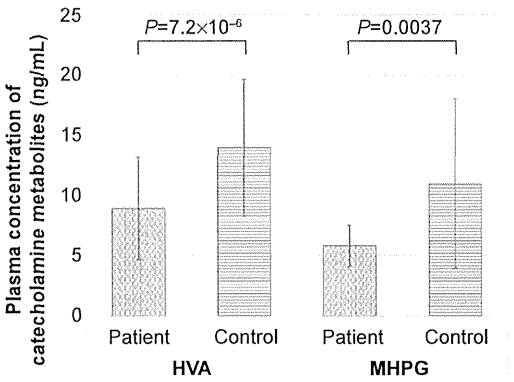

Figure 2 Baseline plasma levels of catecholamine metabolites in the patients with major depressive disorder and the healthy control subjects.
Note: Data are presented as mean ± standard deviation.
Abbreviations: HVA, homovanillic acid; MHPG, 3-methoxy-4-hydroxyphenylglycol.

changes in MHPG or HVA plasma levels. In addition, there was no correlation between the changes in MHPG or HVA plasma levels and the changes in the sub-item scores on the HAMD17. Table 2 provides the COMT Val158Met genotype distributions and the χ^2 and *P*-values for Hardy–Weinberg equilibrium. The demographic data for the patients with MDD did not differ between those with the Val/Val genotype and the Met carriers (Table 3). The baseline plasma levels of MHPG, but not HVA, were lower in the patients with MDD who had the Val/Val genotype than in patients with MDD who were Met carriers (Figure 3). We also compared the MDD patients' baseline scores on the HAMD17 subcategories according to the method of Seretti et al.[13] No differences were detected between the Val/Val group and the Met-carrier group (Table 4).

Next, we investigated the changes in plasma catecholamine metabolites between the two MDD groups. Only patients with MDD and the Val/Val genotype (not Met carriers) demonstrated increased MHPG plasma levels, which were similar to the healthy control subjects after 8 weeks of duloxetine treatment. The HVA plasma levels did not change after 8 weeks of duloxetine treatment for either the Val/Val group or the Met-carrier group (Figure 4).

The baseline plasma HVA levels were significantly higher in the healthy control subjects than in the MDD group, regardless of the COMT Val158Met genotype (Val/Val versus Met carriers). Conversely, the baseline plasma MHPG levels in the healthy control subjects were significantly higher than those in the MDD patients only among subjects with the Val/Val genotype. For Met carriers, the baseline plasma MHPG levels did not differ between healthy control subjects and the patients with MDD (Figure 5A). The plasma MHPG levels increased among the subjects in the MDD group with the Val/Val genotype after 8 weeks of duloxetine treatment and were similar to those of the healthy control subjects with the Val/Val genotype. No other groups demonstrated changes in the levels of plasma catecholamine metabolites between baseline and the end of duloxetine treatment (8 weeks) (Figure 5B). However, there was no significant correlation between the changes in the total HAMD17 scores and the changes in MHPG or HVA plasma levels in the patients with MDD. In addition, there was no correlation between the changes in MHPG or HVA plasma levels and the changes in the HAMD17 sub-item scores in the patients with MDD.

Discussion

This is the first study to investigate the response to duloxetine, the plasma levels of catecholamine metabolites, and the COMT Val158Met polymorphism in Japanese patients with MDD. There was no difference in the response to 8 weeks of duloxetine treatment between the Val/Val group and the Met-carrier group, which suggests that the COMT Val158Met polymorphism is not associated with duloxetine treatment response.

Depression is a common and disabling psychiatric disorder with a complex etiology that includes predisposing risk genes and environmental stressors. Variations in the COMT gene, particularly the Val158Met polymorphism, have been extensively investigated for associations with the clinical phenotypes of depression. Similarly, neurocognitive processes have been examined to evaluate the impact of COMT variants on phenotypes relevant to depression. We observed that clinical phenotypes, such as depression severity and diagnosis, or behavioral endpoints, were less reliably associated with COMT genetic variations. We recently reported that duloxetine

Table 2 Hardy–Weinberg equilibrium of the patients with major depressive disorder and the healthy control subjects

	Genotypes			χ^2	*P*-value	Allele frequency	
	Val/Val	Val/Met	Met/Met			Val	Met
Patients	19 (42.2%)	25 (55.6%)	1 (2.2%)	0.104	0.949	63 (70.0%)	27 (30.0%)
Controls	15 (50.0%)	11 (36.6%)	4 (13.3%)	0.076	0.963	41 (68.3%)	19 (31.7%)

Abbreviations: Val, valine; Met, methionine.

Table 3 The demographic data of all patients with major depressive disorder and respective COMT Val158Met genotypes

	All patients (N=45)	Val/Val (N=19)	Met carrier (N=26)	*P*-value
Age (years)	50.6±15.3	48.6±15.7	52.1±15.1	0.455*
Sex (male/female) (n)	23/22	10/9	13/13	0.861**
DLX max dose (mg/day)	49.3±12.3	50.0±12.9	48.9±12.1	0.760*
Depressive episodes before DLX treatment	1.09±1.70	0.68±1.25	1.39±1.94	0.176*
Baseline HAMD17	21.2±4.9	21.9±5.5	20.7±4.4	0.437*
After 8 weeks HAMD17 score	9.0±5.4	8.3±4.4	9.5±6.1	0.489*
Delta HAMD17 score (0w–8w)	12.2±6.1	13.6±6.2	11.3±5.9	0.212*
Response rate (%)	66.7	68.4	65.4	0.831**

Notes: *P*-values calculated using *Student's *t*-test, **χ^2 test. Data are presented as mean ± standard deviation unless otherwise stated.
Abbreviations: DLX, duloxetine; HAMD17, 17-item Hamilton Rating Scale for Depression; COMT, catechol-O-methyltransferase; Val, valine; Met, methionine; w, weeks.

increased the plasma levels of MHPG among responders (but not nonresponders) in a sample of Japanese patients with MDD.[3] The potent inhibition of the noradrenaline transporter (NAT) by duloxetine is consistent with the increase in plasma MHPG levels in patients with MDD. MDD is a heterogeneous disorder; thus, patients' plasma levels of MHPG and HVA vary widely.[2] Indeed, some studies have reported the plasma levels of MHPG and HVA to be higher in depressed patients than in normal controls, whereas others have shown these levels to be lower in depressed patients than in normal controls.[14]

Considering these findings, it is possible that duloxetine strongly affects noradrenergic neurons in patients with MDD. Noradrenaline is mainly associated with attention, activity, concentration, and pain. Duloxetine might improve MDD symptoms associated with these factors by enhancing noradrenergic neuronal activity. However, no correlation was observed between the changes in plasma MHPG levels and the changes in the HAMD17 sub-item scores. In contrast, Chalon et al[15] reported that duloxetine administration in healthy volunteers for 5 or 6 days significantly decreased urinary MHPG levels. The source of the discrepancy between the results of our study and the results of Chalon et al[15] remains unknown, although differences in subjects and/or sampling points could be major reasons. Although a 1-week washout period was utilized in the current study, some of the patients had been administered SSRIs before duloxetine treatment was initiated. However, duloxetine did not alter the plasma HVA levels of the patients in our study.

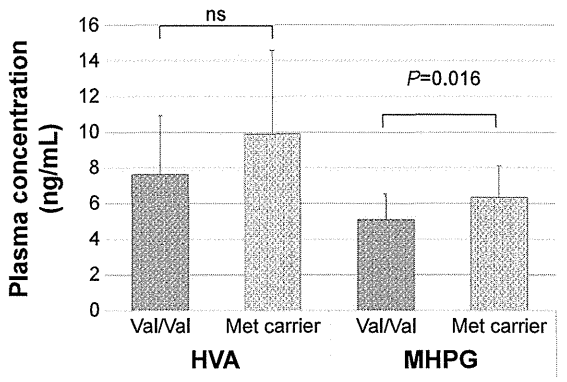

Figure 3 Baseline plasma levels of HVA and MHPG according to the COMT Val158Met genotype in the patients with major depressive disorder.
Note: Data are presented as mean ± standard deviation.
Abbreviations: HVA, homovanillic acid; MHPG, 3-methoxy-4-hydroxyphenylglycol; COMT, catechol-O-methyltransferase; Val, valine; Met, methionine; ns, not significant.

Responses to venlafaxine, another SNRI, in patients with MDD were stratified by COMT genotype (Val158Met) in a previous randomized, double-blind, placebo-controlled clinical trial. Improvements in the depression scores of subjects with the Val/Val genotype were larger than those of patients with the Met/Met genotype, suggesting that venlafaxine may alter noradrenergic flux differentially according to COMT activity.[16] Several previous biochemical, pharmacological, and genetic studies have implicated the COMT gene in the pathogenesis and pharmacological treatment of affective disorders. According to a review by Antypa et al[17] the majority of studies have reported that Met carriers exhibit a better response to SSRIs, SNRIs, tricyclic antidepressants, or mirtazapine than Val/Val carriers. In the present study, no difference in the response to duloxetine was found between the Val/Val group and the Met-carrier group. However, it is not yet possible to form definitive conclusions regarding the COMT Val158Met polymorphism and the response to antidepressants. It is possible that COMT plays a different role in the response to each category of antidepressant (eg, SSRIs, SNRIs, tricyclic antidepressants).

The results of the current study should be interpreted with caution. We cautiously conclude that the plasma MHPG levels of MDD patients after duloxetine treatment are higher in those with the Val/Val genotype than in Met carriers.

Table 4 Comparison of the COMT Val158Met genotype with the subcategories of HAMD17 scores according to the method of Seretti et al[13] at baseline for the patients with major depressive disorder

Seretti et al's category (modified)	Subcategories of HAMD17	Val/Val	Met carrier	*P*-value
Core symptom	1. depressed mood, 2. feelings of guilt, 7. work and activities, 8. retardation: psychomotor, 10. anxiety (psychological), 13. somatic symptom general	11.32±2.83	10.58±2.14	0.323
Sleep	4. insomnia early, 5. insomnia middle, 6. insomnia late	2.89±0.46	2.96±1.59	0.840
Activity	7. work and activities, 8. retardation: psychomotor	4.05±1.39	4.27±1.58	0.637
Psychic anxiety	9. agitation, 10. anxiety (psychological)	2.68±1.34	2.31±1.16	0.318
Somatic anxiety	11. anxiety somatic, 12. somatic symptoms (gastrointestinal), 13. somatic symptoms general	3.74±1.59	3.81±1.16	0.866
Delusion	2. feelings of guilt, 15. hypochondriasis	2.68±1.34	2.35±1.06	0.348

Notes: *P*-values calculated using Students *t*-test. Data are presented as mean ± standard deviation.
Abbreviation: HAMD17, 17-item Hamilton Rating Scale for Depression; COMT, catechol-*O*-methyltransferase; Val, valine; Met, methionine.

However, this finding is independent of improvements in clinical symptoms.

There are several limitations to the current study. First, this study was an open-label trial with a 1-week washout period. Second, the sample was small and heterogeneous, and it included subjects with both moderate and severe depression. Third, the dropout rate was high. Fourth, the plasma levels of MHPG and HVA are considered to reflect only 30%–50% and 10%–20% of the brain dynamics, respectively.[18] Fifth, the 1-week washout period (for former antidepressant therapy) was not sufficiently long. Thus, future studies should be performed to confirm the results of this preliminary study.

Not all of our hypotheses were confirmed. The only confirmed hypothesis was that patients with MDD and the Val/Val genotype had lower plasma MHPG levels than patients with MDD who were Met carriers. In other words, we found that 1) patients with MDD and the COMT Val/Val genotype did not respond better to duloxetine treatment than patients who were COMT Met carriers; 2) patients with MDD and the COMT Val/Val genotype had lower plasma MHPG levels than patients who were COMT Met carriers (Figure 3); and 3) patients with MDD and the COMT Val/Val genotype demonstrated significant increase in MHPG plasma levels compared to COMT Met carriers (Figure 4). The most important finding of the current study was that

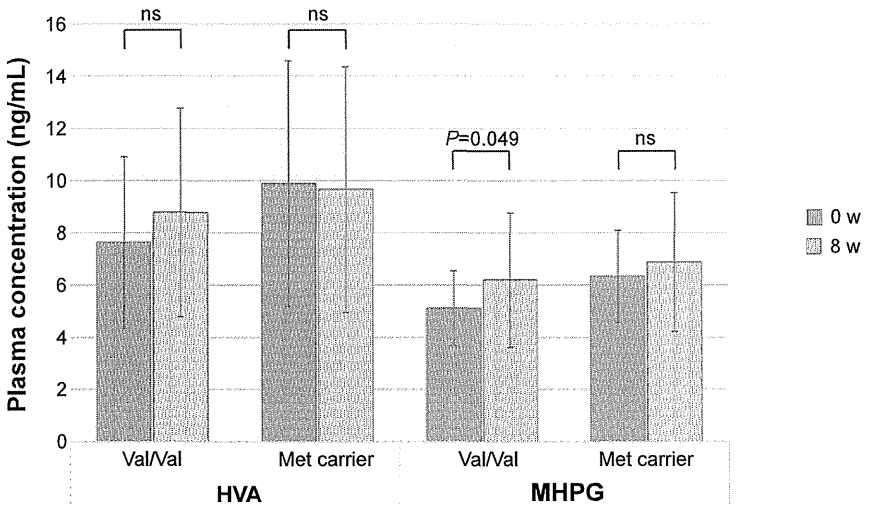

Figure 4 The plasma levels of HVA and MHPG according to the COMT Val158Met genotype at baseline and after 8 weeks of duloxetine treatment.
Note: Data are presented as mean ± standard deviation.
Abbreviations: HVA, homovanillic acid; MHPG, 3-methoxy-4-hydroxyphenylglycol; COMT, catechol-*O*-methyltransferase; Val, valine; Met, methionine; w, weeks; ns, not significant.

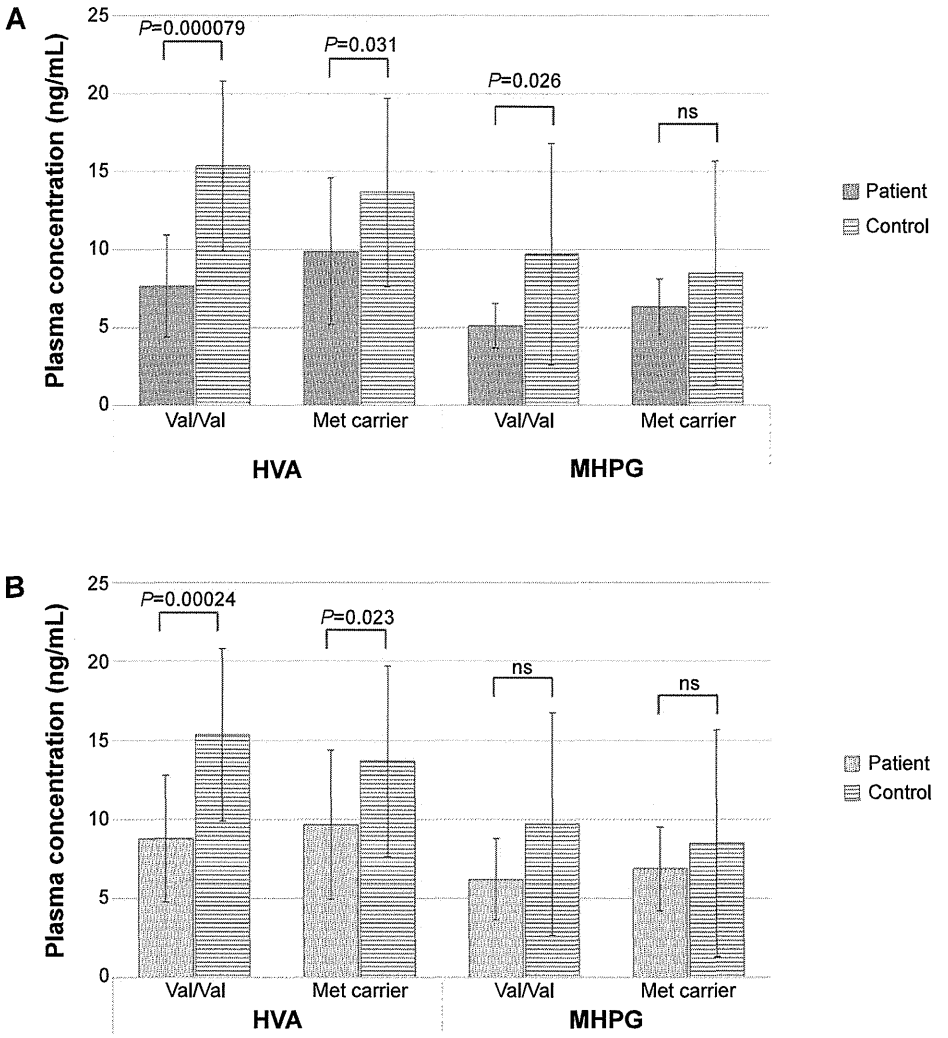

Figure 5 The plasma levels of HVA and MHPG according to the COMT Val158Met genotype in the patients with major depressive disorder and the healthy control subjects.
Notes: (**A**) At baseline. (**B**) After 8 weeks of treatment. Data are presented as mean ± standard deviation.
Abbreviations: HVA, homovanillic acid; MHPG, 3-methoxy-4-hydroxyphenylglycol; COMT, catechol-O-methyltransferase; Val, valine; Met, methionine; ns, not significant.

patients with MDD and the Val/Val genotype recovered to the same plasma MHPG levels as the healthy control subjects when treated with duloxetine. However, this improvement occurred independently of clinical improvement.

Conclusion

The plasma MHPG levels in patients with MDD and the Val/Val genotype were lower than those in patients with MDD who were Met carriers. The MHPG levels in the former group significantly increased (to healthy control levels) after duloxetine treatment. Our results suggest that patients with MDD and the Val/Val genotype are more sensitive to the influence of duloxetine treatment on noradrenergic neurons.

Acknowledgments

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Disclosure

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The potential of SLC6A4 gene methylation analysis for the diagnosis and treatment of major depression

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ABSTRACT

We examined the utility of DNA methylation profiles at the CpG island of SLC6A4 (DMS) as a diagnostic biomarker for major depression (MD). In addition, the relationship between DMS and the serotonin transporter gene-linked polymorphic region (5-HTTLPR) allele, the severity of symptoms, number of early adversities, and therapeutic responses to antidepressants were examined. Genomic DNA was extracted from peripheral blood of Japanese healthy controls and patients with MD before and after treatment. DMS was analyzed using a MassARRAY Compact System. The severity of depression was evaluated using the Hamilton Rating Scale for Depression, and early adversity was evaluated using the Early Trauma Inventory. We were unable to distinguish between and healthy controls, or between unmedicated patients and medicated patients using DMS. The 5-HTTLPR allele had no significant effect on DMS. The methylation rates for several CpGs differed significantly after treatment. Notably, the methylation rate of CpG 3 in patients with better therapeutic responses was significantly higher than that in patients with poorer responses. Although further studies examining the function of specific CpG units of SLC6A4 are required, these results suggest that the pre-treatment methylation rate of SLC6A4 is associated with therapeutic responses to antidepressants in unmedicated patients with MD.

1. Introduction

Major depression (MD) is a common illness worldwide and was the second-leading contributor to global disease burden in 2010 (Vos et al., 2012). Since long-lasting emotional and psychomotor disturbances due to MD can induce functional difficulties at work, school, or home, MD is predicted to become the second-leading cause of disability-adjusted life years in 2020 based on the systematic analyses of population health data (Murray and Lopez, 1997). In addition, MD is a leading cause of suicide and is responsible for 1 million suicide-related deaths every year (WHO, 2012). In this context, early diagnosis and intervention are necessary to prevent worsening of MD and MD-related suicide attempts. However, the diagnostic system for MD, which relies on subjective assessment of patient symptoms using, for example, the Diagnostic and Statistical Manual of Mental Disorders Fourth Edition (DSM-IV) or the International Classification of Diseases Tenth Edition (ICD-10) rather than an objective laboratory test, may be associated with misdiagnoses, poor outcomes in the treatment of MD, and development of refractory depression.

To establish objective diagnostic biomarkers for MD, numerous approaches have been undertaken. For example, Carroll et al. (1981) and Carroll (1982) proposed dexamethasone suppression

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test (DST) results as a biomarker for the diagnosis of melancholia based on dysregulation of the hypothalamo–pituitary–adrenal (HPA) axis in MD, but a major drawback of DST was its modest sensitivity. Subsequently, a refined laboratory test, the combined dexamethasone/corticotrophin-releasing hormone (DEX/CRH) test, was developed (Heuser et al., 1994), although the sensitivity for detection of MD was only 80% at best (Mossner et al., 2007).

We recently proposed that the methylation profile of the promoter region of exon I of the brain-derived neurotrophic factor (BDNF) gene could serve as a valuable diagnostic biomarker for MD (Fuchikami et al., 2011). It is well known that the concordance rate for MD in monozygotic twins is almost 40% (Fu et al., 2002; McGuffin et al., 1996; Sullivan et al., 2000). The heritability of MD is lower than that of bipolar disorder or schizophrenia (Goodwin and Jamison, 2007; Moldin and Gottesman, 1997). These epidemiological studies suggest that gene–environment interactions play a pivotal role in the etiology of MD. With regard to this interaction, growing evidence indicates that the regulation of DNA methylation in response to environmental stimuli plays an important role in the development of stress vulnerability, predisposing to MD under stressful situations (McGowan et al., 2009; Weaver et al., 2004; Zhang et al., 2013). Thus, it is plausible that epigenetic factors could be used for the development of a more sophisticated diagnosis system for MD.

The serotonin transporter (5-HTT) is a major target of antidepressants and the activity of 5-HTT is inhibited by different types of antidepressants. In vivo neuroimaging and postmortem histochemical studies have shown decreased 5-HTT binding density mainly in the prefrontal cortex of patients with MD (Stockmeier, 2003). Whereas a recent in vivo neuroimaging study using positron emission tomography (PET) demonstrated increased 5-HTT binding in the thalamus, insula, and striatum of patients with MD (Cannon et al., 2007), another PET study reported lower 5-HTT binding in the midbrain of patients with MD, particularly those who were unmedicated (Parsey et al., 2006). Based on these findings, it is conceivable that altered 5-HTT function in the brain plays an important role in the pathophysiology of depression.

Furthermore, it is well known that the short (*s*) allele of the 5-HTT gene-linked polymorphic region (5-HTTLPR: 43-bp deletion or insertion in the promoter of exon I) is associated with anxiety-related personality traits (Lesch et al., 1996), and that this polymorphism is associated with decreased expression of the 5-HTT gene in the 5-HTTLPR *s* allele (Bradley et al., 2005). The 5-HTTLPR *s* allele has been reported to be associated with an increased risk of developing depression under stress (Karg et al., 2011).

A recent meta-analysis revealed a significant association between 5-HTTLPR and the clinical response to selective serotonin reuptake inhibitor (SSRI) treatment in terms of both the remission rate and response rate in depressed patients. This suggests that 5-HTTLPR could be a predictor of the response to SSRIs (Serretti et al., 2007). Together, these observations indicate that individual differences in the transcriptional activity of 5-HTT might be involved in the pathophysiology of MD and the response to antidepressant treatment.

With regard to the regulation of 5-HTT expression, the methylation rate of the CpG island at the 5′ region of the SLC6A4 gene have been reported to be associated with the levels of 5-HTT mRNA in human lymphoblast cells (Philibert et al., 2007). In addition, Wang and associates (Wang et al., 2012) reported that in vitro methylation in the promoter of exon I of the SLC6A4 gene in a luciferase-reporter construct suppressed its transcriptional activity. Furthermore, the possibility of a joint effect of 5-HTT methylation and 5-HTTLPR *s* allele carriage on the risk for depression was reported recently (Olsson et al., 2010). These findings suggest that the methylation status of the promoter of exon I of the SLC6A4 gene alters transcription of the SLC6A4 gene, and subsequently leads to the occurrence of MD.

It has also been reported that early adversity is associated with increased prevalence, earlier onset (Widom et al., 2007) or severity of symptoms, treatment resistance of MD (Tunnard et al., 2013; Widom et al., 2007), and elevated DNA methylation across the SLC6A4 gene promoter in subjects without MD as well as in those without psychiatric disorders (Beach et al., 2010, 2011). Furthermore, increased DNA methylation of the SLC6A4 gene has been reported to be associated with bullying victimization in childhood (Ouellet-Morin et al., 2013).

In the present study, we assessed the degree of symptoms and early adversity of patients with MD, analyzed DNA methylation rates of the CpG island in the promoter of exon I of the SLC6A4 gene (DMR), and conducted genotyping of 5-HTTLPR using genomic DNA from the peripheral blood of patients with MD or healthy controls.

First, we examined whether the profile of the SLC6A4 gene was an appropriate diagnostic biomarker for MD. Next, we analyzed the effect of genotype on DMR, the relationship between DMR and the severity of symptoms, and the association between DMR and the number of early adversities. Finally, the effect of antidepressant treatment on DMR and the relationship between DMR and response rates was analyzed by comparing data sets from patients with MD before and after antidepressant treatment to evaluate the potential of DMR as a predictor of the treatment response.

2. Materials and methods

2.1. Subjects

Fifty patients with MD and 50 healthy controls participated in this study. Demographic characteristics of the participants are shown in Table 1. All participants were Japanese. All patients were diagnosed by trained psychiatrists according to DSM-IV criteria, on the basis of unstructured interviews, information from medical records, and the use of a structured clinical interview (the Japanese version of the Mini-International Neuropsychiatric Interview) by a research psychiatrist. The criteria for the selection of samples in this study was as follows: (1) median age (21–62 years old), (2) distribution of sex was almost equal among groups, (3) severity of symptoms were moderate so as to be able to give written informed consent, (4) all patients did not have treatment history or previous depressed episodes. The severity of depression was evaluated using the Hamilton Rating Scale for Depression (HAM-D; Hamilton, 1967; Cusin et al., 2009) and early adversity was evaluated using the Early Trauma Inventory Self Report-Short Form (ETISR-SF; Bremner et al., 2000, 2007).

None of the patients had current or past diagnoses of substance-related disorders or physical diagnoses. Healthy controls were recruited by advertisement. They had no current or past psychiatric or physical diagnoses, and they had no first-degree relatives with MD. Blood samples were collected at Hiroshima University Hospital, Hokkaido University Hospital, Oita University Hospital and Showa University Hospital. Medical treatment was initiated in 50 patients; 40 patients were available for a follow-up interview 6 weeks later, at which time additional blood samples were collected.

This study was approved by the respective ethics committees of Hiroshima University School of Medicine, Hokkaido University School of Medicine, Oita University School of Medicine, and Showa University School of Medicine. All subjects received a description of the study and gave written informed consent.

Table 1
Demographic characteristics of subjects.

Group	Age (years) (mean ± S.D.)	Genotype	HAM-D score (mean ± S.D.)		ETI score (mean ± S.D.)	IR (%) (mean ± S.D.)
			Before	After 6 weeks		
Control (N = 50, 27M/23F)	40.3 ± 10.5	LL: 2, SL: 16, SS: 32				
Major depression (N = 50, 27M/23F)	40.3 ± 10.3	SL: 16, SS: 34	20.6 ± 4.8	8.1 ± 6.6	5.0 ± 3.8	60.2 ± 31.3

M: males, F: females, ETI : Early Trauma Inventory, IR : improvement ratio.
HAM-D: Hamilton Rating Scale for Depression.

2.2. Selection of genomic regions of the SLC6A4 gene for DNA methylation analysis

We chose the CpG island of the SLC6A4 gene as a target for analysis. The target region around the SLC6A4 gene was selected based on a previous report (Olsson et al., 2010). The sequence of the CpG island was identified using the UCSC genome database (http://genome.ucsc.edu/) (chr 17: 28562388–28563186). PCR primers were designed using Epidesigner software (http://www.epidesigner.com/). The schema for the target region used for analysis and the primers used for PCR are shown in Fig. 1.

2.3. Genotyping to generate S- and L-fragments

The sequence of 5-HTTLPR was identified using the UCSC genome database (chr 17: 28564123–28564473) (Fig. 1). PCR primers were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3/input.htm). PCR was performed in a total volume of 5 μL solution containing 10 ng DNA, 2 × Gflex® PCR Buffer, 1 mM $MgCl_2$, 200 μM dNTPs, 0.2 μM of each primer, and 0.125 U of Tks Gflex® DNA polymerase (Takara, Tokyo, Japan) for 30 cycles (98 °C for 10 s, 60 °C for 15 s, and 68 °C for 30 s). The PCR products were then analyzed by 2% agarose gel stained with ethidium bromide. The distributions of genotypes in controls and patients with MD were in Hardy–Weinberg equilibrium (Healthy controls: $\chi^2 = 0$; Patients: $\chi^2 = 1.84$) (Table 1).

2.4. DNA methylation analysis by MassARRAY

All samples were collected between 11:00 AM and 1:00 PM, prior to the participants' lunch. Blood samples (5 mL) were collected and placed in vacuum tubes containing heparin sodium and stored at −80 °C. Genomic DNA was isolated using DNeasy® Blood and Tissue Kits (Qiagen, Hilden, Germany). Genomic DNA (1 μg) was converted with sodium bisulfite using EZ DNA methylation kits (Zymo Research, Orange, CA). The concentration of bisulfite-converted DNA was measured using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA); 10 ng of converted DNA was used for PCR. PCR was performed in a total volume of 5 μL containing 1 μM of each primer, 200 μM dNTP, 0.2 U HotStar Taq DNA polymerase (Qiagen), 2 mM $MgCl_2$, and 10 × PCR buffer (Sequenom, Inc., San Diego, CA, USA). One of two primers in the PCR amplification of the target regions was tagged with a T7 promoter sequence: CAGTAATACGACTCACTATAGGGAGAAGGCT. This included a GGG transcription start and an 8-bp insert (AGAAGGCT) on the 5′ end. The reaction mixture was preactivated for 4 min at 95 °C. DNA was amplified for 45 cycles (95 °C for 20 s, 56 °C for 30 s and 72 °C for 60 s) and the reaction was then continued at 72 °C for 3 min.

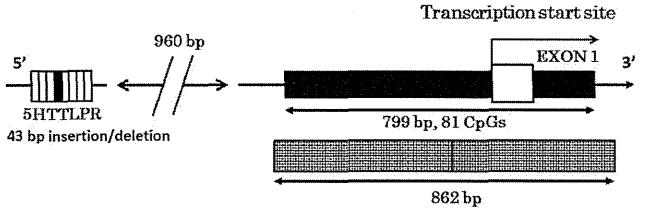

Fig. 1. Schematic representation of the SLC6A4 gene promoter region. Two primers covering the SLC6A4 transcription start site, exon I, part of intron I and the CpG island were used for the DNA methylation study. The target region consists of 862 bp including 81 CpGs. 5-HTTLPR is located 960 bp upstream of the CpG island.

Unincorporated dNTPs were dephosphorylated by adding 1.7 μL DNase free water and 0.3 U shrimp alkaline phosphatase (SAP) (Sequenom). The reaction was incubated at 37 °C for 20 min and SAP was inactivated for 5 min at 85 °C. Subsequently, PCR products (2 μL) were incubated for 3 h at 37 °C with 5 μL of Transcleave mix (Sequenom) for concurrent in vitro transcription and base-specific cleavage.

The resultant 10- to 20-nL products were spotted onto silicon matrix-preloaded chips (SpectroCHIP; Sequenom) using a MassARRAY nanodispenser (Sequenom), and analyzed using a MassARRAY Compact System matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer (MALDI-TOF-MS) (Sequenom).

The spectra's methylation ratios were calculated using EpiTYPER software v1.0 (Sequenom). Triplicate independent analyses from bisulfite-converted DNA samples were undertaken. The method yielded quantitative results for each of the sequence-defined analytic units referred to as CpG units. These methods divided 81 CpG sites in the CpG island into 41 CpG units.

After obtaining the data, initial quality control was performed as follows. Poor-quality and non-valuable data for the quantitative methylation of each CpG unit measured by MALDI-TOF-MS were excluded. Ten CpG units were excluded because their molecular weights were outside the measurable range. In subsequent analyses, data for 2 CpG units were excluded as they were less than 80% of all samples. Data were available for 29 of 41 CpG units in the CpG island and for all samples.

2.5. Statistical analysis

For analysis of the DNA methylation profile, measurements after QC were combined in a data matrix, which was used in a 2-dimensional hierarchical clustering analysis with the "R" software package for statistical computing (available at CRAN, http://cran.r-project.org/). Hierarchical clustering analyses were performed using hclust in the R cluster package, with Euclidean metric and complete linkage. Samples with closer methylation patterns were closely clustered. The Mann–Whitney U test was used to examine differences in the methylation rates of each CpG unit between healthy controls and patients. The Wilcoxon signed-rank test was used to examine differences in the methylation rates of each CpG unit between patients before and after 6 weeks of antidepressant treatment. The significance of each statistical test by Bonferroni correction was set at $P < 0.0017$.

The Spearman rank correlation test was used to examine the correlation between the methylation rate of each CpG unit and total HAM-D scores, total ETISR-SF scores, and improvement ratio (IR:

(HAM-D before treatment − HAM-D after 6 weeks treatment)/ HAM-D before treatment) in patients. The *T*-test was used for subsequent analyses of significance. Significance was set at $P < 0.05$.

3. Results

3.1. DNA methylation profiles in the CpG island of the SLC6A4 gene among all participants

Two-way hierarchical clustering analysis of DMR was undertaken to classify samples and CpG units into clusters according to their similarity, and a dendrogram was used to visualize the results. DNA methylation profiles at the CpG island in the SLC6A4 gene of all subjects are shown in heat map format (Supplemental Fig. 1). At any height of the dendrogram acquired from clustering analysis, it was impossible to distinguish between unmedicated patients and healthy controls (Supplemental Fig. 1).

Next, we compared the methylation rates of each CpG unit between unmedicated patients and healthy controls. There was no significant difference between unmedicated patients and healthy controls at any CpG unit (Table 2).

3.2. The influence of SLC6A4 genotyping for classifying participants

SLC6A4 genotyping analysis demonstrated that 18 healthy controls had *l/l* or *l/s* alleles (an *l* allele) and 32 healthy controls had *s/s* alleles. Similarly, while 16 patients had an *l* allele, 34 patients had *s/s* alleles (Table 1). Two-way hierarchical clustering analysis of DNA methylation profiles could not distinguish samples into any groups associated with diagnosis and/or allele (all participants with an *l* allele vs those with *s/s* alleles, healthy controls with an *l* allele vs those with *s/s* alleles, or patients with an *l* allele vs those with *s/s* alleles) (Supplemental Fig. 2).

We next compared the methylation rates of each CpG unit among all participants by two-factor ANOVA using genotypes (*l* allele and *s/s* alleles) and diagnoses (healthy controls and patients) as fixed factors. Significance after Bonferroni correction was set at $P < 0.0017$. There was no significant effect of genotype on diagnosis. This result indicated that 5-HTTLPR did not affect DMR.

3.3. Correlations between DMR and total HAM-D scores, and DMR and total ETISR-SF scores

Next, we examined correlations between the methylation rates for each CpG unit of the CpG island of the SLC6A4 gene and the severity of depressive symptoms as well as the number of early adversities (Table 3). Regarding the severity of MD, there was a significant positive correlation between the methylation rate for CpG 76 and total HAM-D score ($|r| = 0.30$, $P = 0.03$, power = 0.57). Regarding early adversity, while there was a significant negative correlation between the methylation rate of CpG 3 and total ETISR-SF score ($|r| = 0.32$, $P = 0.02$, power = 0.63), a significant positive correlation was found between the methylation rate for CpG 76 and total ETISR-SF score ($|r| = 0.32$, $P = 0.02$, power = 0.63).

3.4. DNA methylation profiles in the CpG island of the SLC6A4 gene before and after treatment (unmedicated vs medicated patients)

To evaluate the effect of 6-week antidepressant treatment on DNA methylation profiles in the CpG island of the SLC6A4 gene, two-way hierarchical clustering analysis was performed (Supplemental Fig. 3). At any height of the dendrogram acquired

Table 2
The DNA methylation rates of each CpG unit at the CpG island of the SLC6A4 gene in healthy controls and patients with major depression.

	Control	Depression	Depression (6W)	Statistical analysis	
	Rate (%) (mean ± S.E.M)	Rate (%) (mean ± S.E.M)	Rate (%) (mean ± S.E.M)	Con vs Dep (*P*-value)	Dep vs D6W (*P*-value)
CpG 1	7.7 ± 0.4	8.4 ± 0.4	7.7 ± 0.5	0.20	0.68
CpG 3	5.1 ± 0.2	4.9 ± 0.3	6.1 ± 0.3	0.24	0.0004*
CpG 4	4.1 ± 0.3	4.0 ± 0.3	5.7 ± 0.6	0.84	0.03
CpG 10	4.1 ± 0.3	4.0 ± 0.3	5.7 ± 0.6	0.84	0.03
CpG 11	24.2 ± 0.7	24.0 ± 0.6	25.2 ± 0.8	0.50	0.95
CpG 24,25	5.7 ± 0.2	5.2 ± 0.2	4.6 ± 0.2	0.09	0.08
CpG 26,27,28	3.8 ± 0.2	3.7 ± 0.2	3.6 ± 0.2	0.79	0.28
CpG 29	4.6 ± 0.4	4.3 ± 0.7	6.3 ± 1.3	0.23	0.69
CpG 30	5.5 ± 0.9	5.9 ± 0.3	11.8 ± 3.2	0.58	0.17
CpG 31,32	1.6 ± 0.2	4.2 ± 0.2	2.0 ± 0.3	0.69	0.28
CpG 33,34,35,36	4.3 ± 0.2	7.3 ± 0.4	4.1 ± 0.2	0.10	0.29
CpG 37	6.6 ± 0.2	5.9 ± 0.3	5.5 ± 0.3	0.007	0.94
CpG 39,40	4.4 ± 0.1	4.2 ± 0.2	3.8 ± 0.2	0.21	0.10
CpG 41,42	7.3 ± 0.4	7.3 ± 0.4	8.0 ± 0.6	0.73	0.44
CpG 49,50,51,52	6.0 ± 0.4	5.8 ± 0.3	6.5 ± 0.5	0.72	0.13
CpG 55	14.5 ± 0.3	15.0 ± 0.3	15.2 ± 0.4	0.24	0.34
CpG 56	1.3 ± 0.2	1.1 ± 0.2	1.1 ± 0.3	0.51	0.77
CpG 57,58	20.4 ± 0.5	20.8 ± 0.4	21.5 ± 0.5	0.43	0.23
CpG 62	19.9 ± 1.0	20.3 ± 1.0	19.2 ± 1.6	0.59	0.44
CpG 65,66	20.4 ± 0.5	20.8 ± 0.4	21.5 ± 0.5	0.43	0.23
CpG 67,68	24.1 ± 0.7	23.2 ± 0.8	23.8 ± 0.6	0.61	0.67
CpG 70	33.8 ± 1.9	35.2 ± 2.2	34.7 ± 2.7	0.92	0.18
CpG 71,72,73	13.8 ± 0.5	14.6 ± 0.4	13.5 ± 0.4	0.29	0.16
CpG 74	15.4 ± 0.7	16.1 ± 0.7	15.6 ± 0.9	0.63	0.99
CpG 75	49.1 ± 1.8	51.1 ± 1.8	49.3 ± 2.6	0.56	0.20
CpG 76	15.9 ± 0.5	16.3 ± 0.7	16.6 ± 0.7	0.91	0.56
CpG 78	3.3 ± 0.3	3.7 ± 0.4	2.8 ± 0.3	0.80	0.50
CpG 79	9.8 ± 0.8	11.2 ± 0.9	9.8 ± 0.8	0.14	0.59
CpG 80,81	32.3 ± 0.7	33.6 ± 0.8	34.1 ± 1.0	0.38	0.99

Control (Con): healthy controls, depression (Dep): unmedicated patients with MD, Depression6W (D6W); medicated patients with MD.
Statistical analysis: Mann–Whitney *U* test (healthy subjects and unmedicated patients with MD); Wilcoxon ranked signed test (unmedicated patients with MD and medicated patients with MD).
The mean methylation rate and *P*-value are shown.
The asterisks (*) behind of scores indicate statistically significant *P*-value. Significance was set at $P < 0.0017$.

Table 3
Relationships of the DNA methylation rates at the CpG island of the SLC6A4 gene with clinical characteristics in patients with major depression.

	Correlation coefficient		
	HAM-D	ETI	IR
CpG 1	0.26	−0.15	0.08
CpG 3	−0.03	−0.32*	0.36*
CpG 4	0.02	0.008	−0.01
CpG 10	0.02	0.008	−0.01
CpG 11	−0.24	0.25	−0.06
CpG 24,25	−0.09	0.08	−0.008
CpG 26,27,28	0.17	0.05	−0.004
CpG 29	0.11	−0.01	−0.12
CpG 30	−0.16	−0.12	0.15
CpG 31,32	−0.17	0.09	−0.10
CpG 33,34,35,36	0.03	0.14	−0.17
CpG 37	0.06	−0.04	0.18
CpG 39,40	−0.23	0.06	0.11
CpG 41,42	−0.21	−0.19	−0.03
CpG 49,50,51,52	−0.07	0.28	0.07
CpG 55	0.13	0.19	0.05
CpG 56	−0.26	0.18	0.04
CpG 57,58	0.19	0.03	0.12
CpG 62	0.24	0.10	0.05
CpG 65,66	0.19	0.03	0.12
CpG 67,68	0.15	−0.003	0.03
CpG 70	−0.15	−0.17	−0.10
CpG 71,72,73	0.04	0.05	−0.14
CpG 74	0.16	0.19	0.13
CpG 75	−0.07	−0.06	−0.23
CpG 76	0.30*	0.32*	−0.07
CpG 78	0.03	0.17	0.02
CpG 79	0.21	0.06	−0.20
CpG 80,81	0.13	0.11	0.14

ETI: Early Trauma Inventory, IR: improvement ratio, HAM-D: Hamilton Rating Scale for Depression.
Statistical analysis: Spearman rank correlation test.
The correlations between the methylation rates and total HAM-D scores, total ETI scores and IR are shown.
The asterisks (*) behind of several correlations indicate significantly correlations which have statistically significant *P*-values in subsequent analyses by *t*-test.
Significance was set at $P < 0.05$.

from clustering analysis, it was impossible to distinguish between unmedicated patients and medicated patients (Supplemental Fig. 3). We also compared the methylation rates for each CpG unit between unmedicated and medicated patients (Table 2). A significant difference in the methylation rates for CpG 3 ($|Z| = 3.47$, $P = 0.0004$, power = 0.77) was found between unmedicated and medicated patients.

3.5. Relationship between DMR and improvement ratios in response to 6-week antidepressant treatment

To evaluate the potential of DMR as a predictor of treatment response, we analyzed the relationship between pre-treatment methylation rates for each CpG unit and therapeutic responses. The therapeutic responses to antidepressants (paroxetine, fluvoxamine, or milnaciprane) for 6 weeks were defined based on IRs. Correlation analysis was conducted using the IR and the pre-treatment methylation rates for each CpG unit. The pre-treatment methylation rate of CpG 3 showed a significant positive correlation with IRs in MD patients ($|r| = 0.36$, $P = 0.02$, power = 0.64, Table 3).

Two-way hierarchical clustering analysis was performed using samples from patients with an IR of 50% or more, and patients with an IR of less than 50% (Supplemental Fig. 4). At any height of the dendrogram acquired from clustering analysis, it was impossible to classify patients into 2 groups in agreement with the IRs (Supplemental Fig. 4).

Subsequently, we compared the pre-treatment methylation rate of CpG 3 between these 2 groups because it was only changed significantly after antidepressant treatment. The pre-treatment methylation rate of CpG 3 correlated with IRs, and a significant difference was observed ($|U| = 1.98$, $P = 0.047$, power = 0.33).

We determined the relationship between IR and the methylation change of CpG 3 before and after treatment. However, there was no significant correlation (data not shown). Furthermore, we did not identify a significant correlation between IR and ETI (data not shown).

4. Discussion

In the present study, we could not distinguish between healthy controls and unmedicated patients with MD, or between unmedicated and medicated patients with MD based on the results of two-dimensional hierarchical clustering analysis using DNA methylation rates of CpG units in the CpG island of the SLC6A4 gene. Two-way hierarchical clustering analysis and two-factor ANOVA using genotypes (*l* allele and *s/s* alleles) and diagnoses (healthy controls and patients with MD) as fixed factors revealed that 5-HTTLPR itself did not affect DMR. Comparisons of DNA methylation rates for each CpG unit indicated that analysis of the pre-treatment methylation rate of CpG 3 might be useful for determining the therapeutic responses to antidepressants. In addition, correlation analysis using the DNA methylation rate for each CpG unit revealed that DNA methylation rates of several CpG sites were associated with characteristics of patients with MD: CpG 3 and CpG 76 for early adversity in MD patients, and CpG 76 for severity of MD symptoms. We also analyzed the data with respect to the therapeutic response. Although we could not distinguish between patients with high and low therapeutic responses by clustering analysis using all the DNA methylation rates, correlation analysis revealed the pre-treatment DNA methylation rate of CpG 3 correlated with the therapeutic response and an individual comparison of CpG 3 revealed that the DNA methylation rate of CpG 3 was significantly different.

Many epigenetic changes are reversible in response to both external and internal stimuli, indicating a mechanism for interactions of the genome with the environment. Notably, various factors associated with complex diseases, for example nutrition, chemical exposure, and psychosocial stress, have been correlated with epigenetic changes, particularly DNA methylation (Feil and Fraga, 2011). Such findings integrate epigenetic research with population-based epidemiological research investigating not only diagnosis methods (Mill and Heijmans, 2013), but also drug responses and the effect of therapeutic drugs (Duarte, 2013). We recently reported that classification based on DNA methylation profiles of the CpG island in the promoter of exon I of the BDNF gene could be a valuable diagnostic biomarker for MD (Fuchikami et al., 2011). In addition, D'Addario et al. (2013) reported the possible association of changes in DNA methylation of the BDNF promoter with the onset of and/or susceptibility to bipolar disorder, or with mood-stabilizing compounds and antidepressants. In contrast with results of two previous studies, the present study suggested possible associations of DNA methylation of the SLC6A4 gene with the diagnosis of MD, antidepressant treatment, early adversity, and therapeutic responses only when analyses based on data for each specific CpG unit were used, but not when using analyses of all DMR.

In general, an association between DNA methylation and transcriptional activity is considered an important marker of functional relevance (Stranger et al., 2007; Dimas et al., 2009).

With regard to the SLC6A4 gene, Philibert and colleagues reported a significant association between SLC6A4 promoter methylation levels and mRNA levels using peripheral blood from patients with depression (Philibert et al., 2007). However, it is becoming evident that the relationship between DNA methylation

and gene expression is not always tightly linked. In terms of biomarker identification, the fact that methylation is closely associated with a specific pathological condition is thought to be important, even if methylation does not induce gene silencing (Ushijima, 2005). 5-HTT is associated with pathological conditions and the treatment of MD (Lesch and Gutknecht, 2005; Murphy et al., 2008). In this context, the present study only analyzed DNA methylation but not gene expression levels. Future studies examining DNA methylation in the promoter of the SLC6A4 gene together with gene expression will be needed to reveal the functional relevance of DNA methylation of this gene in the pathophysiology of MD.

Thus, although the biological significance of changes in DNA methylation rates for specific CpG units of the SLC6A4 gene is not known, the results of the present study suggest that such information may provide important clinical information regarding MD.

Previous reports described the possibility of a joint effect of 5-HTT methylation and 5-HTTLPR *s* allele carriage on the risk for depression (Olsson et al., 2010) and the effect of CpG islands on DNA methylation by distantly-located SNPs in a fraction of genes (Bell et al., 2011; Soto-Ramirez et al., 2013). Therefore, we evaluated the influence of the 5-HTTLPR allele on DMR. Contrary to the results of Olsson et al. (2010), we found no significant effect on the DNA methylation rates of any CpG unit by genotype. The discrepancy between these studies may be attributed to differences in the tissues (buccal cells vs blood cells) and analytic methods used (Olsson divided the CpG island of the SLC6A4 gene into sub-regions according to co-variation in methylation levels between CpG units).

Changes in the DNA methylation rate of CpG 3 were the most intriguing finding of this study. The DNA methylation rate of CpG 3 correlated negatively with ETISR-SF, and was significantly changed after antidepressant treatment, but correlated positively with therapeutic responses. Several studies have reported that increased DNA methylation of several CpG units within the SLC6A4 gene was associated with early adversity (Ouellet-Morin et al., 2013) and antidepressant treatment (Kang et al., 2013). Although those studies investigated different and smaller regions of the SLC6A4 gene for DNA methylation analyses compared with the present study, they suggested the versatility of DNA methylation of specific CpG units of the SLC6A4 gene induced by environmental factors. In addition, the high prevalence of early adversity in treatment-resistant depressed patients was reported recently (Tunnard et al., 2013). Although the function of CpG 3 methylation of the SLC6A4 gene is unknown, the sequence of changes in the methylation rates at this region (negative correlation with early adversity and positive correlation with response rates) indicates the possible involvement of this factor in the mechanism of resistance to antidepressant treatment.

In summary, analyses using DNA methylation rates for specific CpG units of the SLC6A4 gene, but not analyses based on DNA methylation rates of all CpG units, may be a potential biomarker for the diagnosis, severity of symptoms, early adversity, and history of antidepressant treatment in patients with MD. Notably, the pre-treatment DNA methylation rate for CpG 3 may be associated with antidepressant treatment responses. In addition, the DNA methylation rate for CpG 3 indicated possible involvement in the mechanism of treatment resistance associated with early adversity. Further studies examining the function of these CpG units will help to establish more sophisticated methods for the diagnosis and treatment of MD.

Contributors

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Conflict of interest

The research was conducted in the absence of any commercial or financial relationships.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jpsychires.2014.02.002.

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Maternal Separation Enhances Conditioned Fear and Decreases the mRNA Levels of the Neurotensin Receptor 1 Gene with Hypermethylation of This Gene in the Rat Amygdala

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Abstract

Stress during postnatal development is associated with an increased risk for depression, anxiety disorders, and substance abuse later in life, almost as if mental illness is able to be programed by early life stressors. Recent studies suggest that such "programmed" effects can be caused by epigenetic regulation. With respect to conditioned fear, previous studies have indicated that early life stress influences its development in adulthood, whereas no potential role of epigenetic regulation has been reported. Neurotensin (NTS) is an endogenous neuropeptide that has receptors densely located in the amygdala and hippocampus. Recently, NTS systems have constituted an emerging target for the treatment of anxiety. The aim of the present work is to clarify whether the NTS system is involved in the disturbance of conditioned fear in rats stressed by maternal separation (MS). The results showed that MS enhanced freezing behaviors in fear-conditioned stress and reduced the gene expression of NTS receptor (NTSR) 1 but not of NTS or NTSR2 in the amygdalas of adult rats. The microinjection of a NTSR1 antagonist into the amygdala increased the percentage of freezing in conditioned fear, whereas the microinjection of NTSR1 agonist decreased freezing. These results suggest that NTSR1 in the amygdala may play a role in the effects of MS on conditioned fear stress in adult rats. Moreover, MS increased DNA methylation in the promoter region of NTSR1 in the amygdala. Taken together, MS may leave epigenetic marks in the NTSR1 gene in the amygdala, which may enhance conditioned fear in adulthood. The MS-induced alternations of DNA methylation in the promoter region of NTSR1 in the amygdala may be associated with vulnerability to the development of anxiety disorders and depression in adulthood.

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Introduction

Past clinical studies have shown that exposure to stress during the postnatal development periods is associated with an increased risk for depression, anxiety disorders, and substance abuse later in life [1,2]. Similar to humans, other mammals suffering from early life stress (ELS) in the postnatal period have a vulnerability towards anxiety states and depression-like syndromes [3–7]. These findings suggest that variations in one's early environment may be associated with changes in gene expression and biological function that persist into adulthood. Such "programmed" effects may derive from epigenetic regulation, which causes structural alterations in genomic DNA [4,8].

The conditioned fear stress (CFS) paradigm is based on Pavlovian aversive conditioning. An emotionally neutral stimulus (e.g., a tone, figure, light, or context) is paired with an emotionally potent and innately aversive unconditioned stimulus (e.g., an electric shock) during a conditioning phase. The assessment of

conditioning then involves measuring a conditioned response elicited by the neutral stimulus. CFS is regarded as a psychological stress without physical stimuli and as a simple animal model of anxiety or fear [9–12]. With respect to conditioned fear, previous studies have indicated that ELS continues its influence into adulthood [13,14]; however, no potential role of epigenetic regulation in fear learning and memory caused by ELS has been reported.

Our previous study using a DNA microarray showed that neurotensin (NTS) is the only gene that had its expression changed by CFS. This change in NTS can be overcome by a selective serotonin reuptake inhibitor treatment, which is also effective for the treatment of various anxiety disorders [15]. NTS is an endogenous neuropeptide that closely interacts with monoamine neurotransmitter systems [16], and NTS receptor (NTSR) 1 and 2 are densely located in structures that are important for anxiety, including the amygdala (AMY) and hippocampus (HIP) [17]. The systemic administration of a NTSR1 agonist significantly decreased conditioned footshock-induced ultrasonic vocalization in rats [18], even while decreasing fear-potentiated startling in rats [19]. Moreover, NTSR1 knockout mice showed higher freezing rates than wild-type mice in contextual fear stress situations [11]. However, a role for NTS in conditioned fear induced by ELS has not been documented.

The aim of the present work is to clarify whether the NTS system is involved in the disturbance of conditioned fear in rats stressed by maternal separation (MS), which is one of the most commonly used procedures for inducing ELS in rodents. Our results showed that the MS and NTSR1 antagonist enhanced freezing behaviors in conditioned fear stress. In addition, MS reduced NTSR1 gene expression and increased DNA methylation in the promoter region of AMY.

Materials and Methods

Animals

Adult male Sprague–Dawley (SD) rats, each weighing 230–270 g, were obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan) and were housed in groups of four. Timed pregnant SD rats (Shizuoka Laboratory Animal Center) were delivered on gestation day 14 and were single-housed. All rats were housed in standard animal cages and received ad libitum access to food and water in a temperature-controlled environment (22±1°C) on a 12 h light/dark cycle (light phase: 0600–1800). All procedures were approved by the Hokkaido University Graduate School of Medicine Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals, Hokkaido University Graduate School of Medicine.

MS procedure

MS was conducted according to the method of Plotsky and Meaney [20]. When a litter was born between 0900 and 1700, its day of birth was designated as postnatal day (PND) 0. When a litter was born between 1700 and 0900, its day of birth was designated as PND 1. After the pups were cross-fostered to minimize litter differences, eight males and two females were selected on PND 2. Each litter was assigned for either MS or the animal facility rearing (AFR) groups. Handling and separation were performed from 0930–1230 daily from PND 2 to PND 14. In the MS group, a dam was removed from the cage and placed into an individual cage during the separation period. The pups were then removed from the cage and placed into a clean plastic cage lined with wood-chip bedding. Then, they were taken to another room and placed in an incubator set to maintain an ambient temperature at 27– 30°C for 3 h. At the end of the separation period, the pups were returned to the cage, and then, the dam was returned. Pups in the MS group were permitted to huddle with their littermates during the separation period. AFR rats were briefly handled once a week by an animal care technician. Only male pups were used for the present study. Behavioral, molecular, and neurochemical experiments were performed during the postadolescent period (10–14 weeks old).

Dexamethasone/corticotropin releasing hormone test

The dexamethasone (DEX)/corticotropin releasing hormone (CRH) test was conducted according to the method described by Hatzinger et al. [21]. Surgery was performed under sodium pentobarbital (40 mg/kg, i.p.) anesthesia using aseptic procedures. Rats were chronically catheterized in the jugular vein for subsequent blood sampling six days before the experiment. The catheter was exteriorized at the neck of the animal and filled with sterile saline containing gentamicin (30,000 IU/rat); 0.2 ml was infused into the animal. Rats were weighed at 0700 on the day of the experiment. The jugular venous catheter was connected via free-moving devices (Eicom, Kyoto, Japan) to a plastic syringe filled with sterile heparinized saline (50 IU/ml) at 0800. DEX (Sigma, St. Louis, MO) (30 μg/kg, 0.5 ml/kg) was administered intravenously (i.v.) at 1200. Numerous 0.2-ml blood samples were collected at 1800, 1830, 1900 and 1930 to monitor the effects of the DEX treatment on the basal plasma concentrations of corticosterone during the diurnal acrophase. At 1931, human-CRH (kindly donated from Yoshitomi Pharma, Osaka, Japan) (50 ng/kg, 0.5 ml/kg i.v.) was injected. To assess the CRH-stimulated corticosterone secretion, further blood samples were taken at 1940, 2000, 2020, and 2040. Corticosterone was measured by an enzyme immunoassay using a corticosterone EIA kit (Cayman, Ann Arbor, MI).

Open field test

The open field box (90-cm length × 90-cm width × 40-cm height) was divided into 81 squares of equal size by stripes of black paint, and the lighting at the center of the area was set to 200 lx. The arena was divided into two sections, and the outer edge of the 20-cm width was measured from the walls (outer zone) and the center square (inner zone). Rats were placed singly in the center of the field, and their behavior was recorded for 30 min. The total distance travelled and time spent in the inner zone were assessed using tracking software (LimeLight; Actimetrics, Wilmette, IL). Total movement in the field reflected their general activity, and relative movement in the inner zone was correlated with the anxious state of the rat. All tests were performed between 1400 and 1600 to minimize Circadian influences.

Elevated plus maze

The apparatus consisted of two open arms (50 × 10 cm) and two closed arms (50 × 10 cm) surrounded by 40 cm high side walls that extended from the central platform (10 × 10 cm). The maze was elevated 50 cm above the floor, and the lighting at the center of the area was set to 200 lx. Rats were placed singly on the central platform facing an open arm, and their behavior was recorded for 5 min. The total distance moved in the maze and the number of entries for each arm were assessed using LimeLight (Actimetrics). The total distance moved in the maze was measured as the locomotor activity. The number of entries in the open arms was measured as memory-independent fear because rats innately avoid open spaces [22]. All tests were performed between 1400 and 1600 to minimize Circadian influences.

Footshock sensitivity test

The effects of MS on footshock-induced pain were examined. Four behaviors, including vocalization, limb withdrawal of the forepaw, limb withdrawal of the hindpaw and jumping, were used as indicators of nociception. Generally, each was used as an endpoint in the hot plate procedure [23]. The rats were individually placed in a shock chamber with a grid floor (19 × 22 × 20 cm). After a 5-min adaptation period, the rats were subjected to 15 series of scrambled electric footshocks. Each series had 10-s and 40-s intervals and ranged from 0.4 mA to 3.2 mA in 0.2 mA steps, which are presented in ascending order. The responses of the rats to each shock were recorded, and the minimal intensities of the electric footshocks at which each of the four behaviors appeared were determined.

Condition fear stress paradigm

Rats individually underwent inescapable electric footshocks for a total of 5 min in a shock chamber with a grid floor (30× 24× 30 cm, Med Associates Inc., USA) [24]. Five footshocks (2.0 mA scrambled shock, each of 30 s duration) were delivered at shock intervals of 30 s using an ENV-410 shock generator (Med Associates Inc., USA). Twenty-four hours later, the rats were exposed to the chamber without footshocks to assess their contextual fear memory for 5 min (first exposure), as measured by their freezing behaviors. On the next day, rats were exposed to the chamber for 5 min again (second exposure). During the observation period, the duration of the freezing behavior was recorded, as previously described [24]. Freezing was defined as the absence of all observable movements of the skeleton and the vibrissae, except for movements related to respiration. Rats were classified as either freezing or active according to their behavior during a 5-s period. The percentage score of freezing represents the number of 5-s periods during which the rats froze for 5 s.

Quantitative RT-PCR Analysis

Rats were deeply anesthetized with sodium pentobarbital and decapitated. The brains were removed and washed with ice-cold phosphate-buffered saline (PBS; pH 7.4). Coronal sections with a thickness of 1 mm were cut using a Brain Slicer (Muromachi, Tokyo, Japan) and immersed into dishes containing ice-cold PBS. The regions containing AMY and HIP were dissected carefully with a blade. Total RNA was extracted with the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) from these regions. Total RNA was converted to cDNA with the Quantitect Reverse Transcription kit (Qiagen). Quantitative PCR was performed with the SYBR GreenER qPCR SuperMix for ABI PRISM (Invitrogen) in the ABI PRISM 7600 Sequence Detection System (Applied Biosystems, Foster, CA). All standards and unknown samples were assayed in triplicate. The conditions of PCR were the following: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The sequences of forward and reverse primers used were as follows: AGC TGG TCA TCA ATG GGA AA and ATT TGA TGT TAG CGG GAT CG for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), GCT GAC CGT ATT CCA ACT CC and CAT TGC CAT GAT CGA GGA TA for NTS, AAG CAG GCA CCC TTC ATC T and GGA GGC TGG ATG GTT CTG T for NTSR, CCT GGT GAG ACA CAA GGA TG and ACG ATG GCT CTG AGA AAA CCT G for NTSR2. GAPDH was used as a control. PCR reaction assays for unknown samples were performed simultaneously with the same standard samples (cDNA derived from rat HIP) to construct a standard curve. The relative concentrations of GAPDH and of NTS, NTSR1 or NTSR2 in unknown samples were calculated from this standard curve, and we calculated the ratio of the relative concentrations of NTS, NTSR1 or NTSR2 (already normalized by GAPDH expression) to the relative concentration of GAPDH.

Analysis of DNA methylation in the promoter of NTSR1

Genome DNA of MS and AFR rats were isolated from AMY with a DNeasy Blood & Tissue Kit (Qiagen). A MethylCollector^{TM} Ultra Kit (Active Motif, Carlsbad, CA) was used to enrich CpG methylated DNA fragments. The method was based on the Methylated CpG Island Recovery Assay (MIRA), which utilizes the high affinity of the MBD2b/MBD3L1 complex for methylated DNA. Genomic DNA was digested with Bfa I (New England Biolabs, Ipswich, MA) because the promoter region of NTSR1 contains the recognition site of Bfa I. The MBD2b/MBD3L1 protein-DNA complex was added to the DNA fragments, specifically binding to CpG-methylated DNA. These protein-DNA complexes were then captured with nickel-coated magnetic beads, and subsequent wash steps were performed to remove fragments with little or no methylation (unbound fragment; UF). The methylated DNA was then eluted from the beads in the presence of Proteinase K (eluted fragment; EF). Both UF and EF were purified using a MinElute PCR purification kit (Qiagen). PCR primers were designed to target the three DNA fragments (fragment A, B and C) within the CpG islands, which contain the promoter region of NTSR1 gene. The fragments were located −969 to −674 (fragment A), −673 to −283 (fragment B) and −273 to +263 (fragment C) from the first translational ATG codon. Quantitative PCR was performed with the SYBR GreenER qPCR SuperMix in the ABI PRISM 7600 Sequence Detection System. All samples were assayed in triplicate. The percentage enrichment, which represented the degree of methylation of each fragment, was calculated as follows: enrichment (%) = $2^{CT\ (UF)\ -\ CT\ (EF)}/(1 + 2^{CT\ (UF)\ -\ CT\ (EF)}) \times 100$. The conditions for the PCR were the following: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The primers used were as follows: CCG AGC CAG CTG TAC AAA G and GGC AGC ACA ATC TTC TCC TT for NTSR1-fragment A, CAA GCA GAA GAG GGA GAA CG and TGC TAC GGA CCT CCA GAT TC for NTSR1- fragment B, AAG AAG AGT GGA TCC CTG AGC and TAT GCT GCT TTG TCC TGC AC for NTSR1- fragment C.

Microinjections

Surgery was performed using sodium pentobarbital anesthesia (40 mg/kg, intraperitoneally) under aseptic conditions. The head position was adjusted to place the bregma and lambda on the same horizontal plane in a stereotaxic frame. Naïve rats were implanted with a bilateral 26-gauge guide cannula (Plastics One, Roanoke, VA) directed toward AMY (immediately the central nucleus of AMY). The coordinates were −2.5 mm posterior to the bregma, 4.7 mm bilateral to the midline, and 7.2 mm ventral from the surface of the skull. Dummy stylets were inserted into guided cannulae, and the rats were allowed a 10–12-day post-recovery period. After surgery, the rats were housed individually. During the recovery period, the rats were handled 4 times before the microinjection procedure. PD149163, a specific agonist of NTSR1, was kindly donated by the NIMH Chemical Synthesis and Drug Supply Program (Washington D.C.) and SRI International (Menlo Park, California), and SR48692 was purchased from Tocris Biosciens (Bristol, UK). PD149163 was prepared in 0.9% physiological saline as a 1 mM stock solution, and SR48692 was prepared in DMSO as 1 mM stock solution. They were stored at −20°C. Twenty-four hours, after fear conditioning, rats were injected bilaterally into AMY with 0.5 µl/side of saline or DMSO,

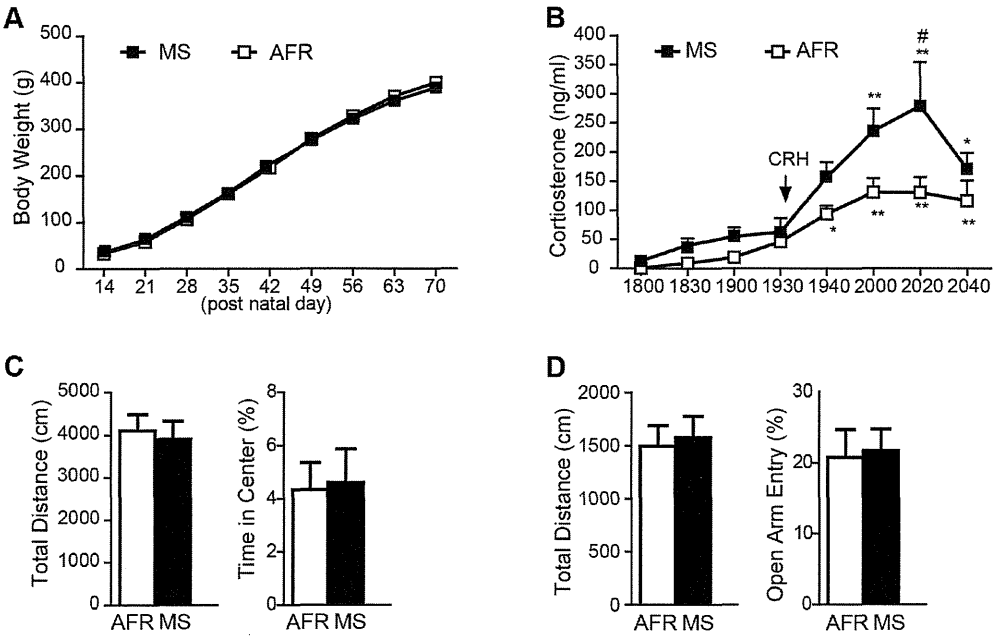

Figure 1. Effect of maternal separation (MS) on body weight, hypothalamic-pituitary-adrenocortical system and anxiety behaviors. *A*, Body weight change of rats in MS and animal facility rearing (AFR) groups (MS, n = 22; AFR, n = 17). *B*, Plasma corticosterone concentrations of MS (n = 8) and AFR (n = 6) rats between 1800 and 2040 h on the dexamethasone/corticotropin-releasing hormone (DEX/CRH) test. All rats were pretreated with DEX (30 μg/kg i.v.) at 1200 h and administered with CRH (50 ng/kg i.v.) at 1931 h. MS rats showed significant increases in plasma corticosterone levels (#$p<0.05$ vs AFR rats, *$p<0.05$, **$p<0.01$ vs baseline). *C*, Open field test. Total distance and % time in center during the 30 min test were analyzed. *D*, Elevated plus maze test. Total distance and % number of open arm entries during the 5 min were analyzed. Error bars represent SEM.

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PD149163 (10, 100, or 1000 μM) or SR48692 (10, 100, or 1000 nM). After the removal of the dummy stylets, bilateral infusions were given simultaneously for 30 sec using 33-gauge stainless steel cannulae projecting 1.0 mm beyond the tips of the guide cannulae. Each injector was connected by polyethylene tubing to a 100-μl syringe that was driven at a rate of 1 μl/min by an infusion pump. Injectors remained in place for an additional 1 min to confine the drug to the target site. Ten minutes after the infusion, the rats were again placed in the shock chamber and observed for 5 min without shocks to assess their contextual fear

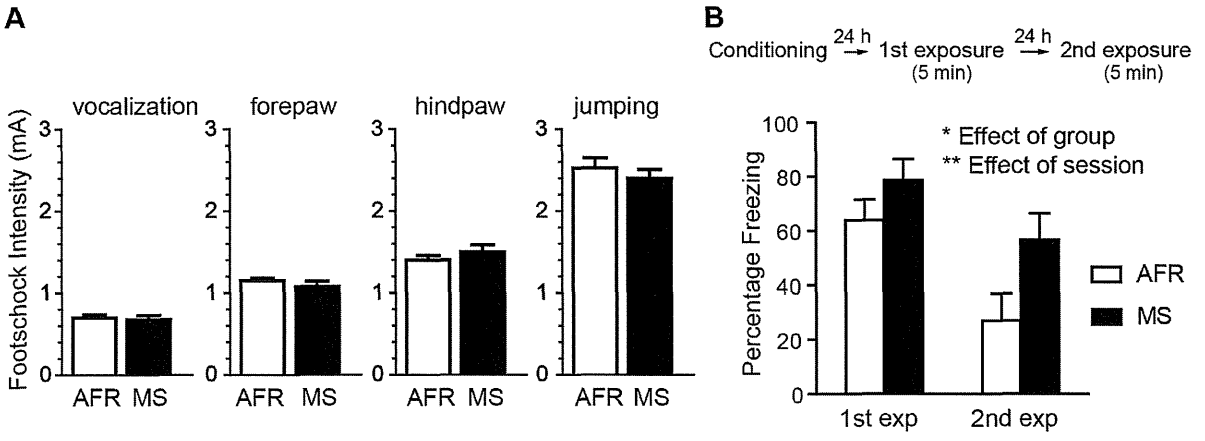

Figure 2. Effect of MS on freezing behavior in conditioned fear stress (CFS). *A*, Sensitivity to pain induced by electrical stimulation. The minimal levels of the current required to elicit the stereotypical response of vocalization, limb withdrawal of the forepaw, limb withdrawal of the hindpaw and jumping were determined. Data are represented as pain thresholds (mA) (n = 8 per each group). *B*, Freezing rates of the MS and the AFR rats in the first exposure of 24 h after and the second exposure of 48 h after the fear conditioning by a 2.0 mA footshock. Two-way repeated ANOVA revealed significant main effects of the group (*$p<0.05$) and session (**$p<0.01$) but no significant interactions between the group and session (n = 10–12 per group).

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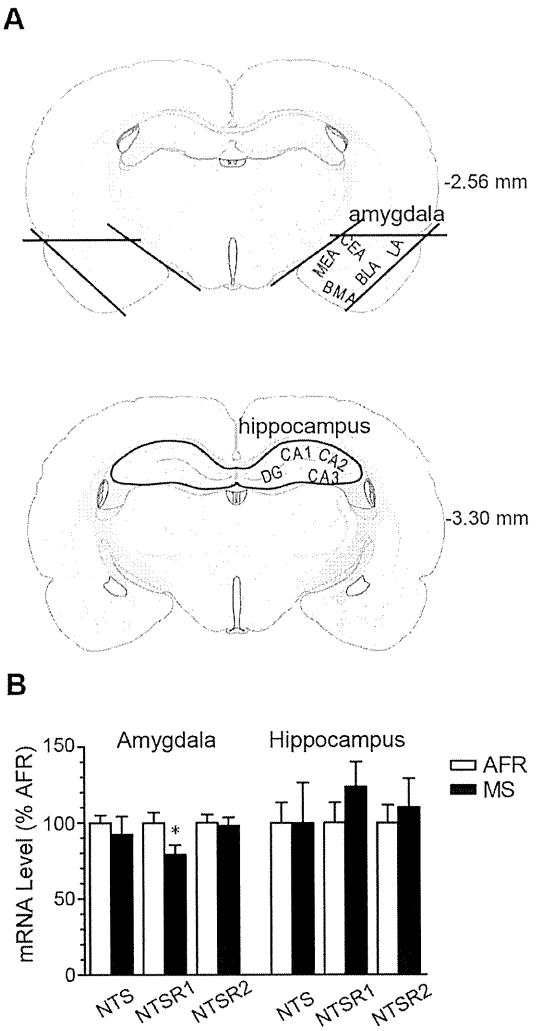

Figure 3. Effect of MS on the mRNA expression of neurotensin receptor 1 (NTSR1). *A*, Dissected regions of the amygdala (AMY) and hippocampus (HIP) (heavy black line) in coronal sections with a thickness of 1 mm from the rat brain atlas of Paxinos and Watson [69] (2.56 and 3.30 mm posterior to bregma). *B*, Comparative mRNA expression levels of neurotensin, NTSR1 and NTSR2 in the AMY and HIP of MS and AFR rats. NTSR1 mRNA levels in MS rats were significantly decreased in AMY compared to AFR rats (n = 7 – 9 per group; $*p<0.05$, $**p<0.01$). Error bars represent SEM.
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memory (first exposure). On the next day, the rats were exposed to the chamber for 5 min again (second exposure). The 0.5-μl injection volume was based on a previous study [25]. The injection was aimed at the central nucleus because this area contains many NTS-immunoreactive neurons [26]. However, the entire region of AMY might be influenced by these drugs, as it has been reported that a volume of 0.5 μl is likely to infiltrate the other areas of AMY [27]. Injection placements were verified immediately after the second exposure session, as described previously [25]. Only the data from animals with the correct placement of the cannulae were analyzed.

Statistics

Data were analyzed using SPSS Statistics version 21 (SPSS, Chicago, IL). Numerical data were analyzed by an unpaired *t*-test, two-way repeated ANOVA or one-way ANOVA, followed by Tukey's post hoc test. In all cases, the significance level was set at $p<0.05$. All data are shown as the means ± S.E.M.

Results

MS affected the hypothalamic-pituitary-adrenocortical (HPA) system but not body weight or anxiety behaviors

No significant difference was found between the AFR and MS groups in body weight at 14 PND, at which point the maternal separation procedure had finished (Fig. 1A). A two-way repeated measure ANOVA revealed that MS had no significant main effect of the group ($F_{(1, 62.5)} = 0.010$, $p>0.05$, Fig. 1A) and no interaction ($F_{(8, 447)} = 1.758$, $p>0.05$, Fig. 1A). The effect of MS on plasma corticosterone levels was examined in the DEX/CRH test (Fig. 1B). A two-way repeated measure ANOVA revealed a significant main effect of group ($F_{(1, 84)} = 5.389$, $p<0.05$) and time ($F_{(7, 84)} = 15.75$, $p<0.001$) but no significant interaction between group and time. The release of corticosterone stimulated by CRH was significantly greater in MS rats than in AFR rats, as was reflected by the higher AUC values (MS: 14650±2584; AFR: 8032±1372; $p<0.05$). Tukey's post hoc test revealed that the maximum rise of corticosterone was significantly greater in MS over AFR rats ($p<0.05$). Basal corticosterone levels between 1800 and 1930 h did not differ between the MS and AFR rats.

We examined the effects of MS on memory-independent anxiety behaviors in the open field test and elevated plus maze test. In the open field test (Fig. 1C), no significant difference was found between the two groups in the total distance moved in the field or in the % time spent in the center field. In the elevated plus maze (EPM) test (Fig. 1D), no significant difference was found between the two groups in the total distance moved in the maze or in the % of entries into the open arms.

MS enhanced freezing behavior in CFS

Figure 2A shows the minimal intensities of the electric footshocks at which pain-related behaviors first appeared. There were no significant differences between the two groups in vocalization, limb withdrawal of the forepaw, limb withdrawal of the hindpaw or jumping. These data indicate that the sensitivity to the footshock was not different between the AFR and MS groups. Next, we examined memory-dependent behaviors in CFS (Fig. 2B). Twenty-four hours after the conditioning session for the 2.0 mA footshock, a two-way repeated ANOVA revealed a significant main effect for the group ($F_{(1, 19)} = 5.25$, $p<0.05$) and session ($F_{(1, 19)} = 37.17$, $p<0.01$) and no significant interaction between the group and session.

MS decreased the mRNA expression of NTSR1 in AMY

AMY and HIP play a central role in fear learning and memory [25,28]. Previous findings have suggested that the NTS system is involved in fear memory processes [18,29,30]. Therefore, we examined the effects of MS on the mRNA expressions of NTS, NTSR1 and NTSR2 in AMY and HIP with quantitative RT-PCR. Figure 3A indicates the dissecting regions as HIP (upper coronal section) and AMY (lower coronal section). The bilateral AMY and HIP of adult rats were dissected, as indicated with heavy black lines. Figure 3B shows the mRNA expression levels of NTS, NTSR1 and NTSR2 in AMY and HIP of MS or AFR rats. In AMY, a significant decrease in mRNA expression of NTSR1 was observed in MS rats ($p<0.05$), but no significant differences

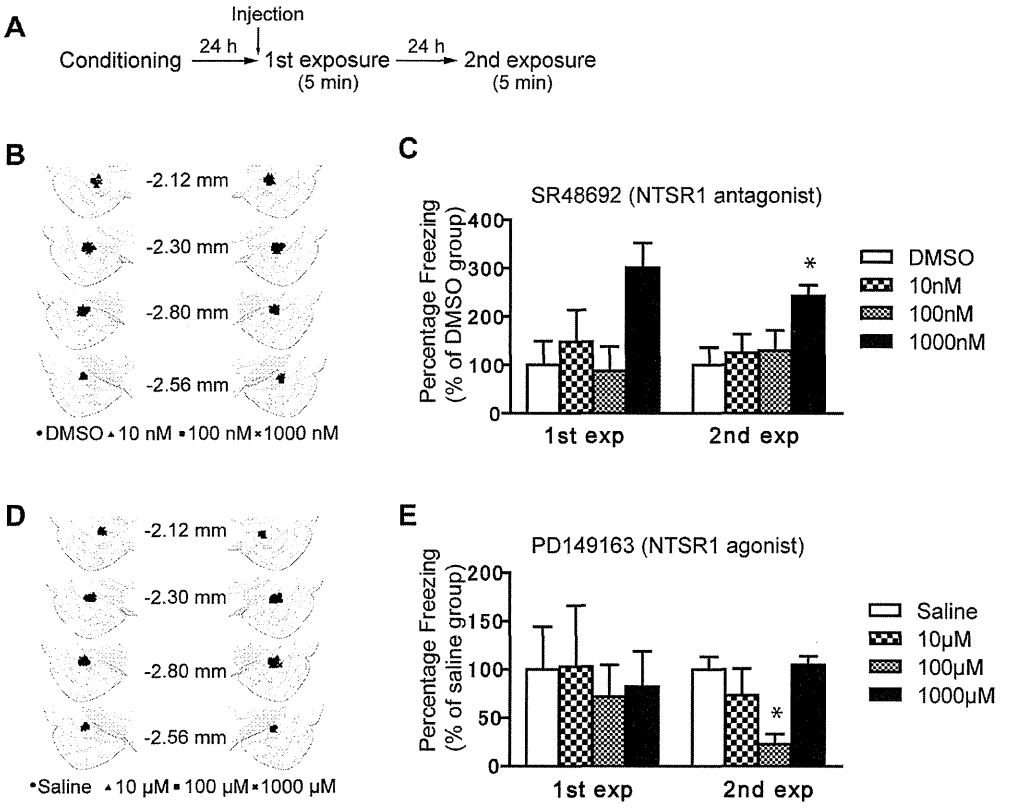

Figure 4. Effect of the NTSR1 agonist and antagonist in AMY on CFS. *A*, Experimental design. *B, D*, Location of the needle tips for intra-AMY infusions of SR48692 (B) and PD149163 (D). Brain structure diagrams of coronal sections through AMY are adapted from the rat brain atlas of Paxinos and Watson [69] (2.12 – 2.56 mm posterior to bregma). *C*, Bilateral infusion of an NTSR1 antagonist (SR48692) dissolved in 0.5μl of DMOSO in AMY produced a significant increase in the freezing rate at a concentration of 1000 nM in the second exposure. *E*, Bilateral infusions of a NTSR1 agonist (PD149613) dissolved in 0.5 μl of saline into AMY produced a significant decrease in the freezing rate at a concentration of 100 μM at the second exposure (n = 7 – 8 rats per all groups; *$p<0.05$ vs. saline or DMSO control). Error bars represent SEM.
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were found between the two groups in the mRNA expressions of NTS or NTSR2. In HIP, no significant differences were found between the two groups in mRNA expressions.

Microinjections of NTSR1 antagonist or agonist in AMY showed opposite effects on freezing behaviors in CFS

To determine whether NTSR1 in AMY is critically involved in CFS, we examined the effects of microinjections of an antagonist or agonist of NTSR1 into AMY of naïve rats on CFS. SR48692, which has a higher affinity for NTSR1 compared to NTSR2 [31], was used as an antagonist of NTSR1. PD149163, which selectively binds to the NTSR1 with no affinity for NTSR2 [32], was used as an agonist of NTSR1. CFS and microinjections of SR48692 or PD149163 were applied, as shown in Fig. 4A. The cannula placements for rats injected with SR48692 and PD1491633 into AMY are shown in Figures 4B and 4D, respectively. Tissue damage was not apparent in either of the treatment groups or in the saline groups.

Figure 4C shows the freezing rate (% the DMSO group) of rats with the bilateral injections of saline or SR48692 (10 nM – 1000 nM) into AMY. One-way ANOVA indicated the significant effects of drug treatment at the first exposure ($F_{(3, 27)} = 3.26$, $p<0.05$) and at the second exposure ($F_{(3, 27)} = 3.19$, $p<0.05$). Tukey's post hoc test showed that all drug treatment groups were not significantly higher than the DMSO group at the first exposure ($p<0.05$), but the freezing rate at a dose of 1000 nM was significantly higher than that of the DMSO group at the second exposure ($p<0.05$). Figure 4E shows the freezing rate (% the saline group) of the rats with bilateral microinjections of saline or PD149163 (10 μM – 1000 μM) into AMY. One-way ANOVA indicated no significant effect of drug treatment in the first exposure ($F_{(3, 25)} = 0.104$, $p>0.05$) but a significant effect in the second exposure ($F_{(3, 25)} = 5.45$, $p<0.05$). Tukey's post hoc test showed that the freezing rate at a dose of 100 μM was significantly lower than that with saline at the second exposure ($p<0.05$). These data indicate that the microinjection of a NTSR1 agonist (PD149163) into AMY disrupted the conditioned freezing and that the microinjection of NTSR1 antagonist (SR48692) into AMY potentiated it.

MS increased DNA methylation in the promoter region of the NTSR1 gene in AMY

MS decreased the expression of NTSR1 mRNA in adulthood (Fig. 3B). Such long-lasting effects are often mediated by epigenetic

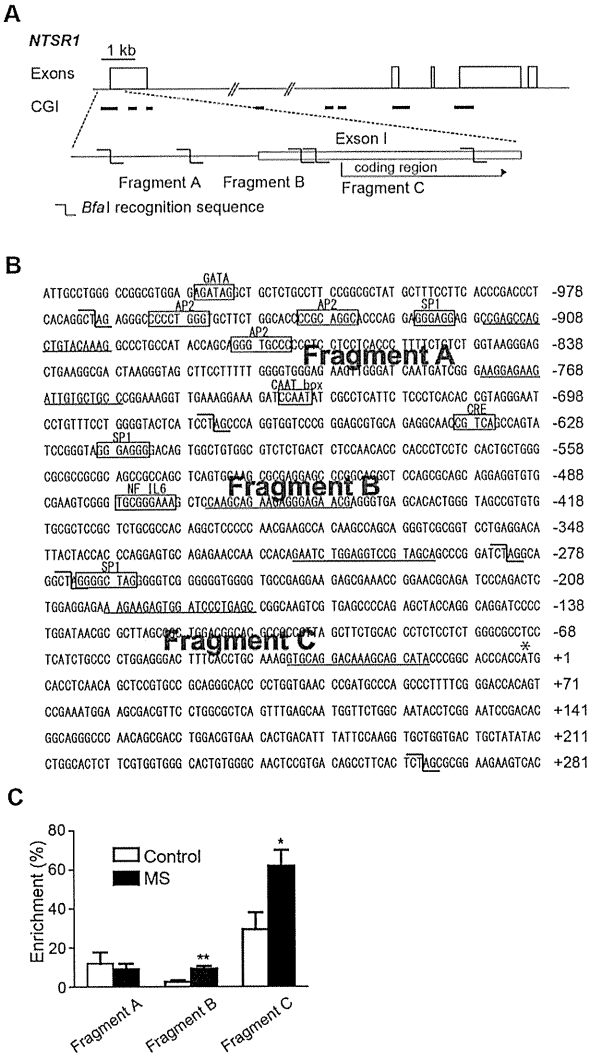

Figure 5. Effect of MS on DNA methylation in the NTSR1 promoter region in AMY. *A*, The diagram of NTSR1 DNA and the locations of the CpG islands (CGI) and fragments A, B and C. *B*, Nucleotide sequence of the promoter region of the rat NTSR1 gene [34]. The hooked shape indicates the Bfa I recognition sites. Underlined sequences indicate the position of the primers of fragments A, B and C. The potential binding sites for transcription factors, GATA, activating protein 2 (AP2), specificity protein 1 (SP1), CAAT box, cAMP response elements (CRE), and nuclear factor-interleukin 6 (NF IL6) are marked by boxes, and their names are indicated above. The coding sequence of the first exon is indicated by an asterisk. *C*, Methylation analysis of NTSR1 promoter region using a MethylCollector Ultra Kit (Active Motif). The percentage enrichment was calculated as follows: enrichment $(\%) = 2^{CT\,(UF)} - {}^{CT\,(EF)}/(1 + 2^{CT\,(UF)} - {}^{CT\,(EF)}) \times 100$. The eluted fragment (EF) represents a DNA fragment with more than five methylated CpG sites, and the unbounded fragment (UF) represents a DNA fragment with four or fewer methylated CpG sites. The % enrichment of fragments B and C was significantly increased in the MS group compared with the AFR group (n = 8 per group; $*p<0.05$ vs. AFR, $**p<0.01$ vs. AFR, unpaired *t*-test).

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mechanisms, including DNA methylation [33]. Therefore, we examined whether MS affected DNA methylation in the promoter region of the NTSR1 gene in AMY.

Figure 5A illustrates a diagram of the NTSR1 gene and the sites of CpG islands. Figure 5B shows nucleotide sequences of the promoter region of NTSR1 and the potential binding sites for following transcription factors: GATA, activating protein 2 (AP2), specificity protein 1 (SP1), CAAT box, cAMP response elements (CRE), and nuclear factor-interleukin 6 (NF IL6) [34]. The coding sequence of the first exon is indicated by an asterisk. A large G+C-rich domain with characteristics in common with a CpG island (64.5% over 700 nt: −648 to 52) extended from the first translation ATG codon (+1) in the promoter region and into part of the coding region of the NTSR1 gene. The promoter region between −662 and −470 from the first translation codon contained strong positive regulatory elements that drove the expression of NTSR1 gene [34]. This region lacked a typical TATA or CAAT box but contained a CRE-like half site and putative Sp1 binding sites, suggesting the possible involvement of these factors in the positive regulation of NTSR1 expression. The locations of the fragments A, B and C are indicated in figures 5A and 5B. The underlined sequences in figure 5B show the position of the primers of fragment A, B and C. The % enrichment represents the degree of methylation of each fragment. The analysis of real time PCR revealed that the % enrichment of the fragments B and C was significantly increased in the MS group compared with the AFR group (unpaired *t*-test; $p<0.01$ and $p<0.05$, respectively) (Fig. 5C).

Discussion

We have shown that MS enhances conditioned fear in adulthood and increases the DNA methylation in the promoter region on the NTSR1 gene in AMY. In addition, the pharmacological blockade of NTSR1 in AMY activated conditioned fear, whereas the activation of NTSR1 disrupted it. These findings suggest that MS may enhance conditioned fear via DNA methylation of the promoter region of NTSR1 in AMY, underlying its decreased mRNA expression.

Our experiment showed that a remarkable increase in corticosterone levels following CRH stimulation could be observed with the DEX/CRH test in MS group, affirming that a long-term dysregulation of the HPA axis is induced by ELS in animal and human studies [33,35–37]. MS did not affect the body weight, anxiety behaviors in the elevated plus maze test or locomotor activity in the open field test. Otherwise, the freezing rate of MS rats was higher than that of AFR rats in the context of exposure without the different sensitivities to pain induced by footshock. Taken together, it has been suggested that MS may disturbance the processes of conditioned fear memory in adulthood. Memory retrieval may initiate two potentially dissociable but opposite processes: reconsolidation and extinction [38–40]. Previous studies have shown that the length of re-exposure determines, in part, which of these two is dominant. Brief re-exposure to a conditioning stimulus seems to trigger a second wave of memory consolidation (reconsolidation), whereas prolonged exposure to the conditioning stimulus leads to the formation of a new memory that competes with the original memory (extinction) [39–46]. However, other characteristics of a memory, such as its age and strength, also affect behaviors after memory retrieval. Hence, our results lead us to hypothesize that MS enhanced the reconsolidation or decreased the extinction of fear memory because our experiments were not sufficient to discriminate between these two processes.

Studies in developmental psychobiology and physiology are replete with examples of the environmental programming of gene expression. Such studies have commonly reported that a variation in the early environment is associated with changes in gene

expression and function that persist into adulthood and thus well beyond the duration of the relevant environmental event [5,6,47]. Our results showed that MS decreased the expression of NTSR1 mRNA in AMY, but not in HIP, in adulthood. In addition, the pharmacological blockade of NTSR1 in AMY increased the freezing rate in the second exposure to CFS, whereas the activation of NTSR1 decreased the freezing rate. These results suggest the involvement of NTSR1 located at AMY in conditioned fear. The systemic administration of an NTSR1 agonist was reported to have an anxiolytic profile in a conditioned footshock-induced ultrasonic vocalization model [48]. NTSR1 knockout mice showed a higher freezing rate than wild-type mice in contextual fear memory [49]. Our study using microinjections of NTSR1 agonist or antagonist to the AMY agrees with these past studies.

It has been shown that NTS and its receptor play a role in pain transmission [50–54]. This may indicate that NTSR1 agonism or antagonism can change the freezing rate in CFS by modulating the pain transmission instead of affecting the fear memory. To exclude this possibility, NTSR1 agonists or antagonists were microinjected before the first exposure, but they did not influence the threshold of pain induced by footshock in the conditioning session. Therefore, it is unlikely that the observed effects on learning fear memory are due to changes in the pain threshold or other nonspecific performance variables associated with memory acquisition. The concentration of PD149163 (NTSR1 agonist) used in this study was in the range of pmol (10 pmol –1000 pmol), comparable to the dose range used by others for intracerebral injections [51,52]. On the other hand, the concentration of SR48692 (NTSR1 antagonist) was in the range of fmol (10 fmol–1000 fmol). Because SR 48692 has an agonistic effect at high doses [55,56], we used a low concentration of SR48692 compared to PD149163, which was also comparable to the dose range used by others for intracerebral injections [29,51,57,58].

We examined the effect of MS on DNA methylation in the promoter region of the NTSR1 gene in AMY. DNA methylations of fragments B and C were significantly increased in the MS group. Fragment B is located 673–283 bp upstream from the first translational codon, which includes the core promoter region of the NTSR1 gene. Increased methylation of CpG dinucleotides, particularly in the 5′-promoter regions, is generally associated with a decrease in gene expression [59]. Therefore, these results suggest that DNA hypermethylation at the promoter region of the NTSR1 gene decreased mRNA expression. Whereas the function of this hypermethylation of the coding regions is not clear, it was difficult to explain why the methylation of the fragment C in MS rats was increased. Taken together, MS enhanced conditioned fear in adulthood, which may be induced from the DNA methylation of the promoter region of the NTSR1 gene in AMY.

Recent studies suggest that the DNA methylation induced by ELS plays a role in the development of psychiatric illness [36,60]. Moreover, human studies have proven that individuals who are exposed to ELS are more likely to develop post-traumatic stress disorder (PTSD) and phobias [61–65]. Our results suggest the possibility that the DNA methylation of the promoter region of NTSR1 gene in AMY may induce a vulnerability to anxiety disorders, such as PTSD and phobia, as disturbances of the fear memory processes play an important role in the development of these diseases [9,66,67]. However, we were unable to verify whether the relationship between the MS-induced down-regulation of NTSR1 mRNA and impaired fear memory processes was causative or correlative. This is one important limitation of this study. To prove this, for example, we would have to confirm the overexpression of NTSR1 in AMY of MS rats recovering from the MS-enhanced conditioned fear memory. The other limitation of this study is that we did not examine the protein levels of NTSR1. Various genes have been reported to be associated with fear memory. For example, the decrease in the GluN2B subtype of the NMDA subunit of the glutamate receptor in AMY would serve to inhibit the reconsolidation of the contextual memory [68]. Thus, it was possible to be associated with other genes than NTSR1 in the processes of fear memory in this study. Additionally, we examined the effect of drug microinjection before the first exposure test to clarify the role of NTSR1 in fear because the effect of drugs on the expression of conditioned fear anticipates the drug's effect on this response in many cases [11]. However, we did not administer the agonist or antagonist of NTSR1 before fear conditioning. Accordingly, future studies to examine the effect of NTSR1 agonism and antagonism administered before fear conditioning or before both fear conditioning and first exposure are needed.

In conclusion, we showed that MS leaves epigenetic marks in the promoter region of the NTSR1 gene in AMY that may enhance conditioned fear memory in adulthood. These results provide the first evidence that differential DNA methylation in the promoter region of NTSR1 gene in AMY may be associated with the conditioned fear memory, although epigenetic alteration is a correlate, and a no cause-effect relation can be established by this study. Because ELS plays an important role in the clinical vulnerability to the development of anxiety disorders and depression in adulthood, our results suggest the hypothesis that manipulating the NTSR1 gene in AMY may have a therapeutic action to improve from these diseases. To clarify this hypothesis, it would be useful to investigate whether the overexpression of NTSR1 in AMY of MS rats can recover MS-enhanced conditioned fear memory.

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Author Contributions

Conceived and designed the experiments: HT SB SN TK. Performed the experiments: HT SB AK NT NS. Analyzed the data: HT AK. Wrote the paper: HT SB SN TI MN TK IK.

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