clark et al., 2003); interestingly, a previous genome-wide scan suggested that this chromosomal region contains a susceptibility locus for schizophrenia (Ekelund et al., 2000) and recent studies have provided additional support for this possibility (Tastemir et al., 2006; Wedenoja et al., 2008, 2009; Idol et al., 2008).

The aim of the present study was to examine the possible association between *SEMA3D* and schizophrenia. *SEMA3D* has a common variant in the coding region due to an A to C base substitution (rs7800072), which results in an amino acid change (701 Lys to Gln). This SNP has previously been examined with regard to brain morphology (assessed with magnetic resonance imaging) in patients with schizophrenia (Gregorio et al., 2009). Although this study failed to find significant alterations in brain morphology, it is still unclear whether this SNP confers susceptibility to schizophrenia. We examined the possible association of schizophrenia with this non-synonymous SNP, plus 8 tagging SNPs encompassing the entire *SEMA3D* gene.

2. Subjects and methods

2.1. Subjects

Subjects were 506 patients with schizophrenia (278 males [54.9%], mean age 44.3 years [SD 14.1]) and 941 healthy controls (334 males [35.5%], mean age 44.8 years [SD 16.3]). All subjects were Japanese, biologically unrelated, and recruited from the same geographical area (Western part of Tokyo Metropolitan). Consensus diagnosis by at least two psychiatrists was made for each patient according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) criteria (American Psychiatric Association, 1994) on the basis of unstructured interviews and information from medical records. The controls were healthy volunteers recruited from the same geographical area. Control individuals were interviewed and those who had a current or past history of psychiatric treatment were not enrolled in the study. The study protocol was approved by the ethics committee of the National Center of Neurology and Psychiatry, Japan. After description of the study, written informed consent was obtained from every subject.

2.2. SNP selection

The tagging SNPs were selected using the phase III version of HapMap (http://www.hapmap.org/cgi-perl/gbrowse/). SNP genotype data for the JPT (Japanese in Tokyo, Japan) were downloaded for the genomic region of *SEMA3D* plus 2 kb 5′ and 2 kb 3′ of this region (chr7q21.11). The most centromeric and telomeric HapMap markers downloaded were rs6944966 and rs11762367, respectively. HapMap markers were analyzed using the Haploview 4.1 system (http://www.broad.mit.edu/mpg/haploview) with the following criteria of marker selection: Hardy–Weinberg (HW) p value cutoff: 0.05; minimum genotypes: 90%; maximum number of Mendelian errors: 1; minimum minor allele frequency: 0.1; minimum distance between tags: 10 kb. Tagging SNPs were selected using the Tagger function implemented in Haploview with the following criteria: pairwise tagging only and r^2 threshold 0.8. We preselected rs7800072 and rs6966472 as markers and used the Tagger function implemented in Haploview to select other markers. As a result, 9 markers were selected as suitable for analysis for *SEMA3D*. SNP rs7800072 is non-synonymous (2141A > C, Lys701Gln). The numbers of base and amino acid positions were according to NM_152754.2 and NP_689967.2, respectively.

2.3. Genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to standard procedures. The SNPs were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay; the assay ID (Applied Biosystems, Foster City, CA) of each SNP was C__15937080_10 for rs2190208, C___7585979_10 for rs1029564, C__33462384_10 for rs17159614, C__31373903_10 for rs12176601, C___2635874_10 for rs6966472, C___2635864_10 for rs17559978, C__33462432_10 for rs17159577, C__33462438_10 for rs17159556, and C__25994972_10 for rs7800072. Thermal cycling conditions for polymerase chain reaction (PCR) were 1 cycle at 95 °C for 10 min followed by 50 cycles of 92 °C for 15 s and 60 °C for 1 min. Genotype data were read blind to the case-control status. Ambiguous genotype data were not included in the analysis.

2.4. Haplotype and statistical analysis

Deviations of genotype distributions from the HW equilibrium (HWE) were assessed with the χ^2 test for goodness of fit. Genotype and allele distributions were compared between patients and controls by using the χ^2 test for independence. These tests were performed with SPSS software ver.11 (SPSS Japan, Tokyo, Japan). Haplotype-based association analyses were performed with SNPAlyze software ver.6.5 (http://www.dynacom.co.jp/e/products/package/snpalyze/about.html). The measures of linkage disequilibrium (LD), denoted as D′ and r^2, were calculated from the haplotype frequency using the expectation-maximization (EM) algorithm. Haplotypes with frequencies of less than 1% were considered to be rare and were excluded from the analyses. All p values reported are two-tailed. We performed 100,000 permutations only for some significant haplotypes (e.g., rs2190208–rs1029564–rs17159614–rs121176601) and 10,000 permutations for the other haplotypes. OR and 95% CI were also calculated. To correct the critical p value for multiple testing, we used the spectral decomposition method of SNPSpD software (http://gump.qimr.edu.au/general/daleN/SNPSpD/) (Nyholt, 2004; Li and Ji, 2005), which considers marker linkage disequilibrium information and generates an experiment-wide significance threshold required to keep the type I error rate at 5%.

3. Results

Genotype and allele distributions of the examined SNPs of *SEMA3D* in patients and controls are shown in Table 1. LD estimates of pairwise SNPs, expressed in D′ and r^2, are presented in Fig. 1. The genotype distributions did not significantly deviate from the HWE in patients and controls for any of the examined SNPs. For the non-synonymous polymorphism of *SEMA3D* (rs7800072), there were significant differences in both genotype ($\chi^2 = 8.7$, df = 2, $p = 0.013$) and allele ($\chi^2 = 7.3$, df = 1, $p = 0.0069$, OR = 0.76, 95% CI 0.63–0.93) distributions between patients and controls (Table 1). Furthermore, with respect to the other 8 SNPs (rs2190208, rs1029564,

Table 1
Genotype and Allelic Distribution of the *SEMA3D* SNPs in Japanese Patients with Schizophrenia, and Controls.

dbSNP ID	position[a]	Inter-SNP distance (bp)	Group	N	Genotype distribution (frequency)			Allele distribution (frequency)		Odds ratio (95% CI)	Chi-square test[b]		
											HWE(df = 1)[c]	GF(df = 2)[d]	AF(df = 1)[e]
rs2190208	9986227				GG	GA	AA	G	A				
	5′ promoter	____	Schizophrenia	494	186 (0.38)	231 (0.47)	77 (0.16)	603 (0.61)	385 (0.39)	0.96	$\chi2 = 0.14$, $p = 0.71$	$p = 0.48$	$p = 0.59$
			Control	930	325 (0.35)	466 (0.50)	139 (0.15)	1116 (0.60)	744 (0.40)	(0.82–1.12)	$\chi2 = 1.79$, $p = 0.18$	$\chi2 = 1.48$	$\chi2 = 0.29$
rs1029564	9974131				AA	AC	CC	A	C				
	intron 1	12096	Schizophrenia	492	334(0.68)	140 (0.28)	18 (0.04)	808 (0.82)	176 (0.18)	0.78	$\chi2 = 0.48$, $p = 0.48$	$\mathbf{p = 0.028}$	$\mathbf{p = 0.011}$
			Control	931	565 (0.61)	324 (0.35)	42 (0.05)	1454 (0.78)	408 (0.22)	(0.64–0.94)	$\chi2 = 0.27$, $p = 0.61$	$\chi2 = 7.17$	$\chi2 = 6.40$
rs17159614	9959778				GG	GA	AA	G	A				
	intron 2	14353	Schizophrenia	495	289 (0.58)	181(0.37)	25 (0.05)	759 (0.77)	231 (0.23)	1.00	$\chi2 = 0.24$, $p = 0.62$	$p = 1.00$	$p = 0.96$
			Control	931	545 (0.59)	339 (0.36)	47(0.05)	1429(0.77)	433 (0.23)	(0.83–1.19)	$\chi2 = 0.38$, $p = 0.54$	$\chi2 = 0.0034$	$\chi2 = 0.0023$
rs12176601	9948019				TT	TA	AA	T	A				
	intron 2	11759	Schizophrenia	493	166(0.34)	244 (0.49)	83 (0.17)	576 (0.58)	410 (0.42)	1.21	$\chi2 = 0.17$, $p = 0.68$	$\mathbf{p = 0.029}$	$\mathbf{p = 0.021}$
			Control	917	375 (0.41)	403 (0.44)	139 (0.15)	1153 (0.63)	681 (0.37)	(1.03–1.41)	$\chi2 = 3.16$, $p = 0.08$	$\chi2 = 7.08$	$\chi2 = 5.35$
rs6966472	9933663				AA	AG	GG	A	G				
	intron 4	14356	Schizophrenia	493	381 (0.77)	103 (0.21)	9 (0.02)	865 (0.88)	121 (0.12)	0.73	$\chi2 = 0.43$, $p = 0.51$	$\mathbf{p = 0.023}$	$\mathbf{p = 0.0075}$
			Control	931	656 (0.70)	252 (0.27)	23 (0.02)	1564 (0.84)	298 (0.16)	(0.59–0.92)	$\chi2 = 0.04$, $p = 0.84$	$\chi2 = 7.59$	$\chi2 = 7.16$
rs17559978	9912136				CC	CT	TT	C	T				
	intron 7	21527	Schizophrenia	499	339 (0.68)	138 (0.28)	22 (0.04)	816 (0.82)	182 (0.18)	0.80	$\chi2 = 2.63$, $p = 0.10$	$\mathbf{p = 0.029}$	$\mathbf{p = 0.025}$
			Control	936	571 (0.61)	322 (0.34)	43 (0.05)	1464 (0.78)	408 (0.22)	(0.66–0.97)	$\chi2 = 0.08$, $p = 0.78$	$\chi2 = 7.11$	$\chi2 = 5.05$
rs17159577	9900238				CC	CT	TT	C	T				
	intron 10	11898	Schizophrenia	494	244 (0.49)	195 (0.39)	55 (0.11)	683 (0.69)	305 (0.31)	1.05	$\chi2 = 2.79$, $p = 0.09$	$p = 0.15$	$p = 0.60$
			Control	934	453 (0.49)	403 (0.43)	78 (0.08)	1309 (0.70)	559 (0.30)	(0.88–1.24)	$\chi2 = 0.77$, $p = 0.38$	$\chi2 = 3.78$	$\chi2 = 0.27$
rs17159556	9886562				GG	GT	TT	G	T				
	intron 10	13676	Schizophrenia	496	372 (0.75)	112 (0.23)	12 (0.02)	856(0.86)	136(0.14)	0.76	$\chi2 = 1.03$, $p = 0.31$	$\mathbf{p = 0.024}$	$\mathbf{p = 0.012}$
			Control	932	635 (0.68)	271 (0.29)	26 (0.03)	1541 (0.83)	323(0.17)	(0.61–0.94)	$\chi2 = 0.21$, $p = 0.65$	$\chi2 = 7.43$	$\chi2 = 6.29$
rs7800072	9863265				AA	AC	CC	A	C				
	exon 17	23297	Schizophrenia	502	342 (0.68)	140 (0.28)	20 (0.04)	824 (0.82)	180(0.18)	0.76	$\chi2 = 1.37$, $p = 0.24$	$\mathbf{p = 0.013}$	$\mathbf{p = 0.0069}$
	Lyn701Gln		Control	934	563 (0.60)	327 (0.35)	44 (0.05)	1453(0.78)	415 (0.22)	(0.63–0.93)	$\chi2 = 0.16$, $p = 0.69$	$\chi2 = 8.67$	$\chi2 = 7.31$

[a] Chromosome position was established from the dbSNP database.
[b] Without Bonferroni's correction.
[c] HWE; Hardy–Weinberg equilibrium.
[d] GF; Genotype distribution frequency.
[e] AF; Allele distribution frequency.

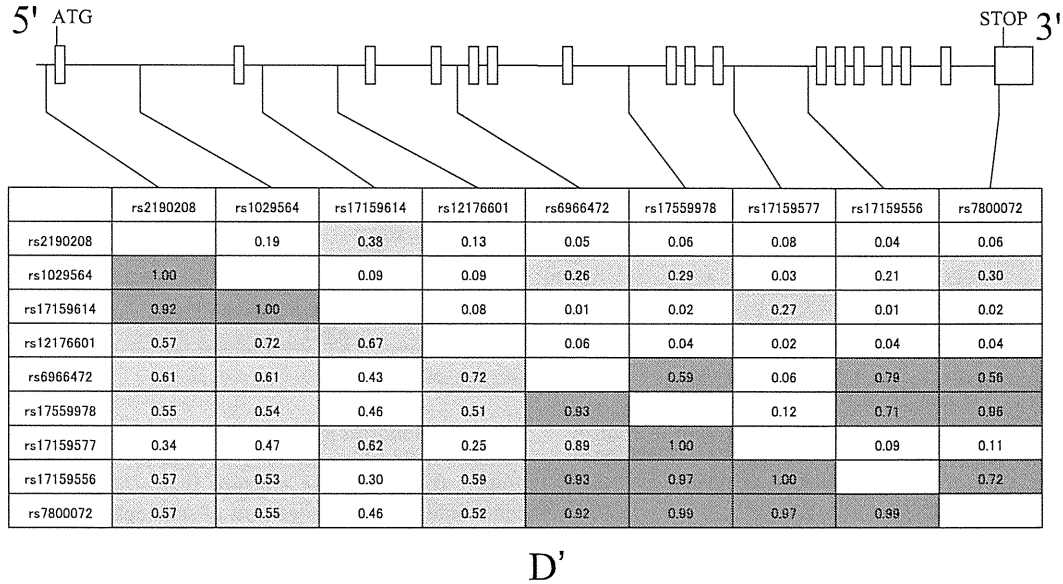

Fig. 1. The genetic structure of *SEMA3D* and location of the examined SNPs. The D′ and r^2 values between paired SNPs are shown in the diagram. The exonic regions are shown as white squares. The intensity of the box color corresponds to the strength of LD or r^2.

rs17159614, rs12176601, rs6966472, rs17559978, rs17159577, and rs17159556), several significant differences in genotype and allele distributions were observed (Table 1). To correct for multiple testing, we calculated the experiment-wide significance threshold required to keep the type I error rate at 5%. As a result, the corrected p value was calculated as 0.0085. The allelic associations with the SNPs rs7800072 (Lys701Gln) and rs6966472 remained significant after the correction (Table 1). Distinguishing between the carriers and the non-carriers with respect to the Gln701 allele for patients and controls, the protective effect became clearer ($p = 0.0033$).

The results of haplotype-based analyses are shown in Table 2. There were significant haplotypic associations of the SNPs in *SEMA3D* when comparing the schizophrenic patients and control subjects. In particular, the four-marker haplotype (rs2190208–rs1029564–rs17159614–rs121176601) showed a statistically significant association with schizophrenia (global permutation $p = 0.00001$). Concerning this haplotype analysis, global p values of 100,000 permutations, which corrected for multiple testing, were also significant. Furthermore, the haplotype frequency of GAGA was significantly higher in schizophrenia patients than in control subjects (0.376 and 0.291, permutation $p = 0.00005$), whereas those of GAGT, AAAA, and GCGA were significantly lower in schizophrenic patients than in controls (0.050 and 0.084, permutation $p = 0.0029$; 0.007 and 0.025, permutation $p = 0.0062$; 0.007 and 0.021, permutation $p = 0.020$, respectively) (Table 3).

When we performed stratified analysis of the data for rs7800072 by sex, a significant association was observed in women ($p = 0.0089$), but not in men ($p = 0.41$) (supplementary Tables 1 and 2). In the haplotype analysis, on the other hand, the four-marker haplotype (rs2190208–rs1029564–rs17159614–rs121176601) showed a statistically significant association in men (global permutation $p = 0.00001$), but was at a trend level in women (global permutation $p = 0.0699$). The haplotype frequency of GAGA

Table 2
Associations with schizophrenia of the 9 SNPs and haplotypes in *SEMA3D*.

SNP No.	dbSNP ID	Allele model	Haplotype p[a]							
		p value	2 Locus	3 Locus	4 Locus	5 Locus	6 Locus	7 Locus	8 Locus	9 Locus
SNP1	rs2190208	0.59								
			0.019							
SNP2	rs1029564	**0.011**		0.10						
			0.029		**0.00001**					
SNP3	rs17159614	0.96		**0.00002**		**0.00005**				
			0.0004		**0.0003**		**0.00007**			
SNP4	rs12176601	**0.021**		**0.0010**		**0.0001**		**0.0003**		
			0.035		**0.0006**		**0.0016**		**0.0007**	
SNP5	rs6966472	**0.0075**		0.053		**0.0004**		**0.0001**		**0.0007**
			0.023		0.098		**0.0001**		**0.0001**	
SNP6	rs17559978	**0.025**		**0.022**		0.061		**0.0004**		
			0.030		**0.025**		0.076			
SNP7	rs17159577	0.60		0.064		**0.024**				
			0.042		0.051					
SNP8	rs17159556	**0.012**		**0.028**						
			0.020							
SNP9	rs7800072	**0.0069**								

[a] global p value.

Table 3
Estimated haplotype frequencies and association significance for *SEMA3D*.

Haplotype	rs2190208	rs1029564	rs17159614	rs12176601	% of individuals					
					Overall	Control	Schizophrenia	$\chi 2$	*p value*	Permutation *p* value
1	G	A	G	A	0.321	0.291	0.376	20.40	**0.0000063**	**0.000050**
2	A	A	A	T	0.207	0.201	0.219	1.21	0.27	0.28
3	G	C	G	T	0.190	0.199	0.172	2.98	0.085	0.089
4	A	A	G	T	0.142	0.143	0.139	0.10	0.75	0.76
5	G	A	G	T	0.072	0.084	0.050	11.23	**0.00080**	**0.0029**
6	A	A	G	A	0.034	0.036	0.031	0.37	0.54	0.59
7	A	A	A	A	0.019	0.025	0.007	10.75	**0.0010**	**0.0062**
8	G	C	G	A	0.016	0.021	0.007	7.55	**0.0060**	**0.020**

	$\chi 2$	*p value*	Permutation *p* value	Replications
Global	46.07	**0.000000085**	**0.00001**	100000

was significantly higher in schizophrenia patients than in control subjects in both men (0.368 and 0.272, permutation $p = 0.00053$) and women (0.384 and 0.302, permutation $p = 0.003$).

4. Discussion

Our results provide the first evidence for the possible involvement of *SEMA3D* in the pathogenesis of schizophrenia. With respect to the non-synonymous (Lys701Gln) polymorphism, we found a significant preponderance of the Lys/Lys genotype and the Lys701 allele in schizophrenia patients compared with control subjects. In the haplotype-based analyses, we also obtained evidence for an association between *SEMA3D* and schizophrenia. Interestingly, the most significant haplotype, rs2190208–rs1029564–rs17159614–rs121176601, does not include rs7800072 (Lys701Gln) (see Fig. 1). Therefore, it is likely that at least one functional polymorphism other than rs7800072, which is in linkage disequilibrium to the haplotype, could be responsible for susceptibility to schizophrenia. In stratified analysis for rs7800072 by sex, the frequency of the Gln701 allele was significantly lower in schizophrenia patients than in control subjects in women (0.17 and 0.23, $p = 0.0088$) (supplementary Table 2). Likewise, this was also lower in men, but was not statistically significant (0.18 and 0.20, $p = 0.41$) (supplementary Table 1). Regarding analysis of the four-marker haplotype (rs2190208–rs1029564–rs17159614–rs121176601), there remained a statistical significance in men (global permutation $p = 0.00001$) and a tendency in women (global permutation $p = 0.0699$). In addition, the frequency of the most major haplotype (GAGA) was significantly higher in schizophrenia patients than in control subjects in both sexes. These inconsistent results between males and females are likely to have arisen from the lack of statistical power after dividing the sexes.

The neurodevelopmental hypothesis of schizophrenia proposes that abnormalities of brain development are involved in the pathogenesis of schizophrenia (Conrad and Scheibel, 1987; Weinberger, 1987; Murray, 1994; Waddington et al., 1998). In early brain developmental stages, a number of semaphorins play important roles in axonal repulsion, axonal attraction, neuronal cell migration, and axon pruning (reviewed in Kruger et al., 2005; Waimey and Cheng, 2006; Halloran and Wolman, 2006; Mann et al., 2007). Indeed, SEMA3D has been shown to act in axon guidance and cell migration during neuronal development (Wolman et al., 2004, 2007; Liu et al., 2004; Liu and Halloran, 2005; Sakai and Halloran, 2006; Takahashi et al., 2009). With respect to neuronal cell migration, neuronal disarray and abnormal migration in the neocortical white matter were reported in postmortem studies of patients with schizophrenia (Jakob and Beckmann, 1986; Akbarian et al., 1993). Regarding pruning, Feinberg proposed that schizophrenia may arise from excessive synaptic pruning during adolescence (Feinberg, 1982; Keshavan et al., 1994). Indeed, decreased density of dendritic spines was observed in the prefrontal cortex of patients with schizophrenia (Garey et al., 1998; Glantz and Lewis, 2000). These findings suggest that variants of *SEMA3D* may contribute to the pathogenesis of schizophrenia through affecting development of neural networks. The genotypic difference based on the Lys701Gln polymorphism of *SEMA3D* might lead to developmental differences in the brain; the Gln701 carriers would exhibit intrinsically greater protective effects against the development of schizophrenia than the Gln701 non-carriers. Although *SEMA3D* has not yet been well-studied, *SEMA3A* has been investigated in detail. In particular, an increase in the expression of SEMA3A has previously been associated with schizophrenia (Eastwood et al., 2003). Moreover, *PLXNA2*, which encodes one of the receptors for class 3 semaphorins, was identified as a candidate gene for schizophrenia in a genome-wide association study (Mah et al., 2006). Currently, this association is also supported by the meta-analysis of Allen et al. (2008). SEMA3A and SEMA3D belong to the same class and share the most similarity with each other of the class 3 semaphorin genes (Luo et al., 1995). These findings further strengthen the evidence for a possible role of *SEMA3D* in the development of schizophrenia.

It is possible that the amino acid change (Lys701Gln) may affect the function of SEMA3D protein and that this results in susceptibility to schizophrenia. Indeed, this is a substitution from a large and basic amino acid (Lys) to a medium-sized and polar one (Gln). This is likely to lead to functional differences between the two types of SEMA3D. One possibility is that this substitution might result in conformational change of SEMA3D and influence its affinity for its receptors. Another possibility is that the Lys701 and Gln701 variants of SEMA3D have different cellular localization. The basic domain of class 3 semaphorins electrostatically interacts with the proteoglycan components of the extracellular matrix (De Wit et al., 2005) and the granule matrix (de Wit et al., 2009). The substitution from the basic Lys701 to the non-basic Gln701 may affect such interactions between SEMA3D and these matrices. Alteration of the extracellular matrix may modify distribution of SEMA3D in neurons, and that of the granule matrix may affect secretion from secretory vesicles. The class 3 semaphorins not only act as axon guidance cues but also have key roles in synaptic formation and function. Therefore, these modified interactions could impact on the establishment of synaptic contacts and the formation of new synapses. Although the amino acid substitution (Lys701Gln) was predicted to be benign by Polyphen (http://genetics.bwh.harvard.edu/pph/) and SIFT (http://sift.jcvi.org/) programs, its actual effects should be elucidated by cell biological or biochemical approaches.

Accumulating evidence suggests that the semaphorins are regulatory factors of tumor progression and modulators of angiogenesis (reviewed in (Neufeld and Kessler, 2008) and (Capparuccia and Tamagnone, 2009)). Recently, SEMA3D was also reported to

possess anti-tumorigenic and anti-angiogenic properties (Kigel et al., 2008). The hypoactivity of *SEMA3D* could be linked to increased incidence of cancer. Previous studies and reviews have partially supported the idea that the incidence of cancer in patients with schizophrenia is reduced compared with the general population (Grinshpoon et al., 2005; Dalton et al., 2005; Catts et al., 2008). It is possible that semaphorins are related to the development of schizophrenia and also contribute to the associated lower incidence of cancer, and this topic warrants further investigation.

In conclusion, we found a significant association between the Lys701Gln polymorphism of *SEMA3D* and schizophrenia. In addition, the haplotype rs2190208–rs1029564–rs17159614–rs121176601, not including the Lys701Gln variant, was shown to be associated with schizophrenia, which suggests that some other polymorphisms of *SEMA3D* play a role in the pathogenesis of schizophrenia. Taking the previous molecular and developmental findings together with the present genetic findings, *SEMA3D* appears to be a promising candidate gene related to susceptibility to schizophrenia.

Conflict of interest

All authors declare no conflict of interest that could influence their work.

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Contributors

T.F. designed the study, performed genotyping of *SEMA3D*, made statistical analysis, managed literature search, interpreted the data, and wrote the manuscript. H.U. and N.Y. took part in genotyping. H.H., M.T., M.I., K.A., and T.H. collected samples and gave comments to the manuscript. H.K. organized recruitment and genotyping of schizophrenic patients and control subjects, and took part in analyzing the data and writing the manuscript.

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Appendix. Supplementary data

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